Natural selection for flowering time in a diverse subterranean clover population sown at five different rainfall sites in southern Australia

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Abstract
A bulk hybrid subterranean clover (*Trifolium subterraneum* L.) population, derived from F_2 seed from 600 crosses, was sown at five locations in southern Australia differing in mean annual rainfall (Wongan Hills, Esperance and Wundowie in Western Australia, Wagga Wagga in New South Wales and Hamilton in Victoria). Seeds were collected from each population after seven years of regeneration under local pasture management conditions. Random samples of 250 seeds from each population and the original sown mixture (OM) were grown as spaced plants in Perth. Flowering time in Perth was highly responsive to selection site location. Mean flowering times were 105.9 ±0.8 for Wongan Hills, 116.4 ±0.6 for Wundowie, 118.5 ±0.9 for Wagga Wagga, 135.2 ±1.0 for Esperance and 145.0 ±1.0 for Hamilton, in comparison with 124.9 ±1.1 for the OM. Flowering time in Perth was significantly related ($p < 0.001$) to mean annual rainfall ($r^2 = 0.95$), mean April-November rainfall ($r^2 = 0.94$) and mean annual maximum temperature ($r^2 = 0.89$) of the selection sites during 1998-2004 and to latitude ($r^2 = 0.72$). The findings demonstrate the use of bulk-hybrid populations as a low-cost breeding method for adaptation of subterranean clover to different environments.

Keywords
Trifolium subterraneum, genetic diversity, bulk-hybrid populations, flowering, plant breeding, adaptation

Introduction
Subterranean clover (*Trifolium subterraneum* L.) is a self-pollinated annual legume native to the Mediterranean basin and surrounding areas (Nichols et al. 2013). It is well adapted to the Mediterranean-type climate of southern Australia, where it has been sown over an estimated 29 million ha (Nichols et al. 2013). Subterranean clover is reliant on self-regeneration for persistence and has two key features for its widespread use: (i) its tolerance of heavy grazing, largely attributable to its prostrate growth habit and its ability to protect against seed predation by burial of its seed-bearing burrs; and (ii) a range of cultivars which differ in flowering time, enabling it to be grown in environments with mean annual rainfall from 300-1200 mm
(Nichols et al. 2013).

A breeding program in Western Australia (WA) commenced for subterranean clover in 1949 and active crossing and selection among progeny continued until 1998. In order to continue a breeding program operating at a low level, the bulk-hybrid population methodology first described by Suneson (1956) was utilised, whereby natural selection was allowed to act on genetically mixed populations to select adapted genotypes. Nichols and Cocks (2006) and Nichols et al. (2009) demonstrated this as an effective method for subterranean clover. They examined changes over 16 years in a bulk-hybrid population, consisting of F_2 seed from 253 crosses sown at a low and high rainfall site, and observed changes in population means in 20 characters at one or both sites. Markedly different populations evolved, with much of this change occurring within the first three years. In particular, flowering time was highly responsive to environment, with rapid selection for early flowering at the low rainfall site and later flowering at the high rainfall site.

This paper examines changes in flowering time after seven years of natural selection under local pasture management conditions in a highly diverse bulk-hybrid subterranean clover population sown at five sites across southern Australia differing in mean annual rainfall. Other traits are also being examined to determine the commercial merit of selected plants and will be reported elsewhere.

Methods
Selection sites sown to the bulk-hybrid population
A bulk hybrid population, consisting of a mixture of F_2 seeds from 600 bi-parental crosses of subterranean clover was sown in May 1998 at five sites across southern Australia differing in mean annual rainfall: Wongan Hills, Wundowie and Esperance in WA, Wagga Wagga in New South Wales (NSW) and Hamilton in Victoria (Vic). Plot size was 12 m x 5 m and sowing rate was 20 kg/ha. The populations at each site were

allowed to set seed and regenerate naturally for seven years under local pasture management practices with regular grazing. Random samples of seed were collected from each site, following senescence, in January 2005. These were threshed, sealed in aluminium foil packets and maintained at 4°C. Seeds from the original bulk-hybrid mixed population (OM) were stored in the same way. Details of selection sites are given in Table 1, along with rainfall and temperature data during the selection period (1998-2004) from the nearest Bureau of Meteorology weather stations.

Table 1. Location of the five selection sites sown to a bulk-hybrid population of subterranean clover, with mean rainfall and temperature data during the 1998-2004 selection period.

	Wongan Hills (WA)	Wundowie (WA)	Esperance (WA)	Wagga Wagga (NSW)	Hamilton (Vic)
Latitude (°S)	30.8433	31.7978	33.8258	35.0531	37.8203
Longitude (°E)	116.7283	116.3494	122.4803	147.3600	142.0686
Mean annual rainfall (mm)	397.7	460.6	633.9	531.0	658.3
Mean April-Nov rainfall (mm)	315.0	411.4	487.4	383.9	512.5
Mean maximum temperature (°C)	25.8	25.5	21.8	22.8	19.2
Mean minimum temperature (°C)	11.5	10.5	12.1	9.2	7.6

Evaluation of evolved populations

In 2018, random samples of 250 seeds from each population and the OM were grown as spaced plants in Perth at the University of Western Australia Field Station (31.9511° S, 115.7928° E). Seeds were scarified as described in Nichols et al. (2009) and sown on 30 April 2018 by germinating in Petri dishes containing a single Whatman No 1 filter paper moistened with 3.5 mL of tap water. These dishes were placed in a germination cabinet at 15°C. At 2-3 days after sowing (DAS), two germinated seedlings of each genotype were planted into hydrated Jiffy-9 peat pots (Jiffy Products Ltd, Norway) in a glasshouse. *Rhizobium leguminosarum* bv. *trifolii* strain WSM1325 (Nodulaid) was applied by watering can (40 mL of inoculum to 9 L of water). Peat pots were watered daily and soluble fertiliser (Thrive, Yates Australia) at a rate of 0.5 g/L was applied weekly. Seedlings were randomly thinned to a single healthy seedling per Jiffy pot at 21 DAS and transferred to outside benches for acclimatisation at 28 DAS. At 35 DAS seedlings were transplanted into the field.

The evaluation site consisted of a loam-dressed sand with a pH (H_2O) of 6.5 in the top 20 cm. The field was rotary hoed and tilled in March. Glyphosate (540 g a.i./L) at 2 L/ha was applied in early April and Basta® (glufosinate-ammonium; 200 g a.i./L) at a rate of 1.5 L/ha was applied one week prior to transplanting. Single superphosphate with potash (6.8% P, 12.4% K and 8.3% S) was drilled in at a rate of 300 kg/ha in early May. The land was smudged to make it level and 12 polyethylene plastic strips were laid (100 m long and 1 m wide), separated by 1.5 m of bare soil. Seedlings were transplanted into 10 cm diameter holes cut into the plastic. A randomised block design with 10 replicates was used. Each plot consisted of 25 plants, spaced 0.8 m apart along the plastic strips. The plastic strips were removed from the field at 90 DAS, when the earliest flowering genotypes had commenced flowering, to allow burr burial. Supplementary overhead irrigation was supplied as required to ensure plants did not suffer moisture stress. Irrigation ceased on November 20 (204 DAS) after all plants had flowered. Plots were hand-weeded.

Measurements

Flowering time was recorded on all plants and determined as the number of days from sowing to the date of the first open flower. Plants were checked for flowering every 3-4 days.

Results

Mean flowering times were 105.9 ±0.8 for Wongan Hills, 116.4 ±0.6 for Wundowie, 118.5 ±0.9 for Wagga Wagga, 135.2 ±1.0 for Esperance and 145.0 ±1.0 for Hamilton, in comparison with 124.9 ±1.1 for the OM.

Flowering time in Perth was significantly related (p <0.001) to mean annual rainfall ($r^2 = 0.95$), mean April-November rainfall ($r^2 = 0.94$) and mean annual maximum temperature ($r^2 = 0.89$) of the selection sites during 1998-2004 and to the latitude of the selection sites ($r^2 = 0.72$) (Figure 1). Interestingly, flowering time was not significantly related to mean annual minimum temperature ($r^2 = 0.22$) of sites (data not shown).

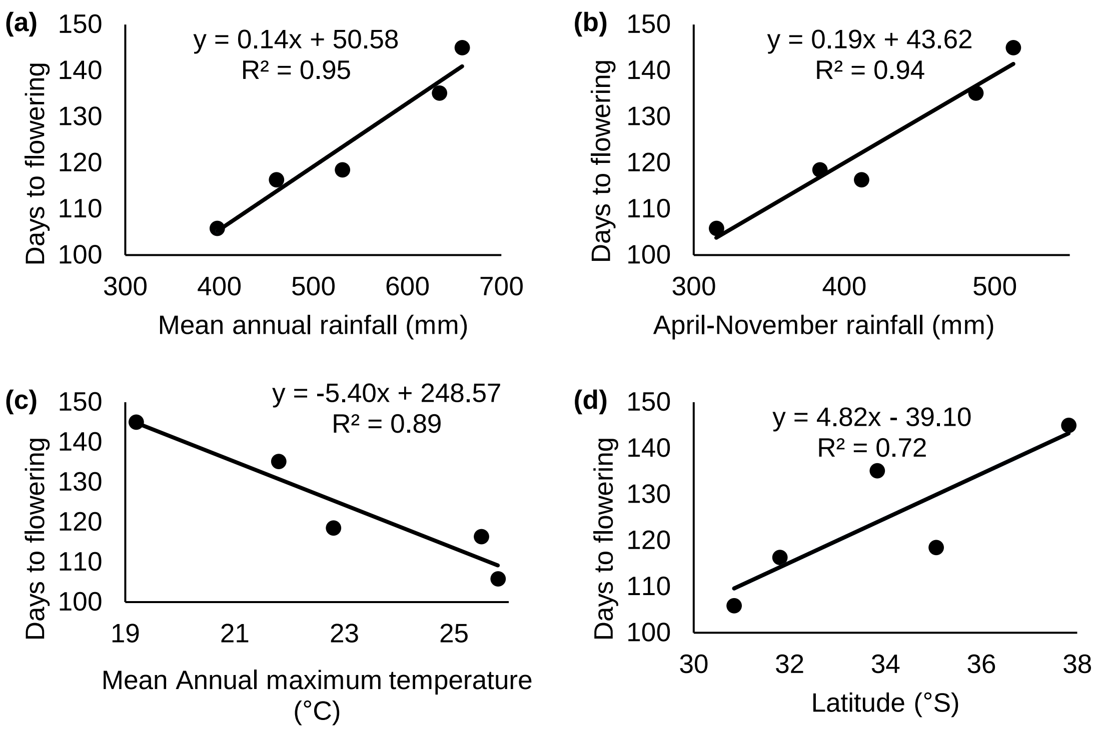

Figure 1. Regressions of flowering times of subterranean clover at Perth in 2018 from 250 random seeds collected at Wongan Hills, Wundowie, Esperance, Wagga Wagga and Hamilton against: (a) mean annual rainfall from 1998-2004; (b) mean April-November rainfall from 1998-2004; (c) mean annual maximum temperature from 1998-2004; and (d) latitude of sites. Plants were derived from a bulk-hybrid F_2 population from 600 crosses, sown in 1998 and allowed to self-regenerate for seven years under grazing. Regression equations and R^2 values are also shown.

Conclusion

This study demonstrated that from one highly variable population, different populations, in terms of flowering time, evolved at the five sites. Furthermore, mean flowering times were related to the amount of rainfall, particularly in the May-November growing season, and to temperature at the sites during the selection process. Thus, early flowering plants were favoured at Wongan Hills, which had the lowest rainfall and was the warmest and most northerly site. By contrast, later flowering plants were favoured at Hamilton, which had the highest rainfall and was the coolest and most southerly site. This confirms previous results of Nichols and Cocks (2006) and Nichols et al. (2009) for a bulk-hybrid population at two sites, which showed strong selection pressure for early flowering at a low rainfall site, while later flowering was favoured at a high rainfall site. It is apparent that genetic segregation in the early generations would have rapidly eliminated the most poorly adapted plants at each site. Thus, late flowering genotypes at the lower rainfall sites would have set little to no seed, while early flowering genotypes at higher rainfall sites would have been outcompeted for light in spring by later flowering ones. Favourable alleles and gene combinations would have accumulated across the genome and been passed on to successive generations. After several generations of selfing, competition would have mainly occurred between homozygous genotypes.

This breeding method was utilised to enable subterranean clover breeding to continue at a low level while funding priorities were directed elsewhere. This followed the proposal of Nichols and Cocks (2006) to use bulk hybrid populations as a low-cost method for breeding subterranean clover. Natural, rather than deliberate, selection acts to select adapted genotypes. The success of the method hinges on parents containing genes for desirable characters, sites being representative of target environments and trial management being representative of typical farm practice. Its main advantages are that it is cheaper to operate and takes no longer than traditional plant breeding methods to produce homozygous lines and requires minimal land with few inputs following sowing. It also allows selection for regional adaptation at sites distant from a main breeding centre. The main disadvantage is that the breeder has limited control over selection direction. Selection may also favour characters relating to survival rather than productivity *per se*.

The genotypes derived from the bulk-hybrid populations are being evaluated for other traits to determine their commercial merit.

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