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# Prolonged isolation and persistence of a common endemic on granite outcrops in both mesic and semi-arid environments in south-western Australia

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## ABSTRACT

**Aim** Granite outcrops may be able to act as refugia for species during adverse climate change, owing to their topographic complexity. We assessed this hypothesis by examining phylogeographical patterns in a common, geographically widespread granite endemic, *Stypandra glauca* (Hemerocallidaceae).

**Location** Granite outcrops of the Southwest Australian Floristic Region, Western Australia.

**Methods** Twenty-four tetraploid individuals of the granite endemic *Stypandra glauca* were sampled from each of 12 granite outcrops: 7 from a mesic environment and 5 from the semi-arid region. Phylogenetic reconstruction and divergence-dating was achieved using Bayesian and parsimony analyses of chloroplast haplotypes from 90 individuals. Nuclear diversity and population differentiation were analysed across all individuals using 10 microsatellite loci.

**Results** *Stypandra glauca* exhibited high (chloroplast) or moderate (nuclear) levels of divergence among, and low diversity within, outcrops. Haplotype diversity was high in both sampling regions, and each haplotype was unique to one outcrop. There was little correlation between geographical and genetic distance. Both nuclear and chloroplast diversity were higher in southern (mesic) outcrops than in northern (semi-arid) outcrops, although the level of chloroplast divergence among outcrops was similar for both climatic regions.

**Main conclusions** The levels of divergence and low diversity revealed in *S. glauca* support a scenario of prolonged isolation and persistence on granite outcrops in both mesic and semi-arid climatic regions, with no evidence of contraction–expansion dynamics across the outcrop network. The higher levels of diversity in the southern populations may result from the maintenance of a larger effective population size in southern regions, which retained more mesic climates during drier glacial periods. Although the climatic conditions differ between outcrops in this study, our results indicate that outcrops in both regions have harboured *S. glauca* throughout climatic changes, accentuating the value of these habitats to biodiversity conservation under future changing climate.

## Keywords

Chloroplast divergence, edaphic endemism, evolutionary history, geoecology, nuclear diversity, phylogeography, Pleistocene refugia, *Stypandra glauca*, Western Australia.

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## INTRODUCTION

Patchily distributed rocky outcrop habitats – such as ultramafic outcrops, banded iron formations and granite inselbergs – are widespread throughout the world (Porembski & Barthlott, 2000; Jacobi & Carmo, 2008; Gibson *et al.*, 2010; Harrison & Rajakaruna, 2011). The edaphic environments associated with such habitats present particular challenges and opportunities for life that are not found in the surrounding landscape, fostering the evolution of endemic plant species with specialist life-history traits (Porembski & Barthlott, 2000; Brady *et al.*, 2005; Yates *et al.*, 2011; Poot *et al.*, 2012). From tropical to temperate regions, these species make a significant contribution to regional species diversity (Brady *et al.*, 2005; Jacobi & Carmo, 2008; Gibson *et al.*, 2012). For example, the ultramafic habitats in the mediterranean-type climate of California only occupy approximately 1.5% of the region, but support 12.5% of the region's endemic flora (Safford *et al.*, 2005). Similarly, in the Southwest Australian Floristic Region (SWAFR) (Hopper & Gioia, 2004), granite outcrops occupy less than 1% of the region, but support *c.* 17% of its vascular native flora (Hopper *et al.*, 1997).

Many rocky-outcrop ecosystems are insular in nature, owing to their often sharply defined edaphic boundaries with the surrounding landscape (Porembski & Barthlott, 2000; Kruckeberg, 2002; Harrison & Rajakaruna, 2011). Specialist traits that allow rocky-outcrop endemics to prosper in these environments, including specialist root morphology, drought tolerance, heavy-metal tolerance, and a tolerance to low levels of macronutrients, may be maladaptive or make species less competitive in other environments, further reinforcing the idea of these ecosystems as edaphic islands (Anacker *et al.*, 2011; O'Dell & Rajakaruna, 2011; Poot *et al.*, 2012). Some authors, however, emphasize the connectivity and collective nature of rocky outcrops linked by dispersal through the aeolian zone (Swan, 1992; Main, 1997), although Spasojevic *et al.* (2012) found that habitat quality was more important than dispersal in maintaining the connectivity of patchy habitats. Interactions between granite outcrops and the atmosphere show that thermal draughts above granite outcrops lift air to considerable heights (Szarzynski, 2000), increasing the likelihood of long-distance seed dispersal.

Phylogeography is a useful tool for investigating the historical range dynamics of species in response to Pleistocene climate changes, but there have been relatively few phylogeographical studies on endemic plant species from patchily distributed rocky-outcrop ecosystems (although see Levy & Neal, 1999; Mayer & Soltis, 1999; Byrne & Hopper, 2008; Duputié *et al.*, 2009; Boisselier-Dubayle *et al.*, 2010; Tapper *et al.*, 2014). Understanding historical patterns of extinction, dispersal and colonization in these habitats is particularly important in an era of anthropogenic climate change that is predicted to have a largely negative impact on the geographical ranges of many species (Thomas *et al.*, 2004). If patchily distributed rocky-outcrop habitats are connected, it is possible that endemic species have been, and will be, able to

contract and disperse from particular outcrops that serve as refugia (Keppel *et al.*, 2012; Damschen *et al.*, 2012). Alternatively, if rocky outcrops are not well connected then endemic species may have become isolated, with persistence through historical climatic change being the major response.

The SWAFR is ideal for investigating the phylogeography of species confined to patchily distributed rocky outcrops. Much of the region is underlain by the Yilgarn Craton and contains numerous granite outcrops of varying size and isolation that occur across a rainfall gradient spanning mesic to semi-arid mediterranean-type climate zones. The region remained free of ice throughout the Pleistocene, although regimes of fluctuating temperature and precipitation during glacial/interglacial cycles have been hypothesized to have had a significant influence on the flora (Hopper, 1979).

Phylogeographical analyses of granite outcrop endemics in the SWAFR have so far failed to find evidence for specific outcrops acting as Pleistocene refugia. Instead, genetic studies have revealed prolonged isolation and persistence on granite outcrops with subsequent divergence, rather than the patterns of contraction and expansion that are typical of refugial dynamics (Moran & Hopper, 1983; Sampson *et al.*, 1988; Yates *et al.*, 2007; Byrne & Hopper, 2008; Levy *et al.*, 2012). Most studies have, however, focused on rare granite endemics confined to a small number of outcrops, suggesting that patterns may be idiosyncratic due to specific aspects of the biology of these species. Recent phylogeographical analysis of the common granite endemic *Kunzea pulchella* (Myrtaceae) revealed similar patterns of isolation and persistence to those observed in rare granite endemics (Tapper *et al.*, 2014). Although *K. pulchella* is common, its distribution is primarily confined to the semi-arid regions of the SWAFR. Phylogeographical analysis of species with distributions that span the region's entire rainfall gradient, including mesic environments, could assess how populations have responded to historical climatic fluctuations in differing climate environments. The greater climatic stability suggested for mesic areas may have facilitated greater population connectivity there than in semi-arid areas (Byrne, 2008; Byrne *et al.*, 2011).

The perennial herb *Stypandra glauca* R.Br. (Hemerocallidaceae) is an excellent focal species for studying phylogeographical patterns in a common and geographically widespread granite endemic of the SWAFR. This generalist, insect-pollinated species grows predominantly along the margins of granite outcrops or in cracks, fissures and shallow depressions on their surface, but also occurs in hillside gullies and along water-courses where granite is present (Russell, 1988). The species is primarily tetraploid, although diploid and tetraploid populations of *S. glauca* have both been identified through chromosome counts (Keighery, 1984; Russell, 1988). No population has been found to contain both diploid and tetraploid individuals, and no morphological or ecological differences associated with ploidy have been noted. *Stypandra glauca* occurs across the entire SWAFR and, as currently circumscribed, occurs in temperate areas of eastern Australia, although the full distribution may represent a spe-

cies complex (Fig. 1; Hopper, 1999). Within the SWAFR, *S. glauca* is common and occurs on the many granite outcrops present across the region. The species occurs across a broader range of climatic conditions than previously studied granite endemics, enabling the effects of mesic and semi-arid environments on connectivity to be investigated.

Here, we investigate whether the common *S. glauca* shows genetic signals typical of contraction and expansion to and from putative refugia, and whether the species exhibits genetic connectivity between outcrops. We focused our investigation on clusters of adjacent rocks in the north and south of the species' distribution in order to test the hypothesis that patterns of connectivity among outcrops differ between the mesic forested environment of the south and the semi-arid rangeland environment to the north.

## MATERIALS AND METHODS

### Collections and DNA analysis

DNA was extracted from leaf material of 24 tetraploid (see Results) *S. glauca* individuals from each of 12 granite outcrops in the SWAFR (Table 1). Plant samples were collected from five semi-arid outcrops and seven mesic outcrops (Fig. 1, Table 1). Leaf material from eight individuals of two *S. jamesii* Hopper populations and one individual of *Dianella revoluta* R.Br. were collected for use as outgroups. *Stypanandra jamesii* is currently the only other recognized species in the genus *Stypanandra* (Hopper, 1999). *Dianella* is related to *Stypanandra*, together forming a polytomy in the phormioid clade within Hemerocallidaceae (Wurdack & Dorr, 2009).

Approximately 40 mg of lyophilized leaf material was ground to a fine powder, and DNA was extracted following Doyle & Doyle (1987) with the inclusion of 1% polyvinylpyrrolidone to the extraction buffer. We undertook analysis of the chloroplast genome using sequence data, and the nuclear genome using microsatellite data. The mode of chloroplast inheritance has not been determined in *Stypanandra* or

the Hemerocallidaceae, but we assumed it to be maternally inherited and thus transmitted through seed, as is common among angiosperms.

Six non-coding cpDNA regions that have been found useful for phylogeographical studies in Australian plants (Byrne & Hankinson, 2012) were trialled in *S. glauca*, and the three intergenic spacer regions that produced the highest sequence quality and nucleotide diversity were selected for further analysis (*psbD-trnT*, *trnQ-rps16* and *trnV-ndhC*). Eight samples were sequenced from each of 12 *S. glauca* and two *S. jamesii* populations, along with one *D. revoluta* sample (see Appendix S1 for details of amplification and sequence alignment).

Ten nuclear microsatellite loci were amplified and scored for 24 individuals from each of 12 populations, using primers and protocols described by Tapper *et al.* (2013). To quantify scoring error, amplification and genotyping was repeated for 5% of samples across all loci, and the results compared in PEDANT 1.0 (Johnson & Haydon, 2007).

### Chloroplast sequence data analysis

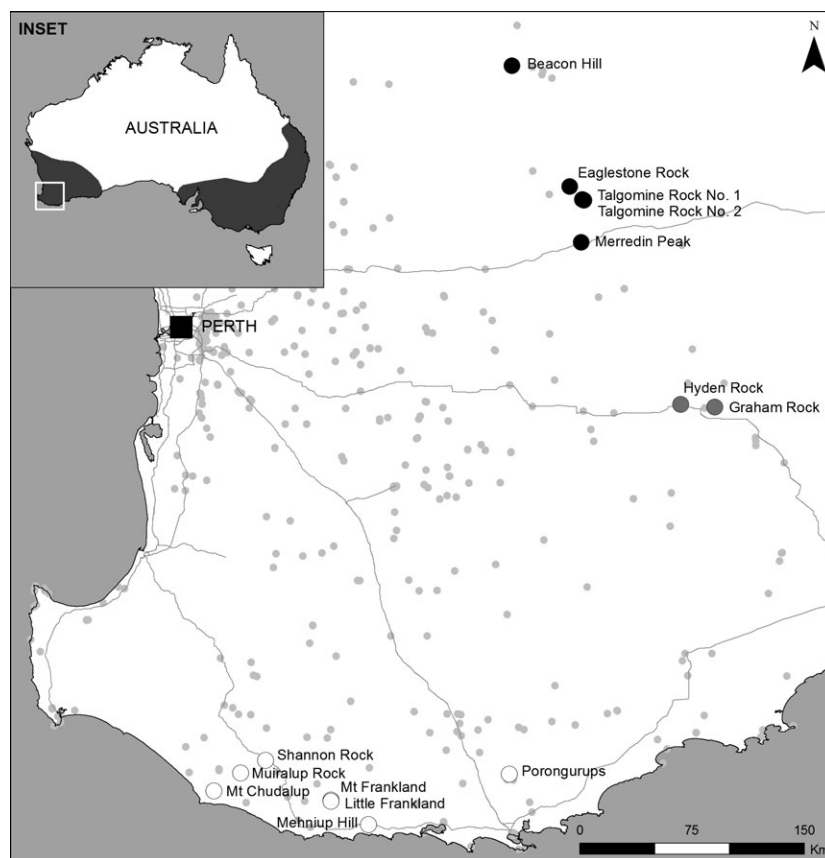
Chloroplast sequences were analysed in DNASP 5.10 (Librado & Rozas, 2009) to identify haplotypes and calculate nucleotide and haplotype diversity. Parsimony analysis was conducted in PAUP\* 4.0b10 (Swofford, 2003) using 1000 heuristic search replicates with the tree bisection–reconnection (TBR) branch-swapping algorithm. Bootstrapping was conducted in PAUP\* using heuristic searches with simple stepwise addition and 1000 replicates. Homoplasy was assessed through the consistency index (CI) and retention index (RI).

Bayesian analysis was conducted in BEAST 1.6.1 (Drummond & Rambaut, 2007) using an uncorrelated lognormal relaxed clock for the rate variation among branches. Two Markov chain Monte Carlo (MCMC) chains were run for 5 million generations, sampling trees every 1000 generations, and adopting the GTR substitution model. The root-height prior was normally distributed and calibrated using a suggested divergence date for *Stypanandra* and *Dianella* of 2.87 million years ago (Ma) (95%

**Table 1** The mean annual rainfall and population locality information of *Stypanandra glauca* and *S. jamesii* samples from south-western Australia used in this study. Mean annual rainfall is derived from the WorldClim database.

Location	Mean annual rainfall (mm)	Locality details	Latitude, longitude
Beacon Hill	312	4.8 km NNE of Beacon	30° 24' 34.15" S, 117° 52' 39.23" E
Eaglestone Rock	315	12.9 km ENE of Nungarin	31° 08' 07.88" S, 118° 13' 26.81" E
Talgomine Rock 1	319	19.2 km W of Nungarin	31° 12' 48.45" S, 118° 18' 0.09" E
Talgomine Rock 2	318	20.5 km W of Nungarin	31° 12' 59.21" S, 118° 18' 34.33" E
Merredin Peak	326	1.9 km NE of Merredin	31° 28' 09.31" S, 118° 17' 33.26" E
Little Frankland	1044	18 km NNE of Walpole	34° 49' 13.87" S, 116° 47' 22.34" E
Mehniup Hill	1087	6 km E of Bow Bridge	34° 58' 04.29" S, 117° 00' 59.10" E
Mount Chudalup	1208	8.6 km NE of Windy Harbour	34° 45' 51.60" S, 116° 05' 11.10" E
Mount Frankland	1053	17.4 km NNE of Walpole	34° 49' 35.13" S, 116° 47' 23.04" E
Muiralup Rock	1128	11.7 km ESE of Northcliffe	34° 39' 26.33" S, 116° 14' 51.75" E
Porongurups	677	Porongurups National Park	34° 39' 53.83" S, 117° 51' 40.17" E
Shannon Rock	1024	21 km SE of Quinninup	34° 34' 55.47" S, 116° 23' 49.11" E
<i>S. jamesii</i> Graham Rock	334	Graham Rock Nature Reserve	32° 27' 35.95" S, 119° 05' 44.18" E
<i>S. jamesii</i> Hyden Rock	333	Wave Rock Reserve	32° 26' 36.18" S, 118° 53' 29.21" E

**Figure 1** Map illustrating the study area in south-western Australia and the distribution of *Stypandra glauca*, with the location of 12 granite outcrops where *S. glauca* was collected and two granite outcrops where *S. jamesii* was collected for genetic analysis. Small grey dots indicate the locations of known populations based on records at the Perth Herbarium (<http://www.florabase.dpaw.wa.gov.au/>). Black circles: populations of the northern sampling region; white circles: populations of the southern sampling region; grey circles: populations of *S. jamesii*. The shaded areas of the inset map indicate the known natural distribution of *S. glauca*.



confidence interval: 0.99–5.79 Ma) from a molecular analysis (*ndhF* and *trnL-trnF*) (M. Crisp, Australian National University, Canberra, pers. comm.), with dating based on the Eocene *Dianella*-like leaf fossil described by Conran *et al.* (2003). The results were imported into TRACER 1.5 (Rambaut & Drummond, 2007) to assess convergence among runs. The maximum product of clade credibilities (MCC) tree was identified in TREEANNOTATOR (Drummond & Rambaut, 2007), with the first 25% of trees discarded as burn-in. A median-joining maximum-parsimony (MJMP) network of haplotypes was constructed in NETWORK 4.6.1.1 (Bandelt *et al.*, 1999), with indels treated as binary characters and epsilon set to zero.

To assess population-size changes and sequence neutrality,  $R_2$  (Ramos-Onsins & Rozas, 2002) and Tajima's  $D$  (Tajima, 1989) were calculated in DNASP across all samples and within each major clade.

### Microsatellite data analysis

Most models that examine genetic diversity or population structure require allele frequencies to be quantified. Partial heterozygotes and the complexity of inheritance patterns in polyploids complicate the estimation of allele frequencies (De Silva *et al.*, 2005). Allele copy number could not be inferred using the relative fluorescence intensity of fragments, because peak-height ratios were inconclusive. Consequently, data sets were phenotypic (describing the identity and presence of observed alleles, but not allele copy numbers) and

analysed in two formats, as in Sampson & Byrne (2012). Firstly, the alleles observed in each individual were combined to produce a multilocus phenotype. Secondly, each microsatellite allele was treated as a variable scored as present or absent, i.e. loci were treated as dominant.

The number of individuals with each multilocus phenotype was determined in GENALEX 6.41 (Peakall & Smouse, 2006). Standard genetic parameters were calculated and Simpson's diversity index, modified for finite sample size, was calculated as the probability that two individuals have a different multilocus phenotype (Robertson *et al.*, 2010). We derived estimates of annual precipitation ( $P_{ann}$ ) for individual outcrops using the WorldClim database (<http://www.worldclim.org/>). Northern outcrops had lower rainfall (mean annual precipitation 312–334 mm, average  $318 \pm 2$  mm) than southern outcrops (mean annual precipitation 677–1087 mm, average  $1031 \pm 64$  mm). We used two-tailed unpaired Student's  $t$ -tests to assess whether diversity differed between regions using STATISTICA 6 (StatSoft, 2001);  $H_O$  was arcsine-transformed for the  $t$ -test.

The binary-coded data was entered into ARLEQUIN 3.5 (Excoffier & Lischer, 2010), where average pairwise differences between populations were calculated and assessed by tests of 1000 permutations. The partitioning of genetic variation was assessed by analyses of molecular variance (AMOVAs) within populations, among populations and between regions, across 1000 permutations. The transformation of codominant markers to binary requires that they be analysed as dominant mark-

ers. There is no accepted methodology for the calculation of genetic similarity between organisms with dominant markers, but it is reasonable to use genetic similarity indices as descriptive measures, because banding patterns in polyploids specify phenotypes (Kosman & Leonard, 2005). Thus, Nei's (1972) genetic distance was calculated on binary phenotypic data using AFLP-SURV (Vekemans *et al.*, 2002), and entered into the NEIGHBOR and CONSENSE programs in PHYLIP 3.69 to produce a neighbour-joining (NJ) tree from 1000 bootstrap replicates (Felsenstein, 1989). The NJ tree cannot be used for inferring genetic relationships between populations but provides a means of comparison to other analyses.

An individual-level distance matrix was calculated following Bruvo *et al.* (2004) in POLYSAT (Clark & Jasieniuk, 2011). This measure considers distances between alleles in the absence of information on allele copy numbers. A principal coordinates analysis (PCoA) of Bruvo's distance (PCoA-BD) was conducted in POLYSAT. For comparison, a second PCoA was generated in GENALEX using Euclidean distances (PCoA-ED) obtained from ARLEQUIN.

Allele frequencies were estimated in POLYSAT using the 'simpleFreq' function, and a pairwise  $F_{ST}$  matrix was calculated. It should be noted, however, that the simpleFreq method does not account for inbreeding, and may underestimate  $F_{ST}$ . Isolation by distance (IBD) was assessed through the correlation of  $F_{ST} / (1 - F_{ST})$  with log-transformed geographical distances using a Mantel test with 9999 random permutations to test for significance. For comparison, the same analyses were conducted using Euclidean distances calculated using ARLEQUIN.

The number of genetically homogenous groups ( $K$ ) and the affinities of individuals to those groups were estimated in STRUCTURE 2.3.3 (Pritchard *et al.*, 2000). This analysis adopted an admixture model of ancestry, the assumption of independent alleles, and a burn-in length of 100,000 followed by 100,000 MCMC replications. Simulations were repeated 10 times for each  $K$  value (1–15). The most probable value of  $K$  was identified using the method of Evanno *et al.* (2005) in STRUCTURE HARVESTER 0.6.92 (Earl & vonHoldt, 2012; <http://taylor0.biology.ucla.edu/structureHarvester/>). All 10 runs for the optimum  $K$ -value were aligned in CLUMPP 1.1.2 (Jakobsson & Rosenberg, 2007) to obtain a similarity coefficient ( $h'$ ). Clustering patterns were visualized in DISTRICT 1.1 (Rosenberg, 2004). The analysis was repeated for populations of each region separately.

## RESULTS

### Phylogeographical analysis

All three cpDNA regions were variable, totalling 4350 base pairs. With the inclusion of 92 binary-coded indels, the sequences encompassed 331 variable sites, of which 159 were parsimony-informative. Six *S. glauca* samples and one *S. jamesii* sample were omitted from the analyses because they consistently failed to produce high-quality data for at

least one region. Analysis revealed 37 unique haplotypes, including 10 unique to *S. jamesii* (Table 2). No haplotype was found on more than one outcrop. Parsimony analysis resulted in 83 equally most parsimonious trees of 402 steps (CI: 0.84; RI: 0.98; Fig. 2).

Nucleotide and haplotype diversity were 0.006 and 0.884, respectively. The number of haplotypes per population ranged from one to four in southern populations, averaging 2.6. Northern populations had up to three haplotypes per population, averaging 1.8 (Table 3). Nucleotide and haplotype diversity were lower in northern populations (0.001 and 0.784, respectively) than in southern populations (0.005 and 0.899, respectively) (Table 3). Tajima's  $D$  was not significant ( $D = 1.910$ ;  $P > 0.05$ ) for the entire dataset, for northern populations ( $D = 0.696$ ;  $P > 0.05$ ) or for southern populations ( $D = 0.906$ ;  $P > 0.05$ ) (Table 3).

Bayesian analysis revealed a divergence into two major clades approximately 875,000 years ago. Northern populations were paraphyletic with respect to Shannon Rock from the south (posterior probability, PP: 1.0; bootstrap support, BS: 100) (Fig. 2). With the exception of Shannon Rock, all southern populations were resolved in one major clade. Samples from Shannon Rock were re-extracted and resequenced to confirm genotypes, and an additional set of samples were analysed, all providing the same phylogenetic result. Both *S. jamesii* populations were nested within the southern clade (PP 0.99). The divergence of *S. jamesii* from *S. glauca* was estimated at approximately 97,000 years ago. Within the predominantly northern clade, the Shannon Rock and Beacon Hill populations diverged approximately 283,000 years ago. Aside from the divergence of the two major clades, and of haplotype H36 (Mount Frankland), all divergences occurred in the middle to late Pleistocene. The 13 poorly supported branches (PP < 0.8) were restricted to more recent divergence events, predominantly between haplotypes within populations. The MJMP network was congruent with Bayesian and parsimony analyses, separating two major clades, and with the exception of haplotype H36 (Mount Frankland), all haplotypes were clustered closely with those of the same granite outcrop (Fig. 3). Talgominie Rock 1 and 2 of the northern region are less than one kilometre apart and possessed related haplotypes. Conversely, two of the southern populations, Mount Frankland and Little Frankland, were highly divergent despite being less than 700 m apart.

### Population genetic analysis

As expected of a predominantly tetraploid species, the majority of individuals (77%) exhibited three or four alleles for at least one locus. Since no population exhibited fewer than three alleles across all samples and loci, no purely diploid populations were identified. Many samples from Little Frankland and Merredin Peak (83% and 63%, respectively) had fewer than three alleles across all loci, but without chromosome counts or flow cytometry, the ploidy of these individu-

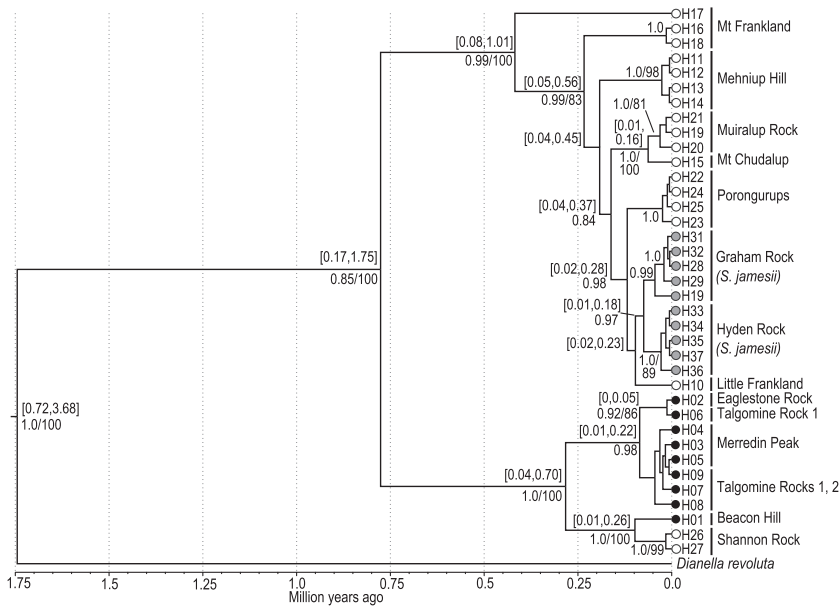
**Table 2** List of haplotypes uncovered in 12 *Stypandra glauca* and two *S. jamesii* populations in south-western Australia via sequencing of *trnV-ndhC*, *psbD-trnT* and *trnQ-rps16* chloroplast intergenic spacer regions. Numbers in parentheses refer to the number of individuals.

Haplotype	Location	GenBank accession numbers		
		<i>trnV-ndhC</i>	<i>psbD-trnT</i>	<i>trnQ-rps16</i>
H01	Beacon Hill (8)	JX875105–JX875108, JX875110–JX875112	JX875199–JX875206	JX875295–JX875302
H02	Eaglestone Rock (8)	JX875121–JX875128	JX875215–JX875222	JX875311–JX875318
H03	Merredin Peak (1)	JX875155	JX875250	JX875345
H04	Merredin Peak (3)	JX875157–JX875159	JX875252–JX875254	JX875347–JX875349
H05	Merredin Peak (4)	JX875152–JX875154, JX875156	JX875247–JX875249, JX875251	JX875342–JX875344, JX875346
H06	Talgomine Rock 1 (1)	JX875187	JX875282	JX875377
H07	Talgomine Rock 1 (7)	JX875184–JX875186, JX875188–JX875191	JX875279–JX875281, JX875283–JX875286	JX875374–JX875376, JX875378–JX875381
H08	Talgomine Rock 2 (1)	JX875195	JX875290	JX875385
H09	Talgomine Rock 2 (5)	JX875192–JX875194, JX875196, JX875197	JX875287–JX875289, JX875292, JX875293	JX875382–JX875384, JX875387, JX875388
H10	Little Frankland (7)	JX875137–JX875143	JX875231–JX875238	JX875326–JX875333
H11	Mehniup Hill (1)	JX875150	JX875245	JX875340
H12	Mehniup Hill (1)	JX875149	JX875244	JX875339
H13	Mehniup Hill (5)	JX875144–JX875148	JX875239–JX875243	JX875334–JX875338
H14	Mehniup Hill (1)	JX875151	JX875246	JX875341
H15	Mount Chudalup (8)	JX875113–JX875120	JX875207–JX875214	JX875303–JX875310
H16	Mount Frankland (1)	JX875129	JX875223	JX875319
H17	Mount Frankland (2)	JX875131, JX875133	JX875225–JX875227	JX875321–JX875323
H18	Mount Frankland (4)	JX875130, JX875132, JX875134, JX875135	JX875224, JX875226, JX875228, JX875229	JX875320, JX875322, JX875324, JX875325
H19	Muiralup Rock (1)	JX875166	JX875261	JX875356
H20	Muiralup Rock (3)	JX875164, JX875165, JX875167	JX875259, JX875260, JX875262	JX875354, JX875355, JX875357
H21	Muiralup Rock (4)	JX875160–JX875163	JX875255–JX875258	JX875350–JX875353
H22	Porongurups (3)	JX875169, JX875170, JX875175	JX875264, JX875265, JX875270	JX875359, JX875360, JX875365
H23	Porongurups (1)	JX875168	JX875263	JX875358
H24	Porongurups (1)	JX875172	JX875267	JX875362
H25	Porongurups (1)	JX875171, JX875173, JX875175	JX875266, JX875268, JX875270	JX875361, JX875363, JX875365
H26	Shannon Rock (1)	JX875179	JX875274	JX875369
H27	Shannon Rock (7)	JX875176–JX875178, JX875180–JX875183	JX875271–JX875273, JX875275–JX875278	JX875366–JX875368, JX875370–JX875373
H28	<i>S. jamesii</i> Graham Rock (3)	KC676233, KC676237, KC676238	KC676254, KC676259, KC676260	KC676277, KC676281, KC676282
H29	<i>S. jamesii</i> Graham Rock (1)	KC676234	KC676256	KC676278
H30	<i>S. jamesii</i> Graham Rock (1)	KC676235	KC676257	KC676279
H31	<i>S. jamesii</i> Graham Rock (1)	KC676236	KC676258	KC676280
H32	<i>S. jamesii</i> Graham Rock (1)	KC676232	KC676254	KC676276
H33	<i>S. jamesii</i> Hyden Rock (2)	KC676240, KC676246	KC676262, KC676268	KC676284, KC676290
H34	<i>S. jamesii</i> Hyden Rock (3)	KC676241–KC676243	KC676263–KC676265	KC676285–KC676287
H35	<i>S. jamesii</i> Hyden Rock (1)	KC676239	KC676261	KC676283
H36	<i>S. jamesii</i> Hyden Rock (1)	KC676244	KC676266	KC676288
H37	<i>S. jamesii</i> Hyden Rock (1)	KC676245	KC676267	KC676289

als cannot be determined. It is likely that these individuals represent tetraploids with homozygous genotypes or null alleles, because neither Keighery (1984) nor Russell (1988) discovered populations with both diploids and tetraploids.

A total of 277 alleles were amplified from 10 loci. The two geographical regions shared 93 alleles (34%). There were 199 unique multilocus phenotypes, of which 194 (98%) were

specific to single individuals. Simpson's diversity index was 0.9997. All four genetic diversity measures were significantly higher in the more mesic southern populations than in the semi-arid northern populations (Table 4): total number of alleles,  $A$  (mean difference: 43.34,  $t = 6.898$ ,  $P < 0.001$ ), average proportion of observed heterozygotes per locus,  $H_O$  (mean difference: 0.311;  $t = 4.580$ ,  $P < 0.001$ ), average



**Figure 2** Maximum-clade-credibility tree from Bayesian phylogenetic analysis of three chloroplast DNA intergenic spacer regions (*trnV-ndhC*, *psbD-trnT* and *trnQ-rps16*) in *Stypandra glauca* and *S. jamesii* from south-western Australia, calibrated using a known root age. Branch lengths are scaled according to time, with nodes representing median age estimates and the numbers in parentheses describing 95% confidence intervals for node ages. Posterior probabilities > 0.8 / parsimony bootstrap support > 80% are illustrated on branches. Black, *S. glauca* haplotypes from the north; white, *S. glauca* haplotypes from the south; grey, haplotypes of *S. jamesii*.

**Table 3** Diversity and neutrality measures based on chloroplast sequence data of 90 *Stypandra glauca* and 15 *S. jamesii* individuals from south-western Australia.

	<i>n</i>	Mean haplotypes per population	Haplotype diversity	Nucleotide diversity	Tajima's <i>D</i>
All samples	105	3.1	0.884	0.006	1.910
Northern clade	46	1.8	0.784	0.001	0.696
Southern clade	59	3.3	0.899	0.005	0.906

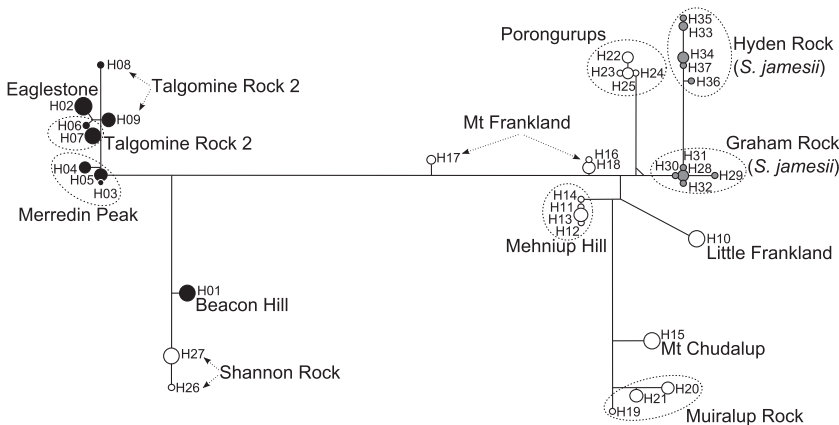
*n*, number of individuals.

number of alleles per individual per locus,  $H'$  (mean difference: 0.464,  $t = 3.454$ ,  $P < 0.01$ ), and average number of allele phenotypes per locus,  $N'_p$  (mean difference: 7.877,  $t = 8.144$ ,  $P < 0.001$ ).

Pairwise  $F_{ST}$  values between southern populations (mean: 0.085) were generally lower than those between populations in the northern region (mean: 0.165) (Appendix S2).  $F_{ST}$  values

among northern populations were similar to those between the regions (mean: 0.197) (Appendix S2). There was support for IBD using  $F_{ST}$  across the entire range, and within the southern region (Table 5), but not for IBD tests based on Euclidean distances (Table 5). Based on AMOVA, almost half (49%) of the total variance was maintained within populations, with near equal amounts of variation among regions and among populations within regions (26% and 25%, respectively).

The NJ tree of Nei's genetic distance indicated divergence between northern and southern outcrops (Fig. 4). The tree supported the division of southern populations into two groupings: Mount Frankland, Little Frankland and Porongurups separate from Mehniup Hill, Shannon Rock, Mount Chudalup and Muiralup Rock. Both PCoAs supported the existence of these three clusters, although the PCoA-ED clustered northern outcrops the most tightly (Appendix S3). PCoA-ED of northern populations only revealed three distinct groups (Fig. 5a). Analysis of the southern region showed the Porongurups population to be distinct, the Mount Frankland and Little Frankland outcrops to be grouped together, and the four remaining outcrops as a separate cluster (Fig. 5b).



**Figure 3** Median-joining maximum-parsimony (MJMP) haplotype network of *Stypandra glauca* from south-western Australia, based on cpDNA sequences. White circles represent southern haplotypes, black circles represent northern haplotypes and grey circles represent *Stypandra jamesii* haplotypes. Circle size in the network is relative to haplotype frequency, and branch lengths indicate the number of mutations between haplotypes.

**Table 4** Nuclear diversity statistics for 12 populations of *Stypandra glauca* in south-western Australia based on analysis of 10 microsatellite loci.

Region/Population	Mean pairwise difference*	<i>n</i>	<i>A</i>	<i>A'</i>	<i>A<sub>p</sub></i>	<i>H<sub>O</sub></i>	<i>H'</i>	<i>N'<sub>p</sub></i>
Northern populations								
Beacon Hill	11.0 ± 0.21	24	61	6.1	3	0.613	1.850	7.9
Eaglestone Rock	7.5 ± 0.19	24	45	4.5	0	0.521	1.638	5.4
Merredin Peak	6.9 ± 0.15	24	51	5.1	9	0.570	1.529	6.6
Talgomine Rock 1	6.5 ± 0.17	24	41	4.1	1	0.479	1.633	5.0
Talgomine Rock 2	5.2 ± 0.14	24	36	3.6	2	0.546	1.629	3.5
Mean ± SE	7.4 ± 0.21	24	46.8		3.0 ± 1.6	0.546	1.656	5.7
Northern total		120	115		25			
Southern populations								
Little Frankland	10.4 ± 0.16	24	90	9.0	5	0.878	2.329	11.1
Mehniup Hill	13.4 ± 0.19	24	76	7.6	6	0.729	1.950	14.0
Mt Chudalup	15.8 ± 0.20	24	79	7.9	8	0.564	1.563	15.2
Mt Frankland	14.1 ± 0.24	24	97	9.7	2	0.848	2.317	11.8
Muiralup Rock	13.8 ± 0.24	24	92	9.2	7	0.817	2.221	13.5
Porongurups	19.1 ± 0.24	24	110	11.0	18	0.704	2.142	15.6
Shannon Rock	15.6 ± 0.22	24	87	8.7	7	0.834	2.317	13.7
Mean ± SE	14.6 ± 0.26	24	90.1		7.57 ± 1.9	0.768	2.120	13.5
Southern total		168	216		126			
Overall total		288	241					

\*Calculated using ARLEQUIN 3.5 (Excoffier & Lischer, 2010); *n*, number of samples; *A*, total number of alleles from all loci; *A'*, average number of alleles per locus; *A<sub>p</sub>*, number of unique alleles; *H<sub>O</sub>*, proportion of observed heterozygotes, averaged over loci; *H'*, number of alleles per locus in an individual, averaged over loci; *N'<sub>p</sub>*, number of different allele phenotypes per locus averaged over loci.

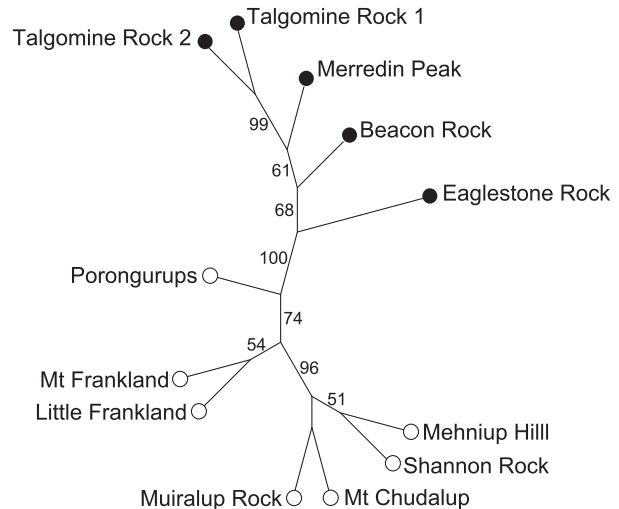
**Table 5** Results of isolation-by-distance tests conducted on populations of *Stypandra glauca* from south-western Australia using Mantel tests of 9999 permutations and based on genetic differentiation (*F<sub>ST</sub>*) or Euclidean distance between populations within and across regions. \**P* < 0.05.

	<i>F<sub>ST</sub></i>		Euclidean distance	
	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>
All populations	0.0002*	0.552	0.188	-0.096
Northern populations	0.440	0.084	0.495	-0.080
Southern populations	0.039*	0.411	0.254	-0.211

Bayesian analysis in STRUCTURE also identified two distinct clusters, corresponding to the two geographical regions, with some admixture evident in Mount Frankland and the Porongurups (Fig. 6a). The two clusters were supported by a peak in  $\Delta K$  at *K* = 2, and a similarity among runs of *h'* = 0.989. When analysed separately, the northern populations were divided into three clusters (*K* = 3, *h'* = 0.996; Fig. 6b) and the southern populations into two clusters (*K* = 2, *h'* = 0.991; Fig. 6c). The three northern and two southern clusters displayed no admixture, consistent with the NJ tree (Fig. 4) and PCoA-ED (Fig. 5a,b).

**DISCUSSION**

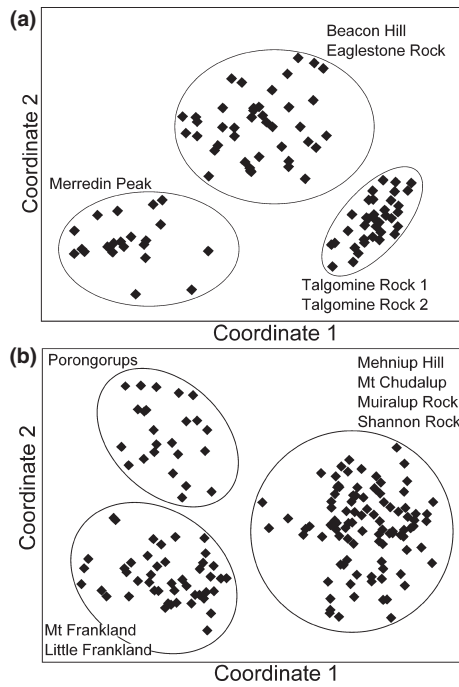
The phylogeographical relationships among populations of *S. glauca* on granite outcrops were similar in the southern mesic and northern semi-arid regions of the species' distri-



**Figure 4** Neighbour-joining tree of Nei's (1972) genetic distance between *Stypandra glauca* populations in south-western Australia based on nuclear microsatellite loci. Black and white circles represent northern and southern populations, respectively. Bootstrap support > 50% is shown on branches.

bution, and are not indicative of dynamics involving contraction to, and expansion from, particular granite outcrops. Rather, *S. glauca* exhibits a genetic signature consistent with a history of prolonged isolation and persistence on specific granite outcrops throughout the Pleistocene climatic oscillations, although outcrops in the mesic region may have maintained higher effective population sizes. The pattern of isolation and persistence previously observed in rare granite





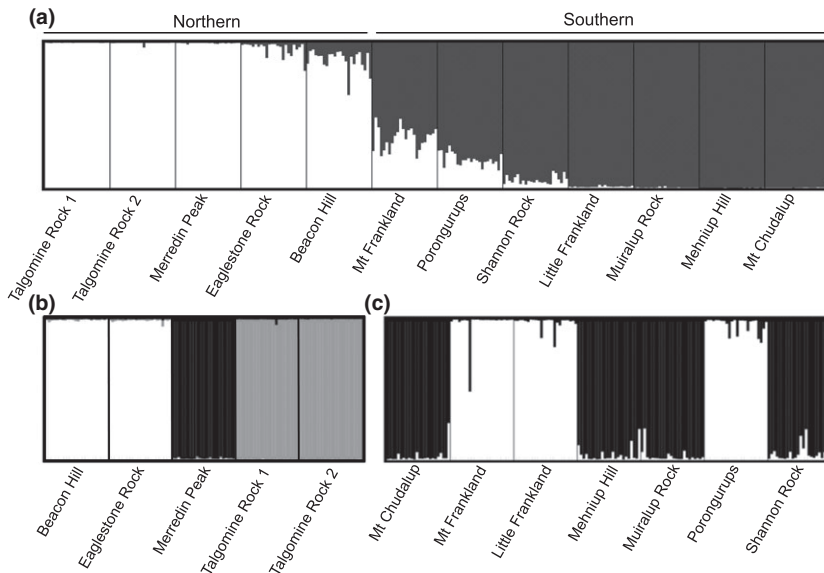
**Figure 5** Principal coordinates analysis (PCoA) of microsatellite diversity based on Euclidean distances (PCoA-ED) between individuals of *Stypandra glauca* from (a) the northern and (b) the southern parts of its range in south-western Australia.

endemics and the widespread *Kunzea pulchella* (Tapper *et al.*, 2014) is also evident in *S. glauca*, which has a wide distribution encompassing both mesic and semi-arid environments in the SWAFR. This result builds on the general patterns of population differentiation that have been observed in some (e.g. Barbará *et al.*, 2007; Boisselier-Dubayle *et al.*, 2010; Moore *et al.*, 2013) but not all (see Duputié *et al.*, 2009) rocky inselberg and serpentine plants, through providing an understanding of the trends in population isolation of edaphic islands in common species and in mesic versus semi-arid environments.

*Stypandra jamesii* was included in this study as an out-group because it is the only other species in the genus. In the chloroplast phylogeny, however, *S. jamesii* was nested within the southern clade of *S. glauca*, indicating that further work and broader sampling is required to determine the relationships between these taxa.

**Isolation and persistence**

Although there are exceptions (Ibrahim *et al.*, 1996; Excoffier & Ray, 2008), expansion from refugia typically leaves a signal of geographically widespread high-frequency haplotypes that occupy central phylogenetic positions and have the highest number of mutational connections to rare haplotypes (Crandall & Templeton, 1993). In contrast to these theoretical expectations, the phylogeographical pattern in *S. glauca* shows low-frequency population-specific haplotypes, suggesting that populations have remained isolated on granite outcrops for long periods. In addition, populations that originated through migration from refugial populations tend to exhibit a pattern of increased nuclear genetic divergence with increasing geographical distance (Byrne, 2008). The genetic patterns of *S. glauca* do not reflect this geographical trend. Aside from comparisons across the two different regions, or for populations less than 2 km apart, there was no correlation between genetic and geographical distance, and the relationships between populations appeared relatively random. Estimates of genetic differentiation were moderate and, although sufficient to prevent inbreeding, were not enough to prevent some effects of drift (Lowe & Allendorf, 2010). These results suggest that populations have diverged independently as a result of prolonged isolation on granite outcrops. Low population diversity, similar variation among regions to that among populations, moderate differentiation, and no strong evidence for IBD within regions, are consistent with relatively low levels of gene flow between outcrops across geographical distances of greater than 2 km.



**Figure 6** Ancestry of *Stypandra glauca* populations in south-western Australia based on microsatellite diversity analysed from codominant polyploidy data using STRUCTURE 2.3.3. Analysis was completed for (a) the entire dataset, as well as separately for (b) the north and (c) the south. The genome of each individual is represented as a single line with shaded segments representing the proportion of each ancestry. Results are an optimal alignment of 10 replicates.

Our investigation of diversity in mesic and semi-arid environments showed a higher level of genetic diversity in mesic populations. Theoretically, higher diversity is expected to be due to larger census population sizes or higher levels of gene flow between populations giving larger effective population sizes, or a combination of the two. Where gene-flow levels are substantial, species typically exhibit a geographical structure to divergence, with adjacent populations more closely related than distant populations. The lower level of genetic differentiation and the presence of IBD in the southern lineage suggest that connectivity through pollen dispersal may have been greater than in the northern region, which is consistent with the greater density of populations in the south than in the north, although the divergence between outcrops in cpDNA suggests limited seed-dispersal. Higher diversity resulting from consistently larger population sizes would also be expected, because greater climate stability through lower-amplitude climatic oscillations in more mesic areas may have enabled larger populations to persist in the south than in the north. Benign climate has been suggested to promote the persistence of small populations, leading to higher levels of species richness in mesic serpentine environments (Anacker & Harrison, 2012).

While rare granite-endemics generally show evidence of isolation and persistence, this study was aimed at a widespread species, occurring across a broad environmental gradient, to enhance the power to detect any genetic signals of expansion and contraction. Despite its widespread nature, *S. glauca* revealed no evidence of range dynamics. Rather, it exhibited phylogeographical patterns consistent with isolation and persistence as seen in the previously studied granite-endemic plants *Kunzea pulchella* (Tapper *et al.*, 2014) and *Eucalyptus caesia* (Byrne & Hopper, 2008) and the ornate dragon lizard *Ctenophorus ornatus* (Levy *et al.*, 2012). The patterns revealed in *S. glauca* and *K. pulchella* illustrate that isolation on granite outcrops is not restricted to rare species, but appears also to be common in widespread granite-endemic species. This pattern of long-term isolation, indicating SWAFR granite outcrops as ancient islands, differs from other phylogeographical studies of inselberg and serpentine endemics, where Pleistocene climatic fluctuations had influenced range dynamics (Mayer & Soltis, 1999; Boisselier-Dubayle *et al.*, 2010; Moore *et al.*, 2013).

### Relationships among regions

Although our sampling strategy was designed to investigate differences in connectivity between rocks within different climatic regions, our analysis also identified some interesting putative phylogenetic relationships among regions. Analyses of cpDNA revealed a divergence of two distinct lineages that was also supported by genetic structure in the nuclear genome. The divergence date of these two lineages was estimated as 875,000 years ago, consistent with major aridification in the middle Pleistocene, and congruent with phylogeographical divergences identified in other widespread generalist species in the SWAFR (Byrne *et al.*, 2002, 2003; Byrne & Hines,

2004; Wheeler & Byrne, 2006). The distinction of two *S. glauca* lineages may, however, be an artefact of sampling from two distant regions; analysis of populations in the intervening area is required to fully understand the phylogeographical patterns within the species.

The *S. glauca* population on Shannon Rock was resolved into the northern clade as sister to the northernmost population, Beacon Hill. Long-distance dispersal appears to be an unlikely explanation. The relatedness of chloroplast haplotypes among these populations, coupled with their geographical separation, suggests a scenario of incomplete lineage sorting and genetic drift, as opposed to recent shared ancestry. The absence of southern haplotypes at Shannon Rock suggests that there is little seed-mediated connectivity between adjacent populations, although more intensive sampling would be needed to confirm this. The similarity of nuclear diversity at Shannon Rock to other populations from the south implies connectivity through pollen dispersal and also supports an explanation of lineage sorting rather than long-distance dispersal.

### Refugia for granite endemics

Granite outcrops have been suggested as refugia during the periods of increased aridity that characterized the Pleistocene (Hopper *et al.*, 1997; Byrne, 2008), owing to their complex topology and capacity to harvest water, which creates mesic microhabitats (Laing & Hauck, 1997; Main, 1997). Although these features facilitate the local persistence of populations on individual outcrops, we were interested in whether the granite network facilitated the contraction and expansion of granite endemics throughout their distribution. Whereas Pleistocene climatic oscillations would be expected to have driven some change in the species' range during arid periods, *S. glauca* did not show evidence of this, and it appears that localized contraction and expansion within outcrops may be a more likely response than local extirpation and recolonization of outcrops across the granite network. Wide physiological tolerances and specialist traits for the granite environment may make granite-endemics resistant to fluctuating climatic conditions. Further, the heterogeneity of granite outcrops may provide greater opportunities for contraction to favourable habitats (localized refugia) during periods of unfavourable conditions, with subsequent expansion if conditions improve.

Our study did not sample at the subpopulation scale needed to detect a genetic signature of expansion and contraction within outcrops, but the persistence of species on granite outcrops throughout the SWAFR indicates that the outcrops must provide heterogeneity and refugial opportunities. Recent work by Schut *et al.* (2014) provides evidence for potential change in population distribution within outcrops under changing climate conditions. Thus, granite outcrops may be important localized refugia for impending anthropogenic climate change, and our studies illustrate the evolutionary potential of populations of both rare and common granite species.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

**Appendix S1** Sequence amplification and alignment.

**Appendix S2** Genetic differentiation between pairs of populations.

**Appendix S3** Principal coordinates analysis of microsatellite diversity.

## BIOSKETCH

The authors are engaged in a multi-disciplinary study on the role of granite outcrops as refugia under future climate change.

Author contributions: All authors conceived the broad ideas and contributed to the writing; M.B., C.J.Y. and G.W.W.-J. designed the study; G.K. and G.W.W.-J. collected samples; S.-L.T. collected the data; S.-L.T. and M.B. analysed the data and led the writing.

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