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Cryptic divergent lineages of *Pultenaea pauciflora* M.B. Scott (Fabaceae: Mirbelieae) exhibit different evolutionary history

MELISSA ANN MILLAR* and MARGARET BYRNE

Science Division, Department of Environment and Conservation, Bentley Delivery Centre, Locked Bag 104, Bentley, WA 6983, Australia

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Genetic structure among disjunct population groups of *Pultenaea pauciflora* was assessed to determine the evolutionary history of this species as a basis for conservation management strategies. Analysis of individuals from all extant populations using 1737 amplified length polymorphism markers revealed two highly divergent genetic entities with strong geographical structuring. Populations located at Narrogin and Brookton clustered together in Bayesian assignment analysis with every individual optimally placed in a single cluster with complete membership. Genetic differentiation between populations in these two areas was very low. Populations at Boddington were highly divergent from those located at Narrogin and Brookton. All individuals from Boddington populations were optimally placed into a second cluster with complete membership. Populations located at Boddington maintain lower levels of allelic diversity, yet greater levels of mean heterozygosity than populations located at Narrogin and Brookton. The degree of genetic differentiation and different patterns of genetic diversity strongly suggest historical divergence and separate evolutionary influences on the two lineages that occur in different ecological habitat. These Evolutionary Significant Units are likely to represent two cryptic sister taxa in the extant populations currently recognized as *P. pauciflora*, and the reassessment of taxonomic and conservation status of both lineages is required.

ADDITIONAL KEYWORDS: conservation genetics – cryptic species – conservation units – evolutionary significant units – fragmentation – genetic diversity.

INTRODUCTION

For many plant species in fragmented landscapes, a combination of historical processes and more recent anthropogenic impacts have interacted to shape the distribution, degree of population disjunction, and size of extant populations. The degree of genetic differentiation among isolated populations and associated reduction in genetic diversity is directly related to the time scale over which evolutionary processes occur, including active selection, mutation, genetic drift, and inbreeding. In general, species with historically isolated population distributions are expected to exhibit high levels of genetic differentiation (Templeton *et al*., 1990; Young, Boyle & Brown, 1996; Thomassen *et al*., 2011). Although such populations may have experienced bottlenecks, persistence through historical climatic fluctuations is likely to have preserved genetic diversity. By contrast, species with isolated population distributions arising from more recent, typically anthropogenic, fragmentation events are expected to exhibit lower levels of genetic differentiation among populations and reduced levels of genetic diversity within small populations. The influence of recent anthropogenic impacts confounds inference of past demographic and evolutionary processes, although investigating patterns of genetic differentiation and genetic diversity among and within plant populations can provide some insight into these factors.

*Corresponding author. E-mail: melissa.millar@dec.wa.gov.au

The biodiversity hotspot of south-west Western Australia (WA) is endowed with a large number of rare endemic plant species with geographically restricted ranges and disjunct or isolated small populations (Coates, 2000; Byrne & Hopper, 2008; Millar, Byrne & Coates, 2010). For some naturally rare taxa, these features are a result of evolutionary processes operating over historical time frames in an ancient landscape. Increased aridification since the Miocene, climatic fluctuations through the Pleistocene, complex patterns of soil mosaics, and the accumulated effects of localized stochastic events are assumed to have led to naturally fragmented species distributions (Hopper *et al*., 1996; Coates, 2000; Hopper & Gioia, 2004; Byrne, 2007; Yates *et al*., 2007). For other restricted species, rarity is the result of more recent anthropogenic disturbance, with humans playing a significant role in shaping current vegetation patterns. Extensive use of fire by Aborigines across the Australian landscape over the last 50 000 years or more has had a substantial impact on the distribution and abundance of some species. More recently, over the last 200 years since European settlement, fire regimes have been altered further, resulting in ongoing changes to species distributions and abundance (Bowman, 1998). More significantly, broadscale clearing for agriculture has resulted in extensive destruction of the natural vegetation, with over 90% of vegetation cleared in some areas. Thus, a high proportion of the remnant vegetation in south-west WA, which includes both naturally rare and historically more abundant species, is now substantially fragmented, persisting as small and highly disjunct populations located in isolated reserves and along roadsides (Hobbs, 1993).

Studies have revealed morphologically cryptic, yet genetically divergent lineages, within a range of nominally described species (Bickford *et al*., 2007), including widespread and restricted species of south-west WA (Byrne, Macdonald & Coates, 1999; Broadhurst & Coates, 2002; Millar, Byrne & O'Sullivan, 2011; Clarke *et al*., 2012). Historically divergent genetic lineages provide unique evolutionary potential that cannot be replaced (Pigeon, Chouinard & Bernatchez, 1997; Losos *et al*., 1998; Fraser & Bernatchez, 2001), and can be considered as Evolutionary Significant Units (ESUs) (Moritz, 1994) that may warrant different management strategies to ensure their persistence (Crandall *et al*., 2000). Knowledge of cryptic divergent lineages or ESUs, as well as patterns of genetic differentiation and genetic diversity within species, can contribute to a wide range of conservation and restoration strategies. Such information can be used to ensure comprehensive approaches to the design and allocation of reserve areas for *in situ* conservation and the collection and use of seed for *ex situ* conservation and translocation (Coates, 2000; Weeks *et al*., 2011). In the light of ongoing climate change, investigation of the evolutionary and demographic processes that shape genetic diversity and structure, and ultimately the long-term adaptive potential of species, is critical to conservation management over the long term. This is especially true for rare plant species with disjunct population distributions and small population size that are experiencing ongoing impacts of anthropogenic disturbance, and for which conservation actions are often urgently required.

Conservation strategies for the rare *Pultenaea pauciflora* M.B. Scott (Fabaceae, Mirbelieae), an endemic plant of the south-west of WA, required knowledge of the evolutionary history of the species given that it occurs in small populations with a highly disjunct distribution. Commonly known as the Narrogin pea, the species was originally known only from very small populations near the town site of Narrogin. As a result of the restricted distribution and number of plants, the taxon was listed as declared rare flora under the Wildlife Conservation Act 1950 (Western Australia), and as vulnerable under the Environmental Protection and Biodiversity Conservation Act 1999 (Commonwealth of Australia). Larger populations of *P. pauciflora* were later discovered in two areas approximately 40–60 km to the north west of Narrogin. The increased species range and greater number of populations and individual plants necessitated a reassessment of the species conservation status. However, populations occur in slightly differing habitat, Jarrah forest or Wandoo/Marri woodland and, given the presence of morphologically cryptic divergent lineages in the flora of south west WA (Coates, 1988; Byrne *et al*., 1999; Broadhurst & Coates, 2002; Millar *et al*., 2011; Clarke *et al*., 2012), analysis of genetic diversity and genetic differentiation among the disjunct populations was required prior to conservation status reassessment. This information will also allow inferences on past evolutionary and demographic histories of the species that will inform appropriate conservation management actions. The prevalence of historically rare species with disjunct geographical populations in south-west WA leads us to hypothesize that *P. pauciflora* will exhibit some genetic differentiation among population groups that may not necessarily be associated with differing habitat, in comparison with the alternative pattern of low genetic differentiation if the species distribution has arisen through recent vegetation clearing for agriculture. We investigated this hypothesis by genotyping individuals from extant populations with highly polymorphic amplified fragment length polymorphism (AFLP) molecular markers.

MATERIAL AND METHODS

STUDY SPECIES AND SAMPLE COLLECTION

The Mirbelieae tribe is a large and diverse group of approximately 690 species largely endemic to Australia (Crisp *et al*., 2005). Species are mainly small, tough, ericoid shrubs confined to the heath understory of sclerophyllous eucalypt dominant open forest and woodland. Flowers are yellow to orange in colour, entomophilous, and largely melittophilous, relying on bees for successful pollination.

Pultenaea pauciflora is a small branching shrub with grey green leaves that bears large yellow flowers (Brown, Thomson-Dans & Marchant, 1998). Individual plants typically live for 10–20 years dependent on environmental conditions. *Pultenaea pauciflora* is known from three population groups of variable size and disjunct geographical distribution (Fig. 1). One population group consists of two small populations (13 and ten plants, respectively) located near the township of Narrogin. Populations occur in a conservation reserve and on a roadside, in white sand over laterite, in association with open woodlands of Wandoo (*Eucalyptus wandoo*), Marri (*Corymbia calophylla*), and Parrot Bush (*Dryandra sessilis*) (Brown *et al*., 1998). A second population group consists of several larger populations of over a few hundred plants in a conservation reserve located 69 km from Narrogin, near the township of Brookton. These populations are also in white sand over laterite in association with open woodlands of Wandoo, Marri, and Parrot Bush. A third population group consists of two medium-sized populations, of less than 100 plants each, on the eastern margin of the main Jarrah (*Eucalyptus marginata*) forest, near the township of Boddington, 59 km from Narrogin and 42 km from Brookton. There are records of a further small population at Narrogin and another at Boddington, although no plants were located at either of these sites in the year of the study. Leaf material for genetic analysis was collected from two populations at each of the three locations (a total of 151 individuals; Table 1).

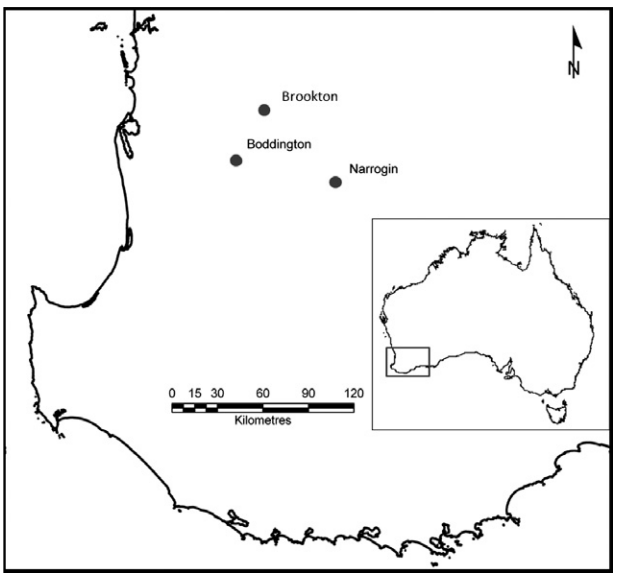

Figure 1. Map of the distribution of known population groups of *Pultenaea pauciflora*, Western Australia. Labelled sites refer to the location of population groups of *P. pauciflora*, not town sites.

DNA EXTRACTION AND GENOTYPING

Genomic DNA was extracted from 50 mg of crushed lypholized leaf material using Qiagen DNeasy plant mini extraction kits in accordance with the manufacturer's instructions (Qiagen Inc.). Trials of 24 AFLP primer combinations were undertaken on four plants from each of the six sampled populations. The four primer combinations giving the strongest, most consistent amplification were selected for further use (Table 2). The AFLP Analysis System I kit was used in accordance with the manufacturer's instructions (Life Technologies), with modification: a double volume digestion with restriction enzymes *EcoR*I/*Mse* was performed using 250 ng of DNA; restriction fragments were ligated to *EcoR*I/*Mse* adapters with overnight incubation and then diluted 1 : 5. Products of selective amplification were diluted 1 : 50 and AFLP fingerprints were resolved using an Applied Biosystems 3730 DNA Analyser (Life Technologies). Individuals were scored for the presence or absence of polymorphic AFLP fragments using GENEMAPPER, version 3.7 (Life Technologies). All amplifications were conducted at the same time and the reproducibility of bands was checked by repeating amplification for 16 (11%) randomly selected samples. One individual from a Narrogin population and two individuals at each of the Brookton and Boddington populations produced inconsistent, poor quality AFLP fingerprints and were excluded from analysis (Table 1). All polymorphic fragments in the range 50–500 bp with a signal strength greater than 200 relative fluorescence units (RFU) were scored for each of the four AFLP primer pairs. Bands were scored as binary data with the presence and absence of bands. Monomorphic bands present in all individuals were not included in the data set. The data for all four primer pairs were combined for further analysis.

STATISTICAL ANALYSIS

We utilized the approaches of Holland, Clarke & Meudt (2008) and Whitlock *et al*. (2008) to optimize

Table 1. Details of six extant populations of *Pultenaea pauciflora* sampled for genetic analysis and diversity measures, including *A*, number of bands; $A_{5\%}$, the number of different bands with a frequency greater than 5%; *P*, percentage of polymorphic bands; A_P, number of private bands; and *H*, mean heterozygosity

Name	Habitat	Population size	Number of plants sampled (number of plants in final analysis)	*A*	$A_{5\%}$	*P*	A_P	*H*
Narrogin 1	Wandoo/Marri woodland	13	13 (12)	587	587	33.8	108	0.037 (0.002)
Narrogin 2	Wandoo/Marri woodland	10	10 (10)	572	572	32.9	106	0.044 (0.002)
Brookton 1	Wandoo/Marri woodland	> 100	32 (30)	906	361	52.2	236	0.030 (0.001)
Brookton 2	Wandoo/Marri woodland	> 100	32 (30)	776	232	44.7	191	0.023 (0.001)
		Total or mean	87 (20.5)	710.25	438	40.9	160.3	0.034 (0.001)
Boddington 1	Jarrah forest	< 100	32 (30)	425	328	24.0	38	0.049 (0.003)
Boddington 2	Jarrah forest	< 100	32 (30)	388	312	22.0	43	0.048 (0.003)
		Total or mean	64 (30)	406.5	320	23.0	40.5	0.048 (0.002)
		Species total or mean	151 (142)	609	399	34.9	120.33	0.038 (0.002)

Values in parenthesis are standard errors.

Table 2. Details of four amplified fragment length polymorphism primer pairs used to fingerprint individuals of *Pultenaea pauciflora*

M primer	E primer	Fluorescent dye	Bands
M-CTC	E-AGC	6-FAM	280
M-CAT	E-ACC	NED	548
M-CTA	E-AAC	VIC	482
M-CAT	E-ACG	PET	427
		Total	1737
		Mean	434.25 (28.52)

The value in parenthesis is the SE.

AFLP fragment scoring parameters. GENEMAPPER was used to investigate the effect of changing the minimum peak height or RFU threshold required for a band to be called present, the minimum fragment length at which a band was scored, and the width of the marker bins in base pairs. This was carried out for the data set for both a low accuracy, high resolution analysis and a high accuracy, low resolution analysis. All downstream analysis was compared using both scenarios and the results varied little for either scenario. The analysis parameters and results for the high accuracy, low resolution analysis are reported because they reduce the risk of introducing errors as a result of more peaks of low quality, they remove small fragments more likely to be homoplaseous, and they reduce the scoring of background noise.

The mean number of fragments per individual was calculated using the AFLP-SURV, version 1.0 (Vekemans *et al*., 2002). Multivariate principal coordinate analysis (PCoA) was conducted to visually compare the grouping of sampled individuals from each of the six populations. A covariance genetic distance matrix was constructed by analysis of molecular variance (AMOVA; Excoffier, Smouse & Quattro, 1992) using GENALEX, version 6.4 (Peakall & Smouse, 2006). The matrix was ordinated in a multidimensional space by PCoA using a standardized data set, and plotted using STATISTICA, version 7.1 (StatSoft, 2001). The hierarchical partitioning of genetic variation was also investigated via AMOVA among and within populations and on a pairwise population basis.

Genetic structure was also investigated using STRUCTURE, version 2.3.2.1 (Pritchard, Stephens & Donnely, 2000), which delineates an optimal number of clusters of individuals, representing biological populations, on the basis of maximizing Hardy–Weinberg equilibrium within clusters. The software can incorporate predefined population location information and is suited to the detection of various patterns of population structure (Evanno, Regnaut & Goudet, 2005; Latch *et al*., 2006). Each band was treated as a separate haploid locus with one locus having either the presence or the absence of a band,

and the second locus of each diploid genotype scored as missing data. Running parameters for the determination of the optimal number of clusters (K) included a burn-in period of 10 000 and 100 000 Markov chain repetitions, population information was used for all individuals (i.e. POPFLAG = 1), and prior population location information was provided by specifying each sampling location as a population (i.e. LOCPRIOR = 1). We used a model without admixture but with correlated allele frequencies, with other parameters set to default values, *sensu* Pritchard *et al*. (2000). Analysis was conducted using ten iterations for each number of clusters (K), for $K = 1$ to $K = 7$. The most likely number of clusters was determined by assessing the *ad hoc* quantity Ln$P(d)$, which is the log of the probability of the data calculated in STRUCTURE, and the variation in Ln$P(d)$, as well as the Evannos' delta K-value (Evanno *et al*., 2005) calculated using STRUCTURE HARVESTER, version 0.6.8 (Earl, 2009). Values of Q, the proportion of the membership to each cluster for a given population, and q_i, the proportion of membership to each cluster for an individual, were assessed and used to assign populations or individuals to clusters.

Genetic differentiation was assessed through determination of pairwise genetic distances between populations and regions using AFLP-SURV software. Allele frequencies were estimated for each individual based on the mean heterozygosity of bands *sensu* Lynch & Milligan (1994). The mating system of *P. pauciflora* has not been investigated although the taxon is expected to be predominantly outcrossed with a degree of self-incompatibility (Gross, 1990; Gross, 1992; Ogilvie, Zalucki & Boulter, 2009). Some deviation from Hardy–Weinberg equilibrium was assumed and a value of $F_{IS} = 0.01$ provided for the analysis. Allele frequencies were calculated using the more accurate Bayesian model with a non-uniform prior distribution of allele frequencies, and pairwise population values of Wrights fixation index (G_{ST}) were obtained. Historical levels of genetic differentiation (G_{ST}) were also determined for populations grouped into appropriate clusters as identified in the STRUCTURE analysis.

Genetic diversity parameters including the number of bands (A), the number of different bands with a frequency greater than 5% ($A_{5\%}$), the percentage of polymorphic bands (P), the number of unique bands (A_P) and levels of mean heterozygosity (H) were calculated for each population, for each identified genetic entity, and for the currently described species overall using GENALEX.

RESULTS

A total of 1737 polymorphic fragments amplified by four AFLP primer combinations were scored with a mean of 434.25 bands for each AFLP primer combination (Table 2).

Genetic structure

Differences in the genetic relationships among the six extant populations of *P. pauciflora* were revealed based on principal component analysis and the degree of genetic differentiation within populations and regions (Fig. 2). Individuals in the Narrogin and Brookton populations clustered together tightly and separately from those in the Boddington populations (Fig. 2) that occupied a greater proportion of the ordination space. The first axis explained 61.00% of the observed variation and separated the Narrogin and Brookton populations from Boddington populations. The second axis explained 12.03% of the variation and illustrated variation among the populations within each region, with most variation observed in populations at Boddington.

An analysis of genetic structure in *P. pauciflora* using STRUCTURE indicated that the six populations were optimally partitioned into two clusters. A plot of mean Ln$P(d)$ against K reached a maximum at $K = 2$ with a low mean standard deviation of Ln$P(d) = 11.14$ (see Supporting information, Fig. S1a). Mean values of Ln$P(d)$ against K fell sharply for greater values of K and mean values of variation in

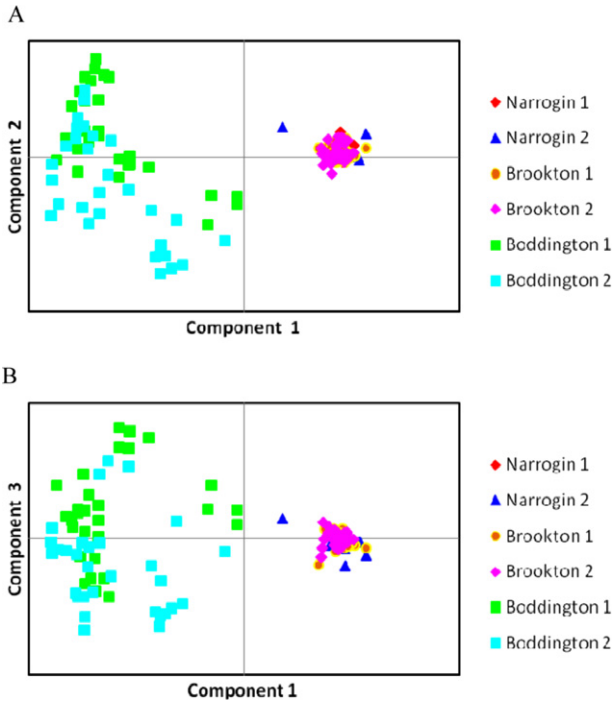

Figure 2. Principal component analysis showing components 1 and 2 (A) and components 1 and 3 (B) for six extant populations of *Pultenaea pauciflora*.

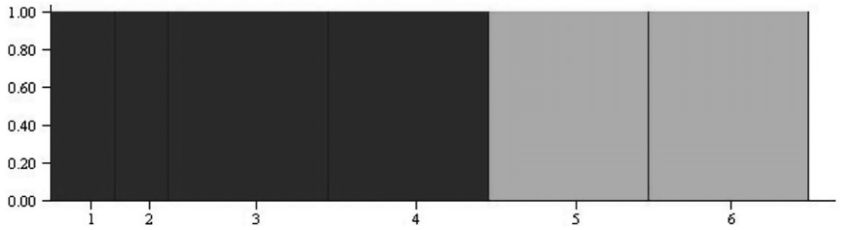

Figure 3. Bar plot representing the identity of sampled individuals of *Pultenaea pauciflora* via assignment to one of two optimal clusters using Bayesian modelling. Each individual is represented as a vertical line partitioned into coloured segments whose length is proportional to the individual coefficients of membership in the two clusters. Cluster one, dark grey; cluster two, light grey. Populations are labelled according to Table 1.

Table 3. Analysis of molecular variance for individuals from six extant populations of *Pultenaea pauciflora*

Source of variation	d.f.	SS	Percentage of total variance	*P*
Among population groups	2	1942.11	26	0.260
Among populations	3	385.54	4	0.059
Among individuals	136	7019.33	70	0.304

Ln$P(d)$ at $K = 3$ or greater were very high [mean SD of Ln$P(d) = 12798.00$ or greater]. Evannos' delta K-value showed a strong peak at $K = 2$ (see Supporting information, Fig. S1b). Given these results, we inferred $K = 2$ to be the optimal number of clusters for the data set. Each population and every individual shared very high proportion of membership values with respect to either of the two clusters (Fig. 3). Cluster one included populations at Narrogin and Brookton and cluster two included populations at Boddington.

Analysis of variance revealed that 30% of genetic variation in *P. pauciflora* was observed among populations and 70% was observed within populations (Table 3). Historical genetic differentiation among populations was moderate: $G_{ST} = 0.1809$. On a pairwise population basis, populations within locations showed a high degree of historical relatedness, although populations at both Narrogin and Brookton were one order of magnitude more related than populations at Boddington ($G_{ST} = 0.0038$ in Narrogin, 0.0051 in Brookton, and 0.0629 in Boddington). Pairwise population values are provided in Table 4. At a regional level, differentiation between Narrogin and Brookton was low ($G_{ST} = 0.0071$), whereas high differentiation was observed between Boddington and both Narrogin ($G_{ST} = 0.2294$) and Brookton ($G_{ST} = 0.2427$). Pairwise genetic differentiation between the two clusters identified by the PCoA and STRUCTURE analyses (i.e. between Narrogin and Brookton, combined, and Boddington) was high ($G_{ST} = 0.2366$). Within the Narrogin/Brookton locations, 2% of genetic variation was observed among populations, whereas, at Boddington, 11% of genetic variation was observed among populations.

Genetic diversity

The mean number of fragments per individual for all populations was 80.4. Measures of allelic genetic diversity within regions were low, and consistent for populations within regions. The lowest levels of allelic diversity were observed in the Boddington populations and the highest in the Brookton populations (Table 1). By contrast, levels of mean heterozygosity were lowest in the Brookton populations and greatest in the Boddington populations. Similar patterns were observed when genetic diversity parameters were calculated separately for each of the two genetic groups identified (i.e. for populations at Boddington and for populations at Narrogin and Brookton). Allelic diversity measures were all lower at Boddington than at Brookton and Narrogin and, in contrast, levels of mean heterozygosity were lower at Brookton and Narrogin compared to Boddington.

DISCUSSION

Genetic analysis of the disjunct populations of *P. pauciflora* has revealed an unexpected pattern of genetic structure in this rare species, given the relative geographical locations of the three extant population groups. High divergence between the Boddington populations and the populations at Narrogin and Brookton is consistent with historical isolation of a divergent lineage. By contrast, the high similarity between the Brookton and Narrogin populations suggests very recent fragmentation. These different genetic patterns are indicative of different evolutionary history among the extant populations of this species even though they have similar levels of

Table 4. Pairwise population genetic differentiation (G_{ST}) among six extant populations of *Pultenaea pauciflora*

Population	Narrogin 1	Narrogin 2	Brookton 1	Brookton 2	Boddington 1	Boddington 2
Narrogin 1	–					
Narrogin 2	0.0038	–				
Brookton 1	0.0097	0.0099	–			
Brookton 2	0.0068	0.0115	0.0051	–		
Boddington 1	0.2577	0.2448	0.2637	0.2874	–	
Boddington 2	0.2684	0.2536	0.2739	0.2987	0.0629	–

geographical separation. These divergent patterns of diversity have implications for the conservation management of populations.

Consistent patterns of high genetic differentiation between extant populations located at Boddington, and those located at Narrogin and Brookton combined, were revealed by each of three different approaches for evaluating genetic structure that were employed. The degree of genetic differentiation indicates that populations at Boddington represent a separate, highly divergent lineage to populations of a second divergent lineage present at Narrogin and Brookton. The two lineages are likely to have been historically isolated (i.e. diverging during the Pleistocene) and can be considered as separate ESUs that may represent cryptic sister taxa, although initial morphological assessment has not identified any obvious morphological differences. This conclusion is further supported by the contrasting patterns of genetic structure and genetic diversity observed within each lineage, which suggests the influence of different evolutionary processes.

The identification of the two divergent lineages in *P. pauciflora* is indicative of historical isolation that is a common feature in the evolutionary history of the flora of south-west WA. Phylogeographical studies have demonstrated that a number of widespread species are comprised of major lineages that have diverged as a result of the influence of climatic fluctuations in the mid Pleistocene, and revealed evidence of widespread population persistence in localized refugia (Byrne, 2007, 2008). Investigations of species with restricted distributions have also revealed divergence that has occurred in historical timeframes. For example, in *Lambertia orbifolia*, populations on the south coast exhibited strong phylogeographical divergence indicating historical separation rather than recent isolation as a result of land clearing (Byrne *et al*., 1999). A high level of divergence was also observed between the two disjunct populations of *Atriplex* sp. Yeelirrie Station in central WA (Clarke *et al*., 2012), and among populations of *Eucalyptus caesia* that have been isolated on disjunct granite outcrops through the Pleistocene (Byrne & Hopper, 2008). Estimates of genetic differentiation between lineages of *P. pauciflora* are consistent with these patterns and indicate that historical fragmentation is also a feature of species inhabiting forest/woodland habitat of south-west WA that is generally considered to have had reasonably continuous populations systems. The identification of divergent lineages in *P. pauciflora* provides another example of the complexity of population structure in species in this biodiversity hotspot.

Further complexity is revealed in *P. pauciflora* with different patterns of genetic structure among the population groups. Although there is strong divergence between the Boddington and the Brookton/Narrogin populations, the similarity between the Brookton and Narrogin populations is striking. The geographical isolation between the Brookton and Narrogin populations is likely a result of contemporary processes associated with more recent (post European settlement) fragmentation through large-scale vegetation clearing for agriculture that was extensive during the 1960s. The level of genetic differentiation is lower than that observed in another fragmented species of the Fabaceae family (Sinclair & Hobbs, 2009) and, surprisingly, is lower than that seen in a number of common and widespread tree species with generally high levels of gene flow and therefore very low levels of population differentiation (Byrne, 1999; Hines & Byrne, 2001; Byrne *et al*., 2003; Wheeler, Byrne & McComb, 2003). This suggests that sufficient gene flow has occurred to maintain genetic connectivity among populations or that the extant plants are recent remnants of a taxon with large effective population size.

It is interesting to note that the genetic relationships among the locations are not congruent with the geographical separation. Genetic differentiation among populations is typically expected to increase with increasing geographical distance between populations as gene flow becomes more restricted, or as a result of intervening features of the landscape that act as barriers to dispersal (Wright, 1943; Loveless & Hamrick, 1984; Manel *et al*., 2003; Storfer *et al*., 2007; Holderegger & Wagner, 2008). This is not the pattern

observed among population groups in *P. pauciflora* where the most geographically distant population groups (Narrogin and Brookton, separated by 69 km) had strong genetic similarity, and the geographically closest population groups (Brookton and Boddington, separated by 42 km) had high genetic differentiation. This pattern indicates different evolutionary history among the populations.

Genetic relationships may be related to environmental factors (Fraser & Bernatchez, 2001; Lenormand, 2002) and a degree of environmental differentiation is evident in that populations of the Narrogin/Brookton lineage occur in mixed Wandoo and Marri woodland, and populations of the Boddington lineage occur in Jarrah forest. These differing habitats are likely to be associated with different edaphic conditions and historical fire regimes, although there is little variation in contemporary climatic conditions (mean maximum temperature of 22.2 °C for Narrogin, 24.2 °C for Brookton, and 21.7 °C for Boddington; mean annual rainfall of approximately 500 mm for Narrogin, 600 mm for Brookton, and 700 mm for Boddington). Although climatic factors may not be a strong influence, differing selection pressures in the different habitats is likely to have played a role is shaping divergence among lineages within *P. pauciflora*.

In addition to genetic divergence estimates that indicated strong historical isolation between the lineages, there was also evidence that genetic diversity was structured differently within each lineage. At the population level, allelic diversity was greatest in the large populations at Brookton, in line with expectations of population genetic theory based on population size (Hamrick & Godt, 1989; Ellstrand & Ellam, 1993; Young *et al*., 1996). However, contrary to such expectations, the smallest populations at Narrogin maintained high levels of allelic diversity similar to the populations at Brookton, and higher than the larger populations of the other lineage at Boddington. These discrepancies in patterns of genetic diversity provide further support that populations of *P. pauciflora* have been influenced by different historical processes rather than contemporary influences of reduced population size.

A number of evolutionary processes that act to shape patterns of genetic diversity may explain the disparate patterns of diversity observed within the lineages of *P. pauciflora*. Variation in chromosome number through polyploidy may be one explanation (Fay, Cowan & Leitch, 2005) and an increased nuclear DNA content would explain the increased numbers of AFLP fragments detected in the Boddington populations. However, although polyploidy has been observed in *Pultenaea* (Sands, 1975), chromosome counts in root tip squashes indicate that all population groups of *P. pauciflora* have the same chromosome number ($2n = 14$; Millar MA, Byrne M, Coates DJ, unpubl. data). Genetic diversity does not always vary greatly with ploidy level (García-Verdugo *et al*., 2009), and chromosome duplication does not appear to be an explanation for different patterns of diversity between lineages in *P. pauciflora*.

Different reproductive systems have been associated with different patterns of diversity in other species with disjunct distributions in south west WA. For example, populations of a sexually reproducing subspecies of *Banksia ionthocarpa*, located near Albany, exhibit high diversity, whereas clonal populations of a second subspecies, near Brookton, show low allelic diversity and greater levels of heterozygosity (Millar *et al*., 2010). Different patterns of sexual reproduction and clonality were also found in geographically separated populations of *Acacia anomola* (Coates, 1988). There was no evidence of clonal reproduction in any populations of *P. pauciflora*, although differences in diversity among the two lineages may reflect other aspects of the breeding system that are not apparent without further investigation.

The level of genetic divergence between the two genetic lineages of *P. pauciflora* suggests they are ESUs that may represent cryptic species. Discrepancies in patterns of genetic diversity among population groups that are indicative of different evolutionary histories provide further support for a consideration of the lineages as separate ESUs, or cryptic sister species. Identification of two divergent lineages increases the complexity of conservation of this species as the two lineages may be assigned different conservation status as a result of their different population sizes, different ecological niches, and different patterns of genetic diversity. A detailed morphological reassessment of populations is recommended and the results of this, in combination with the genetic data, may lead to a taxonomic revision of the species.

The genetic similarity between the originally known populations at Narrogin and the larger populations at Brookton confirm the increase in numbers of the currently recognized *P. pauciflora*. This increase in the number of individuals and populations, and the location of Brookton populations in a conservation reserve, will likely result in an improvement in the conservation status of this lineage. The divergent lineage at Boddington consists of a restricted number of individuals located along road sides and is likely to be identified as threatened and requiring special protection. The main threats to *P. pauciflora* are road maintenance activities, weeds, drought, recreational activities, and inappropriate fire regimes (Durell & Buehrig, 2001). Populations of the genetic lineage at Boddington occur on road verges and damage caused by road maintenance activities, such as grading and road widening, as well

as weed invasion, are identified as the main threats to these populations. Populations of the genetic lineage at Narrogin also occur on road verges and have significantly declined in size over the past 10 years. Drought appears to have caused death in one of the Narrogin populations, and further changes in climatic and hydrological conditions are likely to be detrimental to populations of both lineages (Durell & Buehrig, 2001; DEC, 2008). Inappropriate fire regimes are a threat to all populations and warrants further investigation. *Pultenaea pauciflora* typically re-sprouts after fire and is considered to be an obligate seeder that probably requires fire to break the hard seed coat of soil-banked seeds and trigger germination (Brown *et al*., 1998; Durell & Buehrig, 2001). Repeated high intensity fires at short intervals relative to the maturation period may result in the soil seed bank being exhausted without establishment of reproductively mature plants. Plants of *P. pauciflora* senesce after less than 10 years; thus, too infrequent fires would also reduce population health via a lack of recruitment (Brown *et al*., 1998; Durell & Buehrig, 2001).

CONCLUSIONS

To our knowledge, the present study represents the first investigation of patterns of genetic differentiation and genetic diversity in a member of the native Australian *Pultenaea* genus. Our investigation of six extant populations of *P. pauciflora* identified two morphologically cryptic yet highly divergent genetic lineages. Populations at Boddington represent a separate genetically divergent lineage from populations located at Narrogin and Brookton that are genetically very similar. The present study provides another example of highly divergent evolutionary lineages and highlights the complex evolutionary history of species in the diverse and ancient flora of the biodiversity hotspot in south-west WA. Knowledge of the evolutionary history of species in this region is critical for implementation of effective conservation strategies.

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SUPPORTING INFORMATION

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

Figure S1. (a) Plot of Ln$P(d)$ values and SDs obtained from STRUCTURE analysis at different K-values. (b) Plot of Evannos' delta K-value calculated from Ln$P(d)$ values obtained from STRUCTURE analysis at different K-values.