RESEARCH PAPER
https://doi.org/10.1071/CP21226

CROP & PASTURE SCIENCE

Diversity for morphological traits, flowering time and leaf isoflavone content among ecotypes of *Trifolium subterraneum* L. subsp. *yanninicum* and their relationships with site of origin

Gereltsetseg Enkhbat[A,B,*], Phillip G. H. Nichols[A], Kevin J. Foster[A], Megan H. Ryan[A], Yoshiaki Inukai[B] and William Erskine[A]

For full list of author affiliations and declarations see end of paper

***Correspondence to:**
Gereltsetseg Enkhbat
UWA School of Agriculture and Environment and Institute of Agriculture, The University of Western Australia, 35 Stirling Highway, Crawley, WA 6009, Australia
Email:
gereltsetseg.enkhbat@research.uwa.edu.au

Handling Editor:
Kevin Reed

Received: 29 March 2021
Accepted: 13 July 2021
Published: 1 December 2021

Cite this:
Enkhbat G *et al.* (2021)
Crop & Pasture Science, **72**(12), 1022–1033.
doi:10.1071/CP21226

ABSTRACT

Trifolium subterraneum L. subsp. *yanninicum* is a pasture legume that is widely grown in medium and high rainfall areas of southern Australia and shows waterlogging tolerance. This study investigated diversity within subsp. *yanninicum* corresponding to eco-geographic variables, which may help to identify adapted parents with new traits for genetic improvement. Diversity for 10 morphological traits, flowering time and leaf isoflavone content was investigated using 108 ecotypes derived from wild Mediterranean populations and 10 cultivars, grown as spaced plants. Among the ecotypes, the range of flowering time was 94–149 days after sowing, and contents of formononetin, genistein and biochanin A were 0.05–1.38%, 0.73–2.33% and 0.15–2.10% of dry matter, respectively. Leaf markings also varied considerably. Leaf size and petiole length were correlated at each growth stage. Later flowering genotypes had larger leaves, longer petioles, longer internodes and thicker stems at flowering, but smaller leaves and shorter petioles at both 63 and 88 days after sowing. Contents of genistein and biochanin A were unrelated, but both were negatively associated with formononetin. Flowering time had a weak positive influence on genistein and biochanin A, but a weak negative influence on formononetin. All traits among the ecotypes (except stem diameter and leaf mark crescent size) were significantly correlated with at least one of 22 eco-geographic variables from their collection sites. Precipitation and altitude were more influential than temperature. The study found sufficient diversity to broaden the narrow genetic base of current subsp. *yanninicum* cultivars; however, other agronomically important traits also need to be considered. Further diversity may result from targeted collection, particularly in areas not represented in annual legume genebanks.

Keywords: eco-geographic variables, genetic resources, genotypic diversity, leaf marks, morphological traits, oestrogenic compounds, pasture legume, subterranean clover.

Introduction

Subterranean clover (*Trifolium subterraneum* L.), native to the Mediterranean Basin and surrounding regions, is the most widespread pasture legume in southern Australia, where it has been sown over 29 Mha (Nichols *et al.* 2013; Ghamkhar *et al.* 2015). It is also increasingly sown in other parts of the world with Mediterranean-type climates (Porqueddu *et al.* 2016). A range of cultivars with diverse maturities enables subterranean clover to be grown successfully in different rainfall environments and soil types (Nichols *et al.* 1996; Nichols 2013). Also key to its extensive adoption are attributes that enable it to be simply managed by farmers for high production and persistence. These include: tolerance of heavy grazing due to a prostrate growth habit and ability to bury seed-bearing burrs; ability to self-regenerate after long, dry summers; tolerance to many herbicides; and farmer experience from historical usage (Nichols *et al.* 2013).

Subterranean clover comprises three subspecies, which Katznelson and Morley (1965) classify as: subsp. *subterraneum*, subsp. *brachycalycinum*, and subsp. *yanninicum*. Of these, subsp. *yanninicum*, comprising 10 of the 53 subterranean clover cultivars, is broadly used in

medium and high annual rainfall (450–1200 mm) areas (Nichols *et al.* 2013; PBR 2021), typically in paddocks with poor drainage. Several agronomic and ecological studies show greater persistence of subsp. *yanninicum* than of the other two subspecies on soils prone to waterlogging (Reed *et al.* 1985; Craig 1992; Dear *et al.* 2003). Glasshouse studies confirm its greater waterlogging tolerance (Marshall and Millington 1967; Francis and Devitt 1969; Devitt and Francis 1972; Francis *et al.* 1974; Gibberd and Cocks 1997; Enkhbat *et al.* 2021). Current cultivars of subsp. *yanninicum* comprise a narrow genetic base (Nichols *et al.* 2013), and the diversity within the subspecies has been little studied (Ghamkhar *et al.* 2015). Hence, an understanding of the diversity within subsp. *yanninicum* could lead to a broadening of the genetic base for plant breeding and thereby enable desirable traits from other ecotypes to be utilised.

Diversity for several subterranean clover growth traits have been examined (Piano 1984; Piano *et al.* 1993, 1996; Pecetti and Piano 1997, 2002; Nichols *et al.* 2009; Abdi *et al.* 2020). Flowering time is highly responsive to environment and is considered the most important trait for adaptation of annual legumes (Nichols *et al.* 2007, 2009, 2012; Abdi *et al.* 2020). Petiole length is important for light interception, and large leaves are crucial for biomass accumulation because they increase light capture (Davidson and Donald 1958; Black 1960; Gladstones 1967; Nichols *et al.* 2009, 2013). Long internodes allow growth farther away from the mother plant crown and increased competition and are important particularly when the growing season is longer (Cocks 1992; Nichols *et al.* 2009).

Genetic variation also exists for the isoflavone compounds formononetin, genistein and biochanin A in fresh leaves of subterranean clover (Francis and Millington 1965; Morley and Francis 1968; Nichols *et al.* 2013; Abdi *et al.* 2020). Of these, formononetin is converted to highly oestrogenic compounds in the rumen, causing infertility in ewes (Bennetts *et al.* 1946; Millington *et al.* 1964; Hungerford 1975; Brightling 2006). Collins and Cox (1984) and Davies (1986) suggest that formononetin levels <0.3% on a leaf dry weight basis are 'safe' for sheep reproduction. Breeding programs have aimed to select cultivars with ≤0.20% of leaf dry matter (Nichols *et al.* 2013). Highly heritable morphological traits such as leaf marks are also useful characters for distinguishing subterranean clover genotypes for seed certification (Gladstones 1967; Tan and Collins 1987; Nichols *et al.* 1996; Abdi *et al.* 2020).

Germplasm collected from different sites within the native habitat of subterranean clover provides a source of genetic diversity (Ghamkhar *et al.* 2007, 2015) that can be evaluated for morphological characteristics (Tucak *et al.* 2009). Phenotypic traits can be used to investigate adaptation characteristics by relating them to climatic/eco-geographic variables (precipitation, temperature, latitude, etc.) from their sites of origin (Erskine *et al.* 1989; Piano *et al.* 1996; Mousavi-Derazmahalleh *et al.* 2018; Abdi *et al.* 2020). Understanding phenological adaptations corresponding to eco-geographic variables of collection sites is vital for: (*i*) identifying the distribution and adaptation of genotypes; (*ii*) guiding future germplasm collections and identifying diversity hotspots; and (*iii*) enhancing knowledge on how existing variation is linked with eco-geographic site-of-origin variables (Erskine *et al.* 1989; Hill 1996; Piano *et al.* 1996; Pecetti and Piano 2002; Rosso and Pagano 2005; Ghamkhar *et al.* 2007; Mousavi-Derazmahalleh *et al.* 2018; Abdi *et al.* 2020).

This study investigated diversity of morphological traits, flowering time and leaf isoflavone content corresponding to eco-geographic variables, in order to improve knowledge of diversity among subsp. *yanninicum*. The study tested two hypotheses: (i) variation for morphological traits, flowering time and leaf isoflavone content exists within subsp. *yanninicum*; and (*ii*) these variations are related to eco-geographic variables at the site of origin. These results, and those from other agronomic and physiological studies, will be used to identify adapted parents with new traits for genetic improvement of subsp. *yanninicum* to meet the future needs of farmers.

Materials and methods

Plant materials

Germplasm (seeds) of 154 subterranean clover genotypes from the Australian genetic resource collection of purported subsp. *yanninicum* was obtained from the Australian Pastures Genebank (APG), operated by the South Australian Research and Development Institute. These genotypes consisted of material deemed by the APG to be of sufficient quantity and viability to be made available for research; a further 28 genotypes were unavailable. Genotypes obtained comprised 144 ecotypes, selected as individual genotypes on the basis of morphological uniformity; and 10 commercial cultivars: Yarloop, Larisa, Meteora, Trikkala, Gosse, Riverina, Napier, Monti, Rouse and Yanco. Among the ecotypes, 142 were collected from their native habitats in the Mediterranean Basin, and two (Neuchatel and Yabba North) are naturalised strains from Australia. Of the cultivars, Yarloop is a naturalised strain from the Yarloop district of Western Australia (WA), first commercialised in 1947 (Oram 1989; Nichols *et al.* 1996, 2013), and Meteora (CPI 03927YA) and Larisa (CPI 039313Y) are ecotypes collected in Greece. The other cultivars are all derived from cross-breeding and have Larisa and/or Meteora in their parentage (Nichols *et al.* 2013; PBR 2021). Of the two naturalised strains, Neuchatel is an early flowering mutant of cv. Yarloop from the Waroona district of WA (Gladstones and Collins 1984) and is also an ancestor of all the crossbred cultivars (Nichols *et al.* 2013; PBR 2021), whereas Yabba North was collected from Victoria, Australia (Aitken and Drake 1941).

The subspecies identity of the 144 purported subsp. *yanninicum* ecotypes was determined during the growing season, on the basis of distinguishing morphological features of the three subterranean clover subspecies described by Katznelson and Morley (1965) and Nichols *et al.* (2013). Genotypes were classified as subsp. *yanninicum* if they had all of the following features: corolla length similar to calyx tubes; strong positive geotropism with active burr burial; burrs with few whorls of sterile calyces that covered all or most of the pods; leathery and transversely wrinkled pods; and round to slightly ovoid seeds. All genotypes classified as subsp. *yanninicum* also had glabrous or weakly pubescent stems, petioles and leaf upper surfaces, and the majority had cream–amber-coloured seeds. On the basis of this assessment, it was determined that 36 purported subsp. *yanninicum* ecotypes from the APG had been misclassified. Hence, in total, 118 genotypes of subsp. *yanninicum* (108 ecotypes and 10 cultivars) were examined in this study. Among these, four ecotypes had black-coloured seeds; all other genotypes had the usual amber- or cream-coloured seeds. These genotypes are listed in Supplementary data Table S1.

Geographical and climatic data of collection sites

Geographic coordinates were used to determine accurate locations of collection sites. Associated passport data (latitude, longitude and altitude) recorded by the plant collectors were obtained from the former Australian Trifolium Genetic Resource Centre and the Germplasm Resources Information Network (GRIN) global website (https://apg.pir.sa.gov.au/gringlobal), using numbers allocated by the APG for genotype identities (Supplementary data Table S1). Passport data were available for 90 genotypes of subsp. *yanninicum* and the geographic distribution of their collection sites is shown in Fig. S1.

Standardised temperature and precipitation (BIOCLIM) variables were downloaded for collection sites of known latitude and longitude from the WorldClim (ver. 2) website (http://www.worldclim.org) at 2.5 arc-minutes spatial resolution (Hijmans *et al.* 2005; Ghamkhar *et al.* 2015). These were used for mapping and spatial modelling in the R software package (http://www.worldclim.org/formats1). Definition and codes of the 19 standard BIOCLIM variables are provided in Table S2 (Hijmans *et al.* 2005). Temperature data were divided by 10 to convert to degrees Celsius (http://www.worldclim.org). Table S3 presents a summary of the BIOCLIM variables and associated passport data, and Table S4 provides imported climatic variables for each ecotype.

Germination and transplantation

Prior to sowing, seeds of each genotype were scarified as described in Nichols *et al.* (2009). Seeds were sown on 21 May 2019 by germinating in Petri dishes containing a single Whatman No. 1 filter paper moistened by 3.5 mL tap water. These were placed in a germination cabinet at 15°C. At 2–3 days after sowing (DAS), two germinated seedlings of each genotype were transplanted into hydrated Jiffy-9 peat pots (Jiffy Products, Stange, Norway) in a glasshouse. *Rhizobium leguminosarum* bv. *trifolii* strain WSM1325 (Nodulaid; BASF Australia, Melbourne, Vic.) was applied by watering can (40 mL inoculum to 9 L water).

Peat pots were watered daily to prevent moisture stress, and soluble fertiliser (Thrive; Yates Australia, Sydney, NSW) was applied weekly at a rate of 0.5 g/L. Seedlings were thinned to a single healthy seedling per Jiffy pot at 15 DAS and transferred to outside benches for acclimatisation at 21 DAS. Four healthy seedlings of each genotype (except genotype CPI 039315YB, which had only two replicates) were transplanted into the field at 31 DAS.

Experimental site

The experiment was conducted at The University of Western Australia Field Station at Shenton Park (31°57′S, 115°5′E) under netting to prevent bird damage. The soil at the experimental site is a deep sand with pH(H_2O) 6.5 in the top 20 cm. The field was rotary-hoed and cultivated in March. For weed control, glyphosate (540 g a.i./L) at 2 L/ha was applied in early April, and the field was sprayed with Basta (BASF Australia; a.i. glufosinate-ammonium, 200 g/L) at a rate of 1.5 L/ha 7 days before transplanting the seedlings. Single superphosphate with potash (6.8% P, 12.4% K and 8.3% S) was applied at 300 kg/ha in early May. The area was smudged to level the soil before laying six parallel 1-m-wide, 100 m long polyethylene plastic strips (to restrict weed growth), each separated by 1.5 m of bare soil. Seedlings were transplanted into holes cut into the plastic strips.

The climate at Shenton Park is Mediterranean. The mean maximum and minimum temperatures were 21.9°C and 12.3°C, respectively, with 544.2 mm rainfall during the experimental period (May–November 2019) (www.bom.gov.au). Mean monthly maximum and minimum temperatures and rainfall during the experimental period in the study area are shown in Fig. S2 (www.bom.gov.au). Plants were checked several times each week and supplemental overhead irrigation was supplied as required from 1 October to ensure that plants did not suffer moisture stress, in accordance with Nichols *et al.* (2009) and Abdi *et al.* (2020). Irrigation ceased on 20 November to allow plants to senesce.

Experimental layout and design

A randomised complete block (row–column) design with four replicates was used. Replicates were arranged perpendicular to (i.e. across) the six plastic strips, which acted as columns. Plants were spaced 0.85 m apart along the strips to allow growth without competition for light and moisture and to prevent edge effects. The plastic strips were removed from

the field, to allow burr burial, on 25 August, when the earliest flowering genotypes had just commenced flowering.

In addition to the 154 purported subsp. *yanninicum* genotypes planted in the study, 14 genotypes comprising subspp. *subterraneum* and *brachycalycinum* were included. Thus, the experimental layout consisted of 672 (i.e. 168 × 4) spaced plants and these were arranged in 112 rows across the six plastic strips, with each replicate made up of 28 rows. Only data for the 118 confirmed subsp. *yanninicum* genotypes (108 ecotypes and 10 cultivars) are reported here.

Measurements of growth traits

Measurements were taken on each plant as described in Nichols *et al.* (2009) and Abdi *et al.* (2020). At 62–63 DAS (midwinter), the length of the longest petiole of each plant was measured and the size of the leaf (three leaflets combined) on the longest petiole was measured by using a portable leaf area meter (LI 3000; LI-COR Biosciences, Lincoln, NE, USA).

At 87–88 DAS (end of winter), the length of the longest petiole and leaf size on the longest petiole were measured via the same methods as used for the midwinter measurements. This time was chosen so that all genotypes could be compared simultaneously as late as possible while still in their vegetative phase, before flowering of the earliest genotypes.

The number of days from sowing to the appearance of the first open flower (days to first flowering) was measured, with plants checked every 3 days. The stem (runner) with the first flower was marked with a crossing tag to identify the first flowering node for measurement of morphological traits at first flowering. Leaf size and petiole length on the first flowering node were measured 14–30 days after first flowering. In this case, leaf size was measured by visually comparing leaves with the photographic leaf size plates of Williams *et al.* (1964) and using their conversion formulae as per Nichols *et al.* (2009) and Abdi *et al.* (2020). Internode length and stem diameter were measured between the first and second flowering nodes on the stem with the first flower. Stem diameter was measured with a micrometer (Mitutoyo, Tokyo) with 0.01-mm intervals. These measurements were conducted to compare genotypes at the same physiological stage during the reproductive phase.

Isoflavone contents (formononetin, genistein and biochanin A) were measured by using the semi-quantitative technique of Francis and Millington (1965), as used by Ghamkhar *et al.* (2012), Nichols *et al.* (2009) and Abdi *et al.* (2020). Samples of six leaf discs (6 mm diameter) were collected from healthy, newly fully opened leaves at 71–72 DAS (first and second replicates) and 80–81 DAS (third and fourth replicates) for analysis. Isoflavones were immediately extracted in alcohol and subjected to thin-layer chromatography. Duplicate samples taken for dry weight determination were oven-dried at 60°C for 48 h. Isoflavone contents were calculated as a percentage of dry matter (DM).

Leaf marks were rated at 67 DAS, when they were most prominent owing to cold winter temperatures. Standard semi-quantitative descriptors, based on Nichols *et al.* (1996) and used by Abdi *et al.* (2020), were utilised (Table S5). Briefly, leaf marks are described in terms of 'crescents', 'arms' and 'bands', and variation can take the form of absence or presence, size, shape and position of each (Tan and Collins 1987; Nichols *et al.* 1996). Leaf mark patterns are classified by: (*i*) widths of crescents, which are central triangular markings (usually pale green) that extend from the leaflet centre towards the margins and range in size from a central dot (rating 1) to all the way from margin to margin (rating 4); (*ii*) breadth of arms, which are stripes (usually white or pale green) that extend from the leaflet margins towards the centre and range from narrow (rating 1) to very broad (rating 4); and (*iii*) breadth of bands, which are an alternative marking to crescents and arms, consisting of a pale green stripe extending from margin to margin and varying in breadth from narrow (rating 1) to broad (rating 3). Leaf marks can consist of crescents alone, arms alone, crescents with arms, bands alone or no markings at all. Ratings were taken on plants in only two replicates, owing to the high heritability and consistency of markings among plants of the same genotype (Tan and Collins 1987; Nichols *et al.* 1996).

Statistical analyses

Data were analysed by using one-way ANOVA to compare genotypic variation among means of plant traits, using R software (ver. 3.6.3; The R Foundation, Vienna). This was conducted: (*i*) among all 118 subsp. *yanninicum* genotypes; and (*ii*) within and between ecotype and cultivar groups of subsp. *yanninicum*. Least significant differences (l.s.d.), using Fisher's protected tests, were used to compare means for each measured trait between the ecotype and cultivar groups of subsp. *yanninicum*, and to compare means for each trait among all genotypes.

Pearson correlation coefficients and their levels of significance were calculated between traits for the 118 genotypes of subsp. *yanninicum*. They were also calculated between plant traits and eco-geographic (passport and BIOCLIM) variables of collection sites for all 90 subsp. *yanninicum* genotypes (88 ecotypes and two cultivars) with known collection site information. Broad-sense heritability (H^2) was estimated for each trait across all genotypes of subsp. *yanninicum*, according to Falconer and Mackay (1996).

Results

Diversity among genotypes

Broad variation (Table 1, Table S6) with highly significant genotype differences (Table S7) was observed for all

Table 1. Summary of mean, minimum (Min.) and maximum (Max.) values of morphological traits, flowering time and leaf isoflavone contents for ecotypes and cultivars of *Trifolium subterraneum* subsp. *yanninicum*.

Traits	Ecotypes (n = 108)			Cultivars (n = 10)			Sig. level	H^2 (%)
	Mean	Min.	Max.	Mean	Min.	Max.		
At 63 DAS								
Leaf size (cm^2)	4.0a	2.3	6.8	3.6a	2.4	5.0	n.s.	15
Petiole length (cm)	5.4a	3.7	8.3	5.6a	4.0	6.9	n.s.	10
At 88 DAS								
Leaf size (cm^2)	3.5a	1.9	6.4	3.4a	2.8	4.7	n.s.	29
Petiole length (cm)	6.1a	4.2	9.0	6.5a	5.4	8.1	n.s.	16
At first flowering								
DFF	124a	94	149	119b	101	149	**	92
Leaf size (cm^2)	7.4a	4.0	13.6	6.8a	4.8	9.8	n.s.	27
Petiole length (cm)	9.2a	4.8	14.3	8.9a	6.9	11.3	n.s.	31
Stem diameter (mm)	2.2b	1.6	2.8	2.4a	2.1	2.9	**	20
Internode length (cm)	3.5a	1.3	7.2	2.9b	1.9	4.4	*	36
Isoflavone content (% of dry matter)								
Formononetin	0.73a	0.05	1.38	0.36b	0.09	1.36	***	60
Genistein	1.41b	0.73	2.33	1.75a	1.24	2.08	***	23
Biochanin A	0.64a	0.15	2.10	0.58a	0.25	0.98	n.s.	52
Total isoflavones	2.72a	1.11	4.46	2.69a	1.81	3.10	n.s.	15

Note: Significance level (Sig. level) between mean ecotype and cultivar values for each trait is shown; means with the same letter are not significantly different for each trait ($P > 0.05$; Fisher's l.s.d. test). *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; n.s., not significant ($P > 0.05$). Broad-sense heritability (H^2) across the 118 genotypes of subsp. *yanninicum* is also shown for each trait. Morphological traits were measured at 63 days after sowing (DAS), at 88 DAS, and at first flowering. Days to first flowering (DFF) was recorded as the number of days from sowing to first open flower.

morphological traits, flowering time and leaf isoflavone contents among the 118 genotypes. Ecotypes had greater variation than cultivars for all measured traits.

Flowering time was highly heritable (92%) and ranged from 94 to 149 days (Table 1, Table S6) with highly significant ($P < 0.001$) variation among genotypes (Table S7). Cv. Meteora (149 days) and ecotypes EP059WHITE-F (149 days), CPI 083967 L (148 days), CPI 083948B (147 days) and EP059WHITE-D (147 days) were the last to flower. By contrast, ecotypes Neuchatel (94 days), N4848 (98 days), and DGG041-A, DGG046-B and EP082WHITE (all 100 days) were the earliest flowering. However, mean flowering time of the cultivars was significantly earlier than the ecotypes (Table 1). A frequency distribution of mean flowering time is shown in Fig. 1.

Leaf isoflavone content varied substantially among genotypes (all $P \leq 0.001$), with ranges 0.05–1.38% (of DM) for formononetin, 0.73–2.33% for genistein, 0.15–2.10% for biochanin A, and 1.11–4.46% for total leaf isoflavone content (Table 1, Tables S6 and S7). Heritability was high for both formononetin (60%) and biochanin A (52%) contents, and lower for genistein (23%) and total isoflavone (15%) contents (Table 1). Mean values for formononetin and genistein differed between ecotype and cultivar groups (both $P \leq 0.001$), but not for biochanin A or total isoflavones (Table 1). Highest formononetin contents were found in N4848 (1.38%), cv. Yarloop (1.36%) and EP082WHITE (1.33%). By contrast, CPI 103933, EP059WHITE-F and CPI 039314YB had formononetin contents ≤0.06%. Formononetin content was significantly higher than 0.20% in 65.7% of ecotypes.

For leaf marks, broad variation was found among genotypes (Table S6). The most common leaf mark was a crescent with arms (98 genotypes), followed by a crescent only (11 genotypes) and arms only (eight genotypes); one genotype (CPI 083957N) had no leaf mark. For leaf marks with a crescent, 64% of genotypes had a 2–3 crescent width rating. For leaf marks with arms, 57% of genotypes had a 2–3 arm breadth rating and 31% had a 1–2 arm breadth rating; ecotype N4848 scored 4 because of its very broad arms.

Associations between traits among genotypes

Pearson correlation coefficients between mean values of all variables are shown in Table 2. Leaf size and petiole length were strongly correlated at 63 DAS ($r = 0.51$; $P \leq 0.001$), 88 DAS ($r = 0.66$; $P \leq 0.001$) and first flowering ($r = 0.51$; $P \leq 0.001$). Flowering time was negatively associated with both leaf size ($r = -0.46$; $P \leq 0.001$) and petiole length

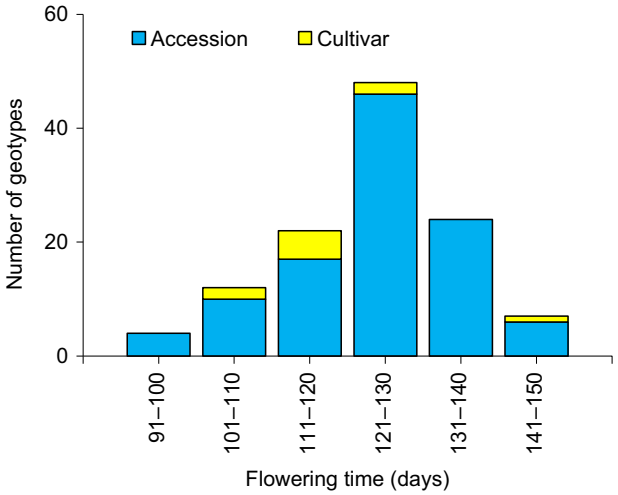

Fig. 1. Frequency distribution of mean flowering time (number of days from sowing on 21 May to first open flower) for 118 *Trifolium subterraneum* subsp. *yanninicum* genotypes, consisting of 108 ecotypes and 10 cultivars, grown at Shenton Park, Western Australia.

($r = –0.31$; $P \leq 0.001$) at 88 DAS. However, at flowering time, later flowering genotypes had longer petioles ($r = 0.29$; $P \leq 0.01$), larger leaves ($r = 0.34$; $P \leq 0.001$), longer internodes ($r = 0.27$; $P \leq 0.01$) and thicker stems ($r = 0.20$; $P \leq 0.05$). Notably, internode length was strongly associated with petiole length at first flowering ($r = 0.63$; $P \leq 0.001$) and at both 63 DAS ($r = 0.45$; $P \leq 0.001$) and 88 DAS ($r = 0.23$; $P \leq 0.05$).

Biochanin A and genistein contents were unrelated, but both, particularly genistein ($r = –0.42$; $P \leq 0.001$), had a negative association with formononetin content (Table 2). Some associations between leaf isoflavone contents and morphological traits and flowering time were found. Flowering time was negatively associated with formononetin content ($r = –0.26$; $P \leq 0.01$) but positively associated with genistein ($r = 0.18$; $P \leq 0.05$) and biochanin A ($r = 0.25$; $P \leq 0.01$) contents. However, there was no relationship between total leaf isoflavone content and flowering time.

For leaf marks, there were several associations with morphological traits, flowering time and leaf isoflavone contents (Table 2). Crescent size increased with flowering time ($r = 0.31$; $P \leq 0.001$), whereas arm breadth decreased ($r = –0.25$; $P \leq 0.01$). Broad arms were also associated with short internode length ($r = –0.33$; $P \leq 0.001$) at flowering and with short petiole length ($r = 0.21$; $P \leq 0.01$) at 63 DAS. Crescent size had weak associations with low formononetin ($r = 0.18$; $P \leq 0.05$) and high biochanin A ($r = 0.22$; $P \leq 0.05$) contents. There was, however, no association between crescent size and arm breadth.

Correlation between traits and eco-geographic variables

Pearson correlation coefficients for 10 morphological traits, flowering time and leaf isoflavone contents with their site of origin variables were calculated for the 90 genotypes with passport data (cvv. Larisa and Meteora and 88 other ecotypes). Correlation coefficients are shown in Table 3 for

Table 2. Pearson correlation coefficients and significance between mean values of 10 morphological traits, flowering time and leaf isoflavone contents for 118 *Trifolium subterraneum* subsp. *yanninicum* genotypes (108 ecotypes and 10 cultivars).

	Traits	n	1	2	3	4	5	6	7	8	9	10	11	12	13	14
At 63 DAS																
1	Leaf size	118														
2	Petiole length	118	0.51***													
At 88 DAS																
3	Leaf size	118	0.53***	0.42***												
4	Petiole length	118	0.29**	0.56***	0.66***											
At first flowering																
5	DFF	118	n.s.	−0.20*	−0.46***	−0.31***										
6	Leaf size	115	0.43***	n.s.	0.24*	n.s.	0.34***									
7	Petiole length	115	0.29**	0.31**	n.s.	0.23*	0.29**	0.51***								
8	Stem diameter	115	n.s.	n.s.	0.24*	0.23*	−0.20*	0.32***	n.s.							
9	Internode length	115	0.35***	0.45***	n.s.	0.23*	0.27**	0.38***	0.63***	n.s.						
Isoflavone contents																
10	Formononetin	118	n.s.	n.s.	0.22*	0.24*	−0.26**	−0.27*	n.s.	n.s.	n.s.					
11	Genistein	118	n.s.	n.s.	n.s.	−0.23*	0.18*	0.25*	n.s.	n.s.	n.s.	−0.42***				
12	Biochanin A	118	n.s.	−0.21*	−0.19*	−0.18*	0.25**	n.s.	n.s.	n.s.	n.s.	−0.24*	n.s.			
13	Total isoflavones	118	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.22*	0.47***	0.58***		
Leaf mark																
14	Crescent size	118	n.s.	n.s.	n.s.	n.s.	0.31***	n.s.	n.s.	n.s.	n.s.	−0.18*	n.s.	0.22*	n.s.	
15	Arm breadth	118	n.s.	−0.24**	n.s.	n.s.	−0.25**	n.s.	−0.19*	n.s.	−0.33***	n.s.	n.s.	n.s.	n.s.	n.s.

Note: n = number of genotypes measured for each trait. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; n.s., not significant ($P > 0.05$). Strength of correlation is shown by increasing intensity of green for positive and increasing intensity of yellow for negative.

Table 3. Pearson correlation coefficients (*r*) and significance for mean values of 10 morphological traits, flowering time and leaf isoflavone contents with 11 eco-geographic variables (passport data and BIOCLIM precipitation variables BIO12–BIO19) for 90 *Trifolium subterraneum* subsp. *yanninicum* genotypes with known passport data.

Traits	Passport data			BIOCLIM precipitation variables							
	Latitude	Longitude	Altitude	BIO12	BIO13	BIO14	BIO15	BIO16	BIO17	BIO18	BIO19
At 63 DAS											
Leaf size	n.s.	0.21*	n.s.	0.30**	0.31**	n.s.	n.s.	0.31**	n.s.	n.s.	0.26*
Petiole length	n.s.	0.36***	n.s.	0.42***	0.40***	0.37***	n.s.	0.41***	0.32***	0.23*	0.40***
At 88 DAS											
Leaf size	n.s.	0.24*	−0.33**	0.26*	0.33**	n.s.	0.35***	0.33**	n.s.	n.s.	0.32**
Petiole length	n.s.	0.21*	n.s.	0.33**	0.33**	n.s.	n.s.	0.34***	n.s.	n.s.	0.35***
At first flowering											
Flowering time	0.22*	n.s.	0.58***	n.s.	−0.22*	0.22*	−0.51***	−0.22*	0.38***	0.51***	−0.21*
Leaf size	0.35***	n.s.	0.29**	n.s.	n.s.	n.s.	n.s.	n.s.	0.21*	0.32**	n.s.
Petiole length	n.s.	n.s.	0.31**	0.33**	0.25*	0.48***	n.s.	0.26*	0.48***	0.45***	0.25*
Stem diameter	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Internode length	n.s.	0.26*	0.29**	0.41***	0.33**	0.53***	n.s.	0.35***	0.52***	0.47***	0.36***
Isoflavone content											
Formononetin	n.s.	0.21*	n.s.	0.26*	0.29**	n.s.	0.25*	0.31**	n.s.	n.s.	0.30**
Genistein	0.33**	−0.31**	n.s.	−0.36***	−0.39***	−0.23*	−0.24*	−0.40***	n.s.	n.s.	−0.41***
Biochanin A	n.s.	−0.44***	0.38***	−0.32**	−0.37***	−0.23*	−0.25*	−0.37***	n.s.	n.s.	−0.45***
Total isoflavone	n.s.	−0.36***	0.22*	−0.26*	−0.30**	n.s.	n.s.	−0.29**	n.s.	n.s.	−0.36***
Leaf mark											
Crescent size	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Arms breadth	n.s.	−0.22*	−0.22*	n.s.	n.s.	−0.29**	n.s.	n.s.	−0.31**	−0.33**	n.s.

Note: Codes and definitions of BIOCLIM variables are given in Table S2. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; n.s., not significant ($P > 0.05$). Strength of correlation is shown by increasing intensity of green for positive and increasing intensity of yellow for negative.

passport data and eight standard BIOCLIM precipitation variables (BIO12–BIO19), and in Table 4 for 11 standard BIOCLIM temperature variables (BIO1–BIO11).

Winter growth

Leaf size and petiole length in midwinter (63 DAS) and late winter (88 DAS) were both correlated positively with longitude, indicating that genotypes from the eastern part of the subspecies range had larger plants in winter than those from the western part. Large leaves at 88 DAS also tended to be associated with low altitudes ($r = -0.33$; $P \leq 0.001$). Leaf size and petiole length during winter were positively correlated with the majority of precipitation variables, particularly annual precipitation (BIO12), and with variables related to winter precipitation (BIO13, BIO16 and BIO19). Petiole length at 63 DAS was also positively correlated with summer precipitation variables (BIO14, BIO17 and BIO18).

Growth at first flowering

High altitude and high summer precipitation (BIO14, BIO17 and BIO18) favoured later flowering genotypes with large leaves, long petioles and long internodes at first flowering (Table 3). Early flowering was associated with high winter precipitation (BIO13, BIO16 and BIO19). Notably, flowering time and both petiole and internode lengths at first flowering were negatively correlated with annual mean temperature (BIO1) and with both winter (BIO6, BIO9 and BIO11) and summer (BIO8 and BIO10) temperatures (Table 4). Stem diameter was not correlated with any eco-geographic variables.

Isoflavone content

The inverse associations between formononetin content and both genistein and biochanin A contents was reflected in their associations with several eco-geographic variables. High formononetin content was associated with easterly longitudes, whereas high genistein and biochanin A contents were both associated with more westerly longitudes. Both total isoflavone and biochanin A contents had positive relationships with altitude, whereas genistein content was associated with latitude. Annual precipitation (BIO12) and variables related to winter precipitation (BIO13, BIO16 and BIO19) were positively correlated with formononetin content but negatively correlated with genistein and biochanin A contents.

Table 4. Pearson correlation coefficients and significance for mean values of 10 morphological traits, flowering time and leaf isoflavone contents with 11 BIOCLIM temperature variables (BIO1–BIO11) for 90 *Trifolium subterraneum* subsp. *yanninicum* genotypes with known passport data.

Traits	BIOCLIM temperature variables										
	BIO1	BIO2	BIO3	BIO4	BIO5	BIO6	BIO7	BIO8	BIO9	BIO10	BIO11
At 63 DAS											
Leaf size	−0.24*	n.s.	n.s.	n.s.	−0.27*	n.s.	n.s.	n.s.	−0.27*	−0.28**	n.s.
Petiole length	n.s.	n.s.	n.s.	n.s.	n.s.	−0.22*	n.s.	−0.25*	n.s.	n.s.	n.s.
At 88 DAS											
Leaf size	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Petiole length	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
At first flowering											
Flowering time	−0.49***	n.s.	−0.31**	n.s.	−0.26*	−0.31**	n.s.	n.s.	−0.44***	−0.40***	−0.44***
Leaf size	−0.37***	−0.22*	−0.30**	n.s.	−0.36***	n.s.	n.s.	n.s.	−0.43***	−0.42***	−0.22*
Petiole length	−0.46 ***	0.23*	n.s.	n.s.	n.s.	−0.45***	0.23*	−0.41***	−0.36***	−0.39***	−0.47***
Stem diameter	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Internode length	−0.47 ***	0.22*	0.24*	n.s.	n.s.	−0.45***	0.21*	−0.39***	−0.38***	−0.42***	−0.46***
Isoflavone content											
Formononetin	n.s.	n.s.	0.31**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.21*
Genistein	n.s.	−0.24*	−0.43***	n.s.	n.s.	n.s.	n.s.	0.26*	n.s.	n.s.	n.s.
Biochanin A	n.s.	n.s.	−0.27*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Total isoflavone	n.s.	n.s.	−0.22*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Leaf mark											
Crescent size	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Arms breadth	0.29**	n.s.	n.s.	n.s.	n.s.	0.25*	n.s.	n.s.	0.25*	0.26*	0.28*

Note: Codes and definition of BIOCLIM variables are given in Table S2. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; n.s., not significant ($P > 0.05$). Strength of correlation is shown by increasing intensity of green for positive and by increasing intensity of yellow for negative.

Leaf marks

Leaf mark crescent size was not correlated with any eco-geographic variable. However, the breadth of leaf mark arms was associated with several variables. Broad arms were associated with both low altitudes and more westerly longitudes (Table 3) as well as drier summers (BIO14, BIO17 and BIO18) (Table 3) and warmer mean annual temperatures (BIO1) and warmer winter (BIO6, BIO9 and BIO11) and summer (BIO10) temperatures (Table 4). By contrast, narrow arms were associated with more easterly longitudes with increasing precipitation.

Discussion

Diversity across genotypes

The wide diversity for all traits measured among subsp. *yanninicum* genotypes supports the first hypothesis of this study. This shows that there is potential to broaden the gene pool for subsp. *yanninicum* in southern Australian pastures by introducing new traits. Larger leaves, longer petioles and longer internodes could provide increased competitive ability (Black 1960; Gladstones 1967; Nichols *et al.* 2009, 2013) beyond that of the current cultivars. Likewise, both the latest and earliest flowering genotypes were found among the ecotypes. A wide range of flowering times enables successful growth of subsp. *yanninicum* in locations with different growing season lengths (Nichols *et al.* 2009). Later flowering genotypes, with their longer vegetative period, have the opportunity to accumulate more biomass with larger leaves and longer petioles, which is reflected by higher spring DM yield, making them well suited to areas with long growing season (Rossiter 1959; Chapman *et al.* 2012; Pecetti and Piano 1993, 1997; Nichols *et al.* 2009; Abdi *et al.* 2020; Pecetti *et al.* 2020). Earlier flowering is crucial for persistence in shorter season environments, because it enables adequate seed production prior to onset of terminal drought (Gladstones 1967; Piano 1984; Nichols *et al.* 1996; Piano *et al.* 1996; Piano *et al.* 1993; Pecetti and Piano 2002). Formononetin content was beyond the level considered safe (i.e. 0.2% of DM) in the majority of ecotypes. Cultivars released since 1975 in Australia have undergone deliberate selection for formononetin levels below this safe level, (Nichols *et al.* 2013), so it was not surprising that the cultivars had a significantly lower level than the ecotypes. Nonetheless, this experiment revealed other ecotypes with low formononetin levels that could be used as alternative parents for developing non-oestrogenic cultivars. Furthermore, the use of high formononetin ecotypes as parents is not excluded, because the high heritability of formononetin content enables ready selection for low formononetin progeny when crossed with a low formononetin parent (Nichols *et al.* 2013).

Correlations between plant traits

This study supports previous findings of Gladstones (1967), Nichols *et al.* (2009) and Pecetti and Piano (1997) that

earlier flowering genotypes tend to have larger leaves and longer petioles during winter growth, whereas later flowering genotypes have larger leaves and longer petioles at flowering. This confirms that leaf growth and petiole elongation of subsp. *yanninicum* increases progressively with subsequent nodes until the first flowering node, similar to observations with subsp. *subterraneum* (Gladstones 1967; Pecetti and Piano 1997).

The observation that earlier flowering genotypes tended to have higher formononetin content, whereas later flowering genotypes tended to have higher biochanin A and genistein contents, agrees with the results of Cocks (1992), Morley and Francis (1968) and Nichols *et al.* (2009) for subsp. *subterraneum*. In the present study, there was no relationship between genistein and biochanin A, whereas studies by Morley and Francis (1968), Abdi *et al.* (2020) and Nichols *et al.* (2013) across all subspecies found a negative relationship. This suggests different biochemical regulatory pathways may be involved for these compounds in subsp. *yanninicum*.

Correlations between plant traits and eco-geographic variables

The second hypothesis, that variation in plant traits is related to the eco-geography of their collection sites, was also supported. Longitude played an important role to discriminate the collection sites and it was well correlated with annual precipitation of the site of origin, supporting Bennett (2000), Bennett and Bullitta (2003) and Ghamkhar *et al.* (2007). The association of large leaf size at the end of winter with low altitude differs from the report of Abdi *et al.* (2020), who found no such association, but is consistent with results from studies of Sardinian (Pecetti and Piano 1997) and Sicilian (Pecetti and Piano 1993) subterranean clover ecotypes.

The differences between the results of this study, which focused on subsp. *yanninicum*, and other studies that focused on the other two subspecies, is most likely due to differences in subspecies distributions, with subsp. *yanninicum* being much more restricted geographically and ecologically than the other two subspecies (Katznelson and Morley 1965; Francis 1976; Ghamkhar *et al.* 2015). The natural habitat of subsp. *yanninicum* is reported to be low-altitude coastal areas (often flat meadows) with high mean annual rainfall (>500 mm) and mild winter temperatures (Katznelson 1966; Francis 1976; Ghamkhar *et al.* 2015). This contrasts with the natural habitats of the other two subspecies, which occur at altitudes up to 2190 m a.m.s.l. (for subsp. *brachycalycinum*) and 2940 m (for subsp. *subterraneum*) and can be found in areas with as little as <300 mm mean annual rainfall (Ghamkhar *et al.* 2015).

This study supports the assertion of Abdi *et al.* (2020) and Pecetti and Piano (1993, 1997) that plants from higher elevations tend to have smaller leaves as an adaptation to cold stress. Earlier flowering genotypes of subsp. *yanninicum* generally had larger leaves in winter, consistent with Pecetti and Piano (1997) and Abdi *et al.* (2020). Notably, ecotypes with longer petioles tended to originate from higher rainfall sites. This is associated with their later flowering time and is possibly an adaptation to submergence.

This study confirms previous studies of annual legumes by Piano (1984), Piano *et al.* (1993, 1996), Pecetti and Piano (2002), Cocks (1992), Nichols *et al.* (2010), Erskine *et al.* (1989) and Ehrman and Cocks 1996 that natural selection for flowering time is strongly influenced by eco-geographic variables (latitude, altitude, precipitation and temperature). Flowering time of subsp. *yanninicum* was correlated with latitude in this study, supporting the results of Ghamkhar *et al.* (2015) and Abdi *et al.* (2020). Flowering time of subsp. *yanninicum* also increased with increasing altitude and rainfall of the collection site, confirming the results of Piano (1984) for Sardinian subterranean clover ecotypes. This is in spite of the restricted elevation of subsp. *yanninicum* compared with the other two subspecies (Ghamkhar *et al.* 2015) and its common position lower in the landscape (Katznelson and Morley 1965; Francis 1976; Piano 1984), which tends to collect more water. The correlations of flowering time with winter and annual rainfall in subsp. *yanninicum* are in agreement with the studies of subterranean clover world (Ghamkhar *et al.* 2015) and core (Abdi *et al.* 2020) collections, Sicilian (Piano *et al.* 1993) and Sardinian (Piano 1984; Piano *et al.* 1996) ecotypes, and a bulk-hybrid subsp. *subterraneum* population (Nichols *et al.* 2009). This can be explained as a plant response to adjust its maturity to soil moisture availability in order to facilitate seedset and burr burial prior to onset of the dry summers experienced in the Mediterranean region (Piano 1984; Piano *et al.* 1993; Pecetti and Piano 2002). Furthermore, later flowering genotypes can remain vegetative and accumulate biomass with larger leaves and longer petioles for a longer period in favourable rainfall environments (Nichols *et al.* 2009). The results of this study also agree with the assertion of Abdi *et al.* (2020) and Pecetti and Piano (1997) that high winter growth is associated with warmer climates.

The observation that sites with high winter rainfall tended to produce subsp. *yanninicum* genotypes with increased formononetin contents and decreased genistein and biochanin A contents contrasts with the results of Abdi *et al.* (2020) and Ghamkhar *et al.* (2015), who found no relationships between rainfall variables and the contents of individual isoflavones or total isoflavone. This difference may reflect the focus of their studies on the other two subspecies of subterranean clover. It also suggests that isoflavone production in subsp. *yanninicum* may respond to soil moisture conditions differently from that in the other two subspecies, and may be related to its superior waterlogging tolerance (Marshall and Millington 1967; Francis and Devitt 1969; Devitt and Francis 1972; Enkhbat *et al.* 2021).

The finding in this study that leaf mark arm breadth for subsp. *yanninicum* was correlated with several eco-geographic parameters at the collection sites was unexpected. Among the subterranean clover core collection, Abdi *et al.* (2020) found an association of arm breadth with soil pH at the collection site, but not with other eco-geographic parameters. However, neither study found any association of crescent size with eco-geographic parameters. Whereas plant growth traits might be expected to have some adaptive relationships with their sites of origin, the significance of the associations between morphological markers and environmental factors is unclear. Abdi *et al.* (2020) suggest the genes controlling them may be linked to other genes controlling adaptive traits that are responsive to different environments. The accumulation of particular regional characteristics such as the predominance of broad leaf mark arms in the western part of the subsp. *yanninicum* range may also be due to genetic drift, as a result of its scant distribution and the difficulty of genetic exchange between widely spaced populations.

Plant breeding implications

This study demonstrated that a range of ecotypes exists that could be used as parents in plant breeding programs to expand the genetic base of subsp. *yanninicum*, which has been heavily reliant on derivatives of the Greek ecotypes CPI 039313Y (cv. Larisa) and CPI 039327YB (cv. Meteora) and the WA naturalised strain, Neuchatel (Nichols *et al.* 2013; PBR 2021). Ecotypes, rather than cultivars, had the maximum values for leaf size and petiole length in both midwinter and late winter and at flowering, indicating the potential to increase the leaf size and petiole length of current cultivars.

Flowering time of the ecotypes spanned that of the cultivars, indicating little scope to breed earlier or later flowering subsp. *yanninicum* cultivars. Indeed, there appears to be no need for earlier or later flowering cultivars in southern Australia. Short-season environments tend not to have prolonged waterlogging and are better suited to subsp. *subterraneum* cultivars, which flower earlier than the subsp. *yanninicum* cultivars (Nichols *et al.* 2013). The marketplace for very late flowering subsp. *yanninicum* cultivars appears to be small, because it tends to overlap with areas suited to perennial legumes such as white clover (*T. repens* L.), which can provide year-round green forage. The drying climate with shorter growing seasons in southern Australia also reduces the need for long-season annuals (Revell *et al.* 2012).

This study focused on diversity for plant growth traits, flowering time and leaf isoflavone content. Other studies are being conducted with this material to identify subsp. *yanninicum* ecotypes with important agronomic traits for adaptation to farming conditions. Such traits include high biomass and seed production potential, hardseededness, resistance to important pests and diseases, and tolerance to waterlogging and other environmental stresses. This will enable selection of parents with desirable traits to be included in crossing programs to improve productivity and persistence of current cultivars.

A major limitation to the continued improvement of subsp. *yanninicum* is the size of its genetic resource, being confined to 55 collection sites and comprising only 2.2% of the world collection of 8812 subterranean clover genotypes from 2993 sites (Nichols *et al.* 2013; Ghamkhar *et al.* 2015). The value of future selection and breeding activities would be enriched by targeted collecting, with detailed passport data, to broaden the genetic diversity of subsp. *yanninicum*. In particular, its genetic resource can be diversified by collecting in regions where it is naturally distributed but which are not yet represented in annual legume genebanks, such as Croatia, Slovenia, Albania and Serbia (Zohary and Heller 1984; Ghamkhar *et al.* 2015).

Study limitations

This study was conducted under highly controlled experimental conditions on undefoliated, weed-free, spaced plants on well-drained and well-fertilised soil. Some aspects of plant performance are likely to differ in swards, particularly under grazing. Further research is also needed to investigate plant performance of different subsp. *yanninicum* ecotypes under waterlogged conditions, particularly because the subspecies is widely reported as having greater tolerance than subspp. *subterraneum* and *brachycalycinum* (Marshall and Millington 1967; Francis and Devitt 1969; Devitt and Francis 1972; Francis *et al.* 1974; Gibberd and Cocks 1997). Other environmental factors such as low temperatures and nutrient deficiencies are also likely to influence plant growth. The common garden conditions of this study did enable estimation of the genetic basis of the measured traits. Time to first flowering, isoflavone contents and leaf markings are highly heritable and values are likely to be transferable between seasons and locations under similar conditions (Nichols *et al.* 2013). However, field trials under typical farming conditions at different sites with diverse climates and soils are crucial for understanding the adaptation and agronomic merits of subsp. *yanninicum* genotypes.

Conclusions

This study demonstrated that although the available genetic resource of subsp. *yanninicum* is relatively small, there is sufficient diversity to broaden the genetic base of the subspecies and develop new cultivars to increase the productivity of pastures in southern Australia and other regions with Mediterranean-type climates. We also conclude that further genetic improvements may result from targeted collecting, particularly in areas not represented in annual legume genebanks.

Supplementary material

Supplementary material is available online.

References

Abdi AI, Nichols PGH, Kaur P, Wintle BJ, Erskine W (2020) Morphological diversity within a core collection of subterranean clover (*Trifolium subterraneum* L.): lessons in pasture adaptation from the wild. *PloS One* **15**, e0223699. doi:10.1371/journal.pone.0223699

Aitken Y, Drake FR (1941) Studies on the varieties of subterranean clover. *Proceedings of the Royal Society of Victoria* 53, 342–393.

Bennett SJ (2000) Genetic variation of five species of *Trifolium* L. from south-west Turkey. *Genetic Resources and Crop Evolution* **47**, 81–91. doi:10.1023/A:1008728905199

Bennett SJ, Bullitta S (2003) Ecogeographical analysis of the distribution of six *Trifolium* species in Sardinia. *Biodiversity and Conservation* **12**, 1455–1466. doi:10.1023/A:1023677726849

Bennetts HW, Uuderwood EJ, Shier FL (1946) A specific breeding problem of sheep on subterranean clover pastures in Western Australia. *Australian Veterinary Journal* **22**, 2–12. doi:10.1111/j.1751-0813.1946.tb15473.x

Black JN (1960) The significance of petiole length, leaf area, and light interception in competition between strains of subterrranean clover (*Trifolium subterraneum* L.) grown in swards. *Australian Journal of Agricultural Research* **11**, 277–291. doi:10.1071/AR9600277

Brightling A (2006) 'Livestock diseases in Australia: diseases of cattle, sheep, goats and farm dogs', (CH Jerram & Associates: Mount Waverley, Vic., Australia)

Chapman SC, Chakraborty SM, Dreccer MF, Howden SM (2012) Plant adaptation to climate change-opportunities and priorities in breeding. *Crop & Pasture Science* **63**, 251–268. doi:10.1071/CP11303

Cocks PS (1992) Evolution in sown populations of subterranean clover (*Trifolium subterraneum* L.) in South Australia. *Australian Journal of Agricultural Research* **43**, 1583–1595. doi:10.1071/AR9921583

Collins WJ, Cox RI (1984) Oestrogenic activity in forage legumes. In 'Forage legumes for energy efficient animal production. Proceedings of a trilateral workshop'. Palmerston North, New Zealand. (Eds RF Barnes, PR Ball, RW Brougham, GC Mearten, DJ Minson) (USDA-ARS: Springfield VA, USA)

Craig A (1992) Pasture production of two cultivars of *Trifolium subterraneum* at Kybybolite, South Australia. *Australian Journal of Experimental Agriculture* **32**, 611–617. doi:10.1071/EA9920611

Davidson JL, Donald CM (1958) The growth of swards of subterranean clover with particular reference to leaf area. *Australian Journal of Agricultural Research* **9**, 53–72. doi:10.1071/AR9580053

Davies LH (1986) Limitations to livestock production associated with phytoestrogens and bloat. In 'Temperate pastures: their production, use and management'. (Eds JL Wheeler, CJ Pearson, GE Robards) pp. 446–456. (Australian Wool Corporation/CSIRO Australia: Melbourne, Vic., Australia)

Dear BS, Sandral GA, Peoples MB, Wilson BCD, Taylor JN, Rodham CA (2003) Growth, seed set and nitrogen fixation of 28 annual legume species on 3 Vertosol soils in southern New South Wales. *Animal Production Science* **43**, 1101–1115. doi:10.1071/EA02141

Devitt AC, Francis CM (1972) The effect of waterlogging on the mineral nutrient content of '*Trifolium subterraneum*'. *Australian Journal of Experimental Agriculture* **12**, 614. doi:10.1071/EA9720614

Ehrman T, Cocks PS (1996) Reproductive patterns in annual legume species on an aridity gradient. *Vegetatio* **122**, 47–59. doi:10.1007/BF00052815.

Enkhbat G, Ryan MH, Foster KJ, Nichols PGH, Kotula L, Hamblin A, Inukai Y, Erskine W (2021) Large variation in waterlogging tolerance and recovery among the three subspecies of *Trifolium subterranean* L. is related to root and shoot responses. *Plant and Soil* 464, 467–487. doi:10.1007/s11104-021-04959-0

Erskine W, Adham Y, Holly L (1989) Geographic distribution of variation in quantitative traits in a world lentil collection. *Euphytica* **43**, 97–103. doi:10.1007/BF00037901

Falconer DS, Mackay TFC (1996) 'Introduction to quantitative genetics', 4th edn. (Pearson/Longman: England)

Francis CM (1976) 'Observations on the ecology of subterranean clover in Greece and Crete: a report based on a seed collection tour 1976', (Western Australian Department of Agriculture: Perth, WA, Australia)

Francis CM, Devitt AC (1969) The effect of waterlogging on the growth and isoflavone concentration of *Trifolium subterraneum* L. *Australian Journal of Agricultural Research* **20**, 819–825. doi:10.1071/AR9690819

Francis CM, Millington AJ (1965) Varietal variation in the isoflavone content of subterranean clover: its estimation by a microtechnique. *Australian Journal of Agricultural Research* **16**, 557. doi:10.1071/AR9650557

Francis CM, Devitt AC, Steele P (1974) Influence of flooding on the alcohol dehydrogenase activity of roots of *Trifolium subterraneum* L. *Australian Journal of Plant Physiology* **1**, 9–13. doi:10.1071/PP9740009

Ghamkhar K, Snowball R, Bennett SJ (2007) Ecogeographical studies identify diversity and potential gaps in the largest germplasm collection of bladder clover (*Trifolium spumosum* L.). *Australian Journal of Agricultural Research* **58**, 728–738. doi:10.1071/AR06359

Ghamkhar K, Isobe S, Nichols PGH, Faithfull T, Ryan MH, Snowball R, Sato S, Appels R (2012) The first genetic maps for subterranean clover (*Trifolium subterraneum* L.) and comparative genomics with *T. pratense* L. and *Medicago truncatula* Gaertn. to identify new molecular markers for breeding. *Molecular Breeding* **30**, 213–226. doi:10.1007/s11032-011-9612-8

Ghamkhar K, Nichols PGH, Erskine W, Snowball R, Murillo M, Appels R, Ryan MH (2015) Hotspots and gaps in the world collection of subterranean clover (*Trifolium subterraneum* L.). *Journal of Agricultural Science* **153**, 1069–1083. doi:10.1017/S0021859614000793

Gibberd MR, Cocks PS (1997) Effect of waterlogging and soil pH on the micro-distribution of naturalised annual legumes. *Australian Journal of Agricultural Research* **48**, 223–230. doi:10.1071/A96074

Gladstones JS (1967) Naturalized subterranean clover strains in Western Australia: a preliminary agronomic examination. *Australian Journal of Agricultural Research* **18**, 713–731. doi:10.1071/AR9670713

Gladstones JS, Collins WJ (1984) Naturalized subterranean clover strains of Western Australia. Technical Bulletin No. 64. Western Australian Department of Agriculture, Perth, WA, Australia.

Hijmans RJ, Cameron SE, Parra JL, Jones PG, Jarvis A (2005) Very high resolution interpolated climate surfaces for global land areas. *International Journal of Climatology* **25**, 1965–1978. doi:10.1002/joc.1276

Hill MJ (1996) Defining the white clover zone in eastern mainland Australia using a model and a geographic information system. *Ecological Modelling* **86**, 245–252. doi:10.1016/0304-3800(95)00059-3

Hungerford T (1975) 'Diseases of livestock', (McGraw-Hill: Sydney, NSW, Australia)

Katznelson JS (1966) Report on seed collection tour in Greece, Yugoslavia and northern Italy. Technical Pamphlet No. 101. Volcani Institute, Israel.

Katznelson J, Morley FHW (1965) A taxonomic revision of sect. *Calycomorphum* of the genus *Trifolium*. I. The geocarpic species. *Israel Journal of Botany* **14**, 112–134.

Marshall T, Millington A (1967) Flooding tolerance of some Western Australian pasture legumes. *Australian Journal of Experimental Agriculture* **7**, 367–371. doi:10.1071/EA9670367

Millington AJ, Francis CM, McKeown NR (1964) Wether bioassay of annual pasture legumes. II. The oestrogenic activity of nine strains of *Trifolium subterraneum* L. *Australian Journal of Agricultural Research* **15**, 527.

Morley FHW, Francis CM (1968) Varietal and environmental variations in isoflavone concentrations in subterranean clover (L.). *Australian Journal of Agricultural Research* **19**, 15–26. doi:10.1071/AR9680015

Mousavi-Derazmahalleh M, Bayer PE, Nevado B, Hurgobin B, Filatov D, Kilian A, Kamphuis LG, Singh KB, Berger JD, Hane JK, Edwards D, Erskine W, Nelson MN (2018) Exploring the genetic and adaptive diversity of a pan-Mediterranean crop wild relative: narrow-leafed lupin. *Theoretical and Applied Genetics* 131, 887–901. doi:10.1007/s00122-017-3045-7

Nichols PGH (2013) Development of R&D pre-breeding plans for annual legumes. Report preperad for Meat & Livestock Australia for

project P.BE.0028. Available at https://www.mla.com.au/research-and-development/reports/2014/development-of-rd-plans-for-annual-legumes/
Nichols PGH, Collins WJ, Barbetti MJ (1996) Registered cultivars of subterranean clover: their characteristics, origin and identification. Bulletin No. 4327. Agriculture Western Australia, Perth, WA, Australia.
Nichols PGH, Cocks PS, Francis CM (2009) Evolution over 16 years in a bulk-hybrid population of subterranean clover (*Trifolium subterraneum* L.) at two contrasting sites in south-western Australia. *Euphytica* **169**, 31–48. doi:10.1007/s10681-009-9906-7
Nichols PGH, Loi A, Nutt BJ, Evans PM, Craig AD, Pengelly BC, Dear BS, Lloyd DL, Revell CK, Nair RM, Ewing MA, Howieson JG, Auricht GA, Howie JH, Sandral GA, Carr SJ, De Koning CT, Hackney BF, Crocker GJ, Snowball R, Hughes SJ, Hall EJ, Foster KJ, Skinner PW, Barbetti MJ, You MP (2007) New annual and short-lived perennial pasture legumes for Australian agriculture: 15 years of revolution. *Field Crops Research* **104**, 10–23. doi:10.1016/j.fcr.2007.03.016
Nichols P, Loi A, Nutt B, Snowball R, Revell C (2010) Domestication of new mediterranean annual pasture legumes. In 'Sustainable use of genetic diversity in forage and turf breeding'. pp. 137–141. (Springer: Dordrecht, Netherlands)
Nichols PGH, Revell CK, Humphries AW, Howie JH, Hall EJ, Sandral GA, Ghamkhar K, Harris CA (2012) Temperate pasture legumes in Australia – their history, current use, and future prospects. *Crop & Pasture Science* **63**, 691–725. doi:10.1071/CP12194
Nichols PGH, Foster KJ, Piano E, Pecetti L, Kaur P, Ghamkhar K, Collins WJ (2013) Genetic improvement of subterranean clover (*Trifolium subterraneum* L.). 1. Germplasm, traits and future prospects. *Crop & Pasture Science* **64**, 312–346. doi:10.1071/CP13118
Oram RN (1989) 'Register of Australian herbage plant cultivars', (Australian Herbage Plant Registration Authority, Division of Plant Industry, CSIRO: Melbourne, Vic., Australia)
PBR (2021) Plant Breeders Rights database. IP Australia, Australian Government, Canberra, ACT, Australia. Available at https://www.ipaustralia.gov.au/plant-breeders-rights [Accessed 12 January 2021]
Pecetti L, Piano E (1993) Influence of the environment of origin on early dry matter yield and plant morphology in subterranean clover. In 'Management of Mediterranean shrublands and related forage resources'. REUR Technical Series 28. pp. 20–23. (FAO: Rome, Italy)
Pecetti L, Piano E (1997) Leaf size variation in subterranean clover (*Trifolium subterraneum* L. *sensu lato*). *Genetic Resources and Crop Evolution* **45**, 161–165. doi:10.1023/A:1008616706588
Pecetti L, Piano E (2002) Variation of morphological and adaptive traits in subterranean clover populations from Sardinia (Italy). *Genetic Resources and Crop Evolution* **49**, 189–197. doi:10.1023/A:1014795116603
Pecetti L, Carroni AM, Annicchiarico P (2020) Performance and adaptability of subterranean clover pure lines and line mixtures of different complexity across contrasting Mediterranean environments. *Field Crops Research* **256**, 107907. doi:10.1016/j.fcr.2020.107907
Piano E (1984) Preliminary observations on the structure and variability of Sardinian populations of subterranean clover. *Genetica Agraria* **38**, 75–90.
Piano E, Spanu F, Pecetti L (1993) Structure and variation of subterranean clover populations from Sicily, Italy. *Euphytica* **68**, 43–51. doi:10.1007/BF00024153
Piano E, Pecetti L, Carroni AM (1996) Climatic adaptation in subterranean clover populations. *Euphytica* **92**, 39–44. doi:10.1007/BF00022826
Porqueddu C, Ates S, Louhaichi M, Kyriazopoulos AP, Moreno G, Pozo A, Ovalle C, Ewing MA, Nichols PGH (2016) Grasslands in 'Old World' and 'New World' Mediterranean-climate zones: past trends, current status and future research priorities. *Grass and Forage Science* **71**, 1–35. doi:10.1111/gfs.12212
Reed KFM, Schroder PM, Eales JW, McDonald RM, Chin JF (1985) Comparative productivity of *Trifolium subterraneum* and *T. yanninicum* in south-western Victoria. *Australian Journal of Experimental Agriculture* **25**, 351–361. doi:10.1071/EA9850351
Revell CK, Ewing MA, Nutt BJ (2012) Breeding and farming system opportunities for pasture legumes facing increasing climate variability in the south-west of Western Australia. *Crop & Pasture Science* **63**, 840–847. doi:10.1071/CP12160
Rossiter RC (1959) The influence of maturity grading on total yield and seed production in strains of *Trifolium subterraneum* L. grown as single plants and in swards. *Australian Journal of Agricultural Research* **10**, 305. doi:10.1071/AR9590305
Rosso BS, Pagano EM (2005) Evaluation of introduced and naturalised populations of red clover (*Trifolium pratense* L.) at Pergamino EEA-INTA, Argentina. *Genetic Resources and Crop Evolution* **52**, 507–511. doi:10.1007/s10722-005-0777-z
Tan BH, Collins WJ (1987) Multi-allelic nature of the locus controlling leaf marking in subterranean clover. *Australian Journal of Agricultural Research* **38**, 547–558.
Tucak M, Cupic T, Popovic S, Stjepanovi M, Gantner R, Meglic V (2009) Agronomic evaluation and utilization of red clover (*Trifolium pratense* L.) germplasm. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca* **37**, 206–210. doi:10.15835/nbha372
Williams R, Evans L, Ludwig L (1964) Estimation of leaf area for clover and lucerne. *Australian Journal of Agricultural Research* **15**, 231–233. doi:10.1071/AR9640231
Zohary M, Heller D (1984) 'The genus *Trifolium*', (The Israel Academy of Science and Humanities: Jerusalem, Israel)

Data availability. The data that support this study are available in the article and accompanying online supplementary material.

Conflicts of interest. The authors declare no conflict of interest. Megan Ryan is an Associate Editor of *Crop and Pasture Science*, but was blinded from peer-review process for this paper.

Declaration of funding. This research was conducted as part of the Annual Legume Breeding Australia (ALBA) Joint Venture between PGG Wrightson Seeds and UWA. The project is under the International Collaborative Program in Agricultural Sciences between Nagoya University and UWA. G.E. is supported by an Australian Government Research Training Program (RTP) scholarship and a Science Industry PhD Fellowship from the Government of Western Australia. Kevin Foster was funded by the UWA-MLA DC project P.PSH.1171.

Acknowledgements. The Australian Pastures Genebank, operated by the South Australian Research and Development Institute, provided seed of all genotypes, and the collection site data were obtained from the former Australian Trifolium Genetic Resource Centre (ATGRC) of the Department of Primary Industries and Regional Development (DPIRD), WA. We thank Bradley Wintle for his advice and assistance in the field. We gratefully acknowledge Michael Blair, Rowan Hungerford, Rosemarie Lugg, Robert Jeffery and Elise Robinson for technical help.

Author affiliations
[A]UWA School of Agriculture and Environment and Institute of Agriculture, The University of Western Australia, 35 Stirling Highway, Crawley, WA 6009, Australia.
[B]Graduate School of Bioagricultural Science at Nagoya University, Furo-cho, Chikusa-ku, Nagoya, Japan.