Diversity within subterranean clover and biserrula for persistence traits with potential use in New Zealand hill country

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Abstract

As part of an investigation into the potential of alternative forage species and/or germplasm in New Zealand's hill country and the traits required for their success, two F2 populations, their original parents, and 34 cultivars of subterranean clover (*Trifolium subterranean*) were evaluated for hardseededness in Australia. The 34 cultivars were also screened for burr burial strength and flowering time. Microsatellite markers were also used to investigate their association with these traits. The polygenic nature of hardseededness was confirmed and markers associated with this trait were detected and traits for New Zealand's hill country were identified. Additionally, a core collection of 30 (two cultivars and 28 wild) biserrula (*Biserrula pelecinus*) accessions was screened for flowering time, growth habit and leaflet size. Also, a subset of five accessions of biserrula exhibited traits that are desirable for hill country, namely, mid-late flowering and adaptation to low-medium rainfall.

Keywords: biserrula, flowering time, hardseededness, hill country, subterranean clover

Key messages

- Molecular markers may help in selection for future soft-seeded subterranean clovers
- Low and high burr burial should be screened in subterranean clover core collection for a balance between persistence and economically and environmentally viable seed harvest
- Biserrula is a new candidate species for summer dry hill country and five accessions of this species have desirable traits for this region.

Introduction

There are many constraints to pasture productivity in the hill country of New Zealand, including the effect of slope on the infiltration of water into an often shallow soil profile, which reduces rainfall effectiveness and total plant available water. This results in plant moisture stress. This type of drought is not necessarily due to the lack of precipitation but rather the high rate of water flow and/or a high evaporation/transpiration ratio (Feldhake & Boyer 1990; Lambert & Roberts 1976; Maxwell *et al.* 2010; Radcliffe & Lefever 1981). At the forefront of solutions to this problem is the introduction of new species, or new germplasm of current species, that are more likely to tolerate this environment, as past introductions have had varying success (Barker *et al.* 1993; Charlton & Belgrave 1992; Nichols *et al.* 2016).

Subterranean clover (*Trifolium subterraneum*) (sub clover) is the best annual self-regenerating and predominantly self-fertile/inbred clover species in grass-based perennial pastures of New Zealand (Lucas *et al.* 2015) and has been recommended for New Zealand's moist and dry hill country (Charlton & Stewart 1999; Dodd *et al.* 1995; Suckling *et al.* 1983). Many traits have been investigated to increase productivity of sub clover in the hill country, with seed set and grazing-tolerant morphology shown to be highly desirable (Chapman & Williams 1990; Widdup & Pennell 2000). However, a subset of three sub clover traits are likely to be the main persistence traits in the hill country. The first is reduced hardseededness, or softer seeds, leading to stronger seedling regeneration. In the warm, temperate Australian environment there is a marked decline in hardseededness over summer so that by autumn it has fallen to a low level, allowing most seed to germinate (Collins *et al.* 1984). However, in cooler climatic conditions of New Zealand, this does not seem to be the case (Lucas *et al.* 2015; Smetham & Ying 1991), although Chapman and Williams (1990) argued that hard seed plays only a minor role in population regeneration in subterranean clover. They suggested re-establishment of the populations each year was almost solely dependent on seed inputs during the previous spring. The second trait is late flowering, resulting in a longer period of vegetative production in the field (Chapman & Williams 1990; Smetham & Jack 1995). The third trait is the degree of burr burial. This is an important trait as burial protects the seeds in dry conditions where the hook structures of peduncle contract and help to draw the burr into the ground

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(Nichols *et al.* 2013; Smetham 2003; Yates 1958). While high burr burial will be useful for farmers who want subterranean clover to regenerate year after year in the field, it is potentially a negative trait for seed growers because it impedes seed harvest.

Currently there are 45 registered cultivars of subterranean clover in Australia with a broad range of flowering time and hardseededness (Nichols *et al.* 2013). Microsatellite DNA markers have been used to discriminate 41 of these cultivars at a single seed level (Ghamkhar 2014). These markers have also been successfully used to investigate associations with important traits such as hardseedness, based on data from two F_2 (second generation) populations (Ghamkhar *et al.* 2012).

Historically, the most widely used cultivars in New Zealand are Mount Barker, Woogenellup, Tallarook and, less frequently, Riverina. However, an accurate trait and climate match study may result in better cultivar selection from the wider pool of available cultivars. Further, the core collection of subterranean clover germplasm (Ghamkhar *et al*. unpublished data) can be used as a important resource to find accessions with potential adaptation to New Zealand's hill country (Nichols *et al.* 2016).

Another promising annual inbreeding pasture legume for hill country is biserrula (*Biserrula pelecinus*). However, there are strict regulatory requirements that must be satisfied before introducing it into field experiments (Nichols *et al.* 2016). A core collection of 30 accessions of this species has been developed (Ghamkhar *et al.* 2013). This provides a broad diversity for discovering germplasm with potential adaptation to summer dry hill country environments.

The aim of the study reported here was to analyse genetic variation among cultivars and accessions of subterranean clover and biserrula, respectively, in the context of traits known to be of importance for New Zealand hill country. Selected attributes, which have been previously identified as being desirable in hill country are also explored in current cultivars/germplasm. These results will provide valuable information for future cultivar development for the targeted regions.

Methods

Plant material

Data were obtained from sub clover and biserrula material used by Faithfull (2008), Banik *et al.* (2013) and Ghamkhar *et al.* (2013), briefly described below.

Subterranean clover populations

Two F_2 populations, 92S05 and 92S80 (Table 1) (Ghamkhar *et al.* 2012), and their parents were sown in a glasshouse and transplanted into the field at Medina Research Station, Western Australia (32.23°S, 115.80°E) 6 weeks after sowing.

Seed preparation and screening in subterranean clover

Seeds were counted using a Contador seed counter (Pfeuffer GmbH, Kitzingen, Germany) into three replicates of 100 seeds. Screening of hardseededness was conducted in the laboratory using the method of Quinlivan (1961), which is designed to simulate the conditions of seed on the soil surface during summer in the Western Australian wheatbelt. Although the absolute amount of seed softening under this system is likely to be much higher than under New Zealand field conditions, other studies comparing field and laboratory seed softening (Norman *et al.* 2006) suggest the relative differences between varieties are likely to be valid in New Zealand. In brief, seeds were immersed in water and ice cube trays at 15°C for 48 hrs. Imbibed (soft) seeds were removed and the remaining hard seeds were placed in a cabinet with a diurnally fluctuating temperature range of 15/60ºC. Germination tests were conducted at weeks 8 and 16. Final hardseed percentages were calculated as per Equation 1: Final hardseed (%) = Hard seed remaining / (Initial seed – Initial soft seed)

Molecular markers and subterranean clover cultivars

Burrs of 34 cultivars (Table 2) grown under irrigated, well-fertilised conditions were collected from 1 m rows at South Perth, Western Australia (31.98° S, 115.86° E). DNA was extracted from fresh leaf samples at a three-leaf stage or older of 24 cultivars using a Nucleon PhytoPure Plant DNA Extraction Kit (GE Healthcare, Buckinghamshire, UK). A total of 25 SSR primers (Ghamkhar *et al.* 2012) were used to test their

Table 1 Characteristics of the F2 populations 92S05 and 92S80 and their parents.

Character	Population 92S05 (size: 214)		Population 92S80 (size 221)	
	Denmark	DGI007	Woogenellup	Daliak
Origin	Sardinian ecotype	Italian ecotype	Naturalised WA strain	Naturalised WA strain
Status	Cultivar	-	Cultivar	Cultivar
Flowering time (days)	142	86	130	98
Hardseededness (0-10)	2	8	1	7

discrimination power among the 24 cultivars. Each set of cultivars and primers were run through a PCR cycle as follows: 94°C for 5 minutes and then 35 cycles of 94°C for 50s, 60°C for 30s and 72°C for 50s.

Biserrula

Data for flowering time of 30 accessions of biserrula were obtained in the field and annual rainfall was recorded from the original collection site (Ghamkhar *et al.* 2013).

Table 2 List of 34 subterranean clover cultivars and their scores for hardseededness (HS, 1 (soft) – 10 (hard)), burr burial (BB, 1 (weak) – 9 (strong)), and flowering time (FT, in days measured in Perth from an early May sowing) (Faithful 2008; Nichols *et al.* 2013).

Cultivar	Subspecies	HS	BB	FT
Antas*	brachycalycinum	4	1	134
Bacchus Marsh*	subterraneum	1	2	132
Clare	brachycalycinum	2	1	130
Coolamon*	subterraneum	7	7	132
Daliak*	subterraneum	7	7	98
Dalkeith	subterraneum	9	9	97
Denmark	subterraneum	2	5	142
Dinninup*	subterraneum	7	7	114
Dwalganup*	subterraneum	7	7	83
Esperance	subterraneum	5	6	120
Geraldton	subterraneum	8	7	93
Gosse	yanninicum	4	5	126
Goulburn	subterraneum	6	5	143
Green Range*	subterraneum	4	4	128
Izmir	subterraneum	10	7	78
Junee	subterraneum	6	6	126
Karridale	subterraneum	2	6	139
Larisa	yanninicum	2	6	140
Leura	subterraneum	2	5	147
Losa*	subterraneum	5	8	97
Meteora	yanninicum	6	5	148
Mount Barker	subterraneum	1	3	137
Napier	yanninicum	6	6	140
Northam	subterraneum	7	9	78
Nuba	brachycalycinum	4	1	146
Nungarin*	subterraneum	10	6	77
Riverina	yanninicum	4	6	119
Rosedale	brachycalycinum	8	1	114
Seaton Park	subterraneum	6	7	110
Trikkala	yanninicum	2	6	112
Urana	subterraneum	10	7	104
Woogenellup	subterraneum	1	3	130
Yarloop*	yanninicum	2	6	110
York	subterraneum	9	5	110

*Cultivars not used in the molecular analysis

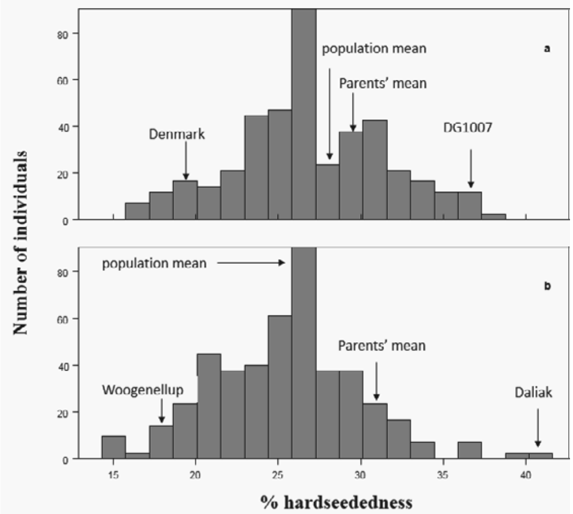

Figure 1 % hardseededness in the two populations of subterranean clover: a) population 92S05, b) population 92S80.

Other phenotypic data were obtained from the same accessions in the glasshouse (Banik *et al.* 2013) (Table 3). The geographical and agro-morphological traits of each accession were categorised as below. Annual rainfall: low: ≤ 350 mm, medium: >350 and <550 mm, high: ≥550 mm. Early flowering: ≤ 77 days, mid-flowering: >77 and <120 days, and late flowering: ≥120 days. Visual scoring was used for leaflet size and growth habit.

Data analysis

For the sub clover segregating populations, GENSTAT v. 5.1 statistical package (Rothamsted Experimental Station, UK) was used to generate trait BLUPs, adjusted means for each line and parent, and to compare BLUPs. Histograms were drawn using 'R' (R Development Core Team) and 95% confidence intervals. For the sub clover cultivars and biserrula core collection, principal components analysis (PCA) was conducted using NTSYSpc v. 2.2 (Exeter Software, Setauket, NY) to investigate correlations between traits/variables. Principal coordinate analysis (PCoA) was used to project hardseededness into the molecular data using NTSYSpc v. 2.2.

Results

Subterranean clover

Both sub clover F_2 populations showed a broad range of hardseededness. Mean hardseededness of the population 92S05 (26.7%) was not significantly different (P<0.67) to the mean of the two parents (27.9%). However, in the 92S80 population, the population mean of 25.4% was significantly lower (P<0.05) than the mean of the two parents (29.4%) (Figure 1). This indicates a tendency towards less hardseededness in population 92S80 than in population 92S05.

Hardseededness data was also obtained for all 34 cultivars (Figure 2). The results of PCA enabled us to group 23 cultivars into five main categories and the other 11 as non-categorised, based on hardseededness, flowering time and burr burial (Figure 3). The categories were as follows: a late flowering group of ssp. *brachycalycinum* with weak seed burial; a late flowering group from Sardinia (although Mt Barker is a naturalized strain from South Australia, but its pre-Australian origin is unknown); a group of soft seeded and late flowering cultivars; a group of early flowering West Australian cultivars with strong seed burial; and a group of belonging to ssp. *yanninicum*.

Results from PCoA suggested that four molecular markers may be associated with hardseededness based on their reasonable association with hardseeded cultivars (Figure 4).

Biserrula

A PCA analysis of five variables in biserrula identified five distinct groups in its core collection. These groups were categorised as: accessions with small leaves, prostrate habit, and mid flowering time; accessions from medium to high rainfall regions; early flowering accessions; accessions with large leaves, late flowering time and Moroccan origin, and accessions from low to medium rainfall regions with mid to late flowering time (Fig. 5). No significant correlation was found among the studied traits.

Table 3 Country of origin and agro-morphological traits of accessions of the biserrula core collection (Banik *et al.* 2013; Ghamkhar *et al.* 2013).

Number	Name	Origin	Annual Rainfall (mm)	FT* (days)	Leaflet size	Growth habit
1	cv. Casbah	Morocco	450	105	Large	Prostrate
2	cv. Mauro	Italy	450	116	Medium	Prostrate
3	2004ERI1PEL	Eritrea	450	56	Medium	Semi-prostrate
4	2004ERI37PEL	Eritrea	450	49	Small	Semi-prostrate
5	2004ERI38PEL	Eritrea	550	54	Medium	Semi-prostrate
6	2004ERI56PEL	Eritrea	600	52	Medium	Semi-prostrate
7	91FRA4PEL	France	650	125	Large	Semi-prostrate
8	139026	Greece	450	117	Medium	Prostrate
9	139049.2	Greece	650	98	Medium	Prostrate
10	GEH71PEL	Greece	450	>77	Large	Prostrate
11	GEH77PEL	Greece	450	>77	Large	Prostrate
12	139058	Greece	800	124	Large	Prostrate
13	2005GRC77PEL	Greece	450	117	Large	Prostrate
14	2006ISR20PEL	Israel	700	120	Medium	Prostrate
15	143267aA	Italy	600	119	Medium	Prostrate
16	143267bA	Italy	600	119	Large	Prostrate
17	143464A	Italy	400	106	Large	Prostrate
18	143467	Italy	450	117	Medium	Prostrate
19	143469A	Italy	550	122	Large	Prostrate
20	143474	Italy	300	118	Small	Prostrate
21	93ITA45PELA	Italy	550	121	Small	Prostrate
22	138972A	Morocco	400	117	Large	Prostrate
23	139362	Morocco	250	111	Medium	Prostrate
24	139363	Morocco	250	123	Medium	Prostrate
25	2006MAR22PEL	Morocco	550	111	Large	Semi-prostrate
26	2006MAR29PEL	Morocco	250	115	Medium	Prostrate
27	143415A	Spain	350	116	Large	Prostrate
28	2004ESP19PEL	Spain	150	82	Large	Semi-prostrate
29	2004ESP39PEL	Spain	400	130	Large	Prostrate
30	2004ESP64PEL	Spain	450	146	Medium	Prostrate

*Flowering time

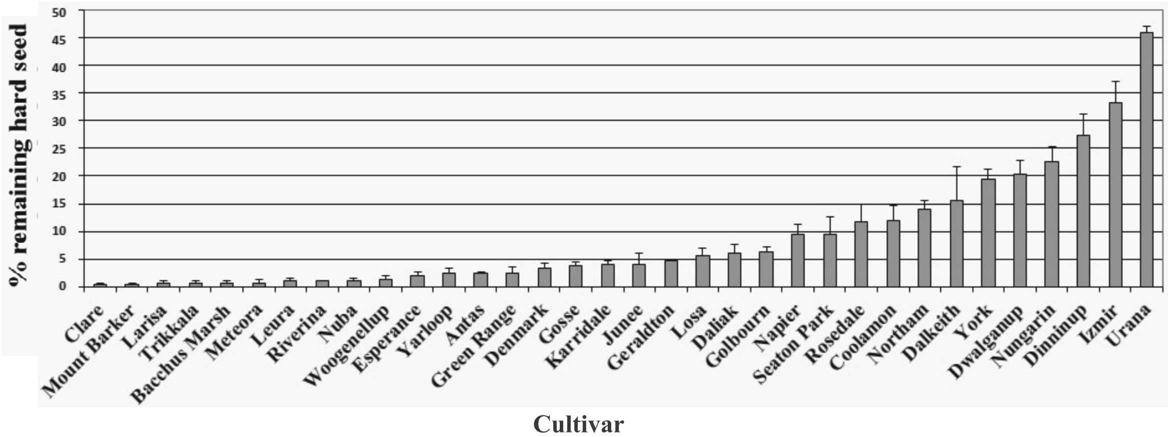

Figure 2 % hard seed remaining after 16 weeks in subterranean clover cultivars.

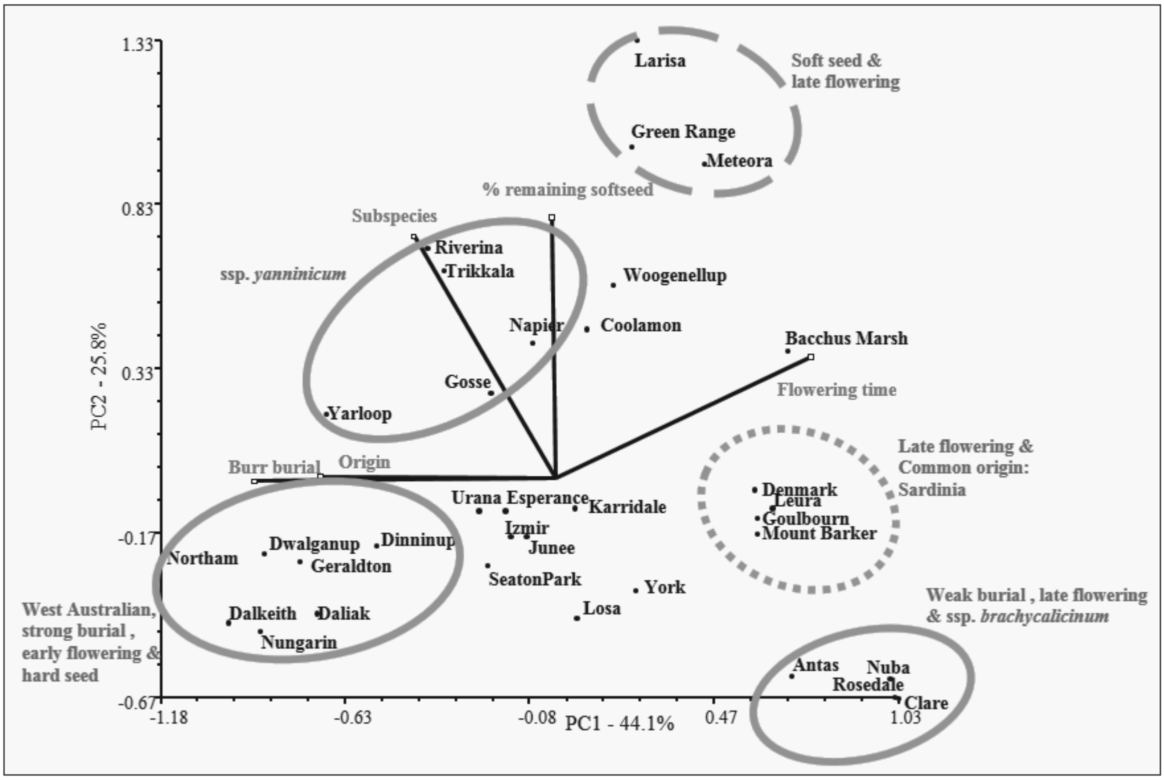

Figure 3 Principal components analysis of agronomic data and categorisation of subterranean clover cultivars with percentage of variation explained by each principal component. The dashed ellipse highlights cultivars with soft seeds and late flowering features. The dotted ellipse highlights cultivars with late flowering and Sardinian origin. Traits of these two groups might potentially be useful in hill country in the future.

Discussion

Subterranean clover

In subterranean clover, high levels of hardseededness impede optimal growth in the summer dry hill country due to poor seedling germination. Hardseededness appears to be a polygenic trait based on relatively normal distribution of data along with evident grouping. Salisbury & Halloran (1983) and Ghamkhar *et al.* (2012), also showed that hardseededness to be a polygenic trait. Further investigation into the data set showed evidence of transgressive segregation (formation of extreme phenotypes) in both populations, by the number of lines showing lower % hardseededness than the more softseeded parent (cv Denmark in 92S05 and cv Woogenellup in 92S80). Transgressive segregation for hardseededness was also found by Rieseberg *et al.* (1999).Transgressive

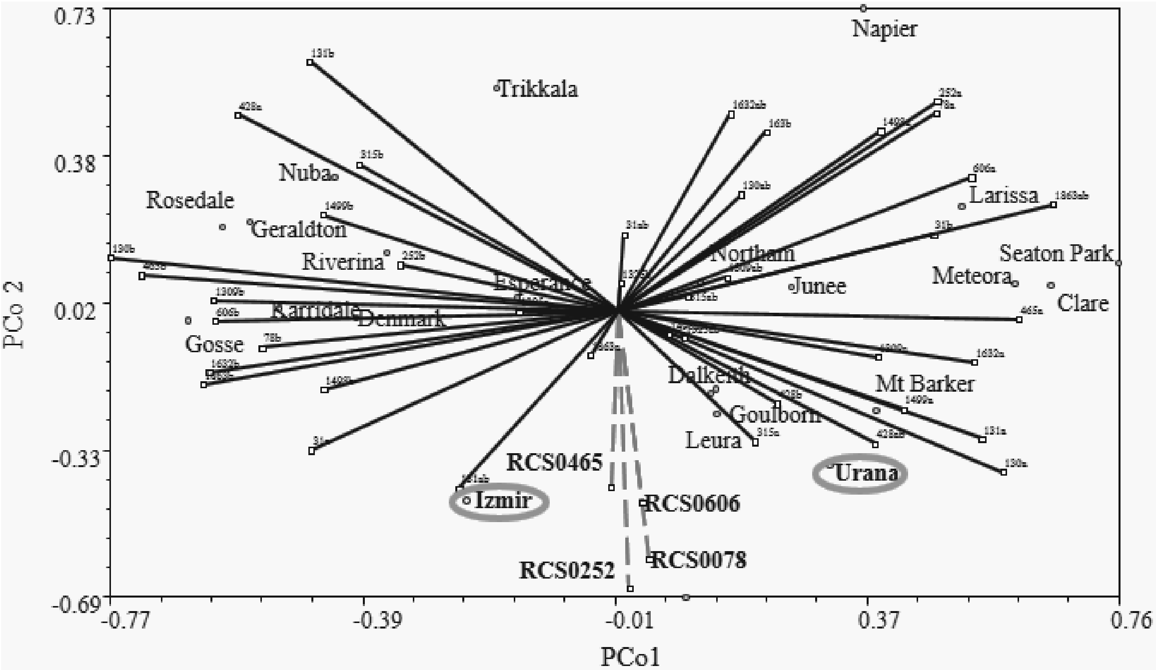

Figure 4 Principal coordinate analysis of molecular markers and cultivars. Cultivars Urana and Izmir are the most hardseeded cultivars among the 34 cultivars studied.

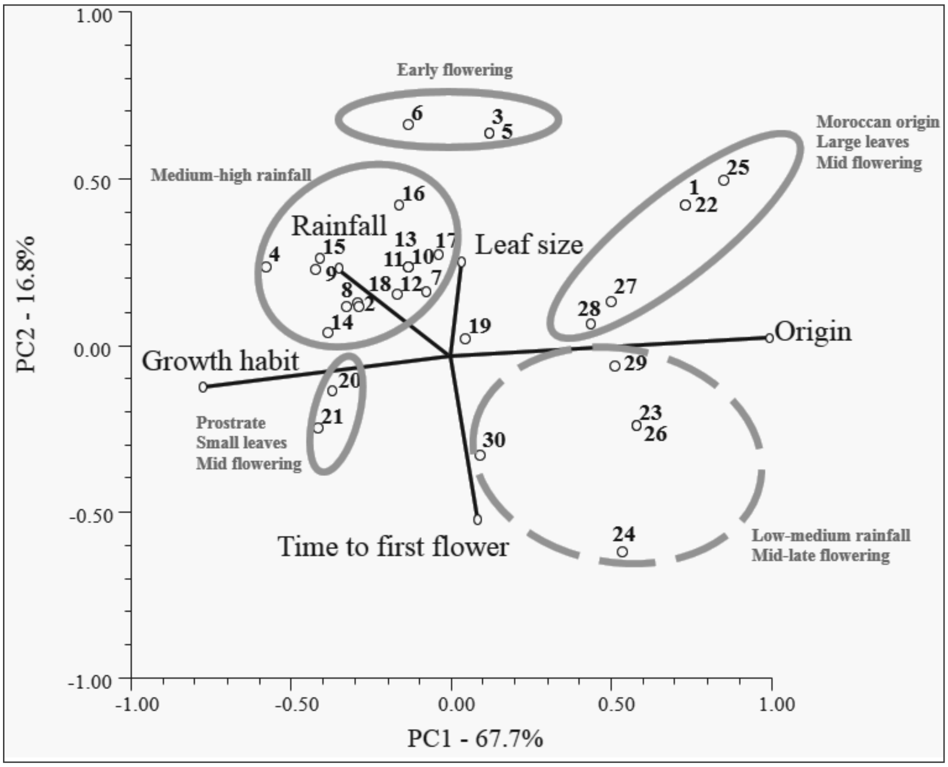

Figure 5 Principal components analysis of agronomic and ecogeographic data and categorisation of accessions with percentage of variation explained by each principal component. The dashed ellipse suggests cultivars most suitable for New Zealand hill country summer dry climate.

phenotypes in segregating hybrid populations reported here may have been caused by epistatic effects or over-dominance. If significant, these heterotic effects could be used in the development of hybrids. The significant difference between the parent and population means in population 92S80 suggests that softseededness is a dominant trait, supporting Kilen & Hartwig (1978) finding. Dominance is difficult to measure in a polygenic trait, however, this significant variation indicates that there may be major genes having a dominant effect, influencing the distribution of the population mean. Estimating the magnitude of additive genetic variation for hardseededness in both populations will provide a basis for future design of breeding strategies.

Currently, two main cultivars, Mount Barker and Tallarook, are widely naturalised in New Zealand's hill country (Lucas *et al.* 2015; Macfarlane & Sheath 1984; Smetham 2003). These cultivars were introduced decades ago and in the case of Mount Barker, up to 90 years ago (Saxby 1956). However, in Australia, Mt Barker has poor persistence in summer dry areas, while Tallarook is susceptible to disease (subterranean clover stunt virus (SCSV)) (Nichols *et al.* 2013) and has high formononetin content, which can cause ewe infertility in clover-dominant swards. Therefore, in Australia, Mount Barker has been replaced by cultivars Leura and Denmark (and more recently by Rosabrook), and Tallarook by Leura. Woogenellup and Riverina are two other cultivars that more recently have been tried in the hill country, but Riverina is much earlier flowering than the other three and also belongs to subspecies *yanninicum*, with no tolerance to drought.

Any future cultivar for New Zealand's winter wet hill country must have traits similar to the common traits of the group of Larisa, Green Range and Meteora in Figure 3; i.e. softseededness and late flowering. However, this is not a cultivar recommendation and by no means suggests that these cultivars are the best for New Zealand's hill country in their current form. The second group of interest may be the late flowering cultivars from Sardinia comprising Denmark, Goulburn, Leura and Mount Barker, although the origin of Mount Barker is not known. These cultivars have an average to strong seed burial and Goulburn is a hardseeded cultivar. Denmark and Leura were also recommended by Widdup & Pennell (2000) for their late-flowering, small leaves, dense crowns and tolerance of moderate-high grazing pressure traits. The third group, comprises cultivars Antas, Nuba, Rosedale and Clare all belonging to subspecies *Brachycalycinum* and having a weaker burr burial, which might be of potential interest to seed companies, and late flowering time. Further research into this group might help in developing cultivars with easier seed harvest while having minor effect on persistence. Some newer cultivars not studied here should also be tested in New Zealand.

Flowering time in subterranean clover is highly heritable and also polygenically determined, with additive effects accounting for most of the variance (Davern *et al.* 1957; Salisbury *et al.* 1987). Dominance for earliness was observed by Salisbury *et al.* (1987), while transgressive segregation towards earlier flowering was reported by Ghamkhar *et al.* (2012).

When a breeding programme is developed to address the regional needs of the hill country for specific cultivar(s), the molecular markers identified in this study and also marker loci in the genomic regions identified by Ghamkhar *et al.* (2012) will prove useful. However, it will require further studies to confirm the association of these markers to hardseededness and/or the percentage of remaining hard seeds. Such markers would be of value for screening progenies of crosses for hardseededness to facilitate time and cost efficiencies in cultivar development.

The genetics of burr burial is not well known and is still an unexplored area of research. Support from seed companies to find a balance between easier seed harvest and quality seed production and persistence will initiate genetic and phenotyping studies on the proposed candidate groups and the core collection of subterranean clover for future cultivar development. However, as the grazing pressure in typical hill country at flowering removes runners and aerial seed, a change in grazing management will be needed to allow non-burying types to set seed. Further studies in the field in New Zealand's diverse hill country will be needed to confirm the suggestions made in this study.

Biserrula

Initial experiments to identify the most promising lines of biserrula for future cultivar development in New Zealand will need to proceed in contained environments due to quarantine issues. It is not known if there are any soft-seeded types of biserrula. The effect of hardseededness needs to be studied to determine the fit of biserrula in New Zealand. These studies must focus on the group from mid-medium rainfall regions with mid-late flowering time comprising five accessions: 139362, 139363, 2006MAR29PEL, 2004ESP39PEL and 2004ESP64PEL (Figure 5). As in sub clover, there is a second group of interest in biserrula which comprises five accessions from Morocco with large leaves and mid flowering time. This late flowering group will add the large leaf trait to future cultivars.

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