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Disjunct, highly divergent genetic lineages within two rare *Eremophila* (Scrophulariaceae: Myoporeae) species in a biodiversity hotspot: implications for taxonomy and conservation

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Effective conservation management should target appropriate conservation units, but evolutionarily and genetically divergent lineages within nominal taxa are often unrecognized. The south-western Australian biodiversity hotspot may harbour many cryptic taxa, as it contains many plant species with naturally fragmented population distributions. Using microsatellite markers, we tested the hypothesis that disjunct population groups in the rare species *Eremophila microtheca* and *E. rostrata* (Scrophulariaceae: Myoporeae) are highly genetically divergent and represent separate evolutionarily significant units (ESUs). Chromosome counts indicated that all individuals assessed were diploid ($2n = 36$). Genetic differentiation among disjunct population groups was highly significant ($P < 0.001$) for both *E. microtheca* ($F_{ST} = 0.301–0.383$; $D_{est} = 0.756–0.774$) and *E. rostrata* ($F_{ST} = 0.325–0.346$; $D_{est} = 0.628–0.660$), and was similar to their differentiation from allied species. These results, including high incidences of private alleles, suggest historical divergence among cryptic taxa within *E. microtheca* and *E. rostrata*. Population groups in *E. rostrata* have recently been taxonomically recognized as two subspecies. Our study suggests that *E. microtheca* should also be reassessed as two taxa or considered as two ESUs, and the southern occurrence should be listed as Critically Endangered. We suggest a precautionary approach for flora in this and similar landscapes, whereby historically wide geographical disjunctions are assumed to indicate separate units for conservation.

ADDITIONAL KEYWORDS: conservation units – cryptic species – *Eremophila microtheca* – *Eremophila rostrata* – ESU – evolutionarily significant units – fragmentation – genetic diversity – south-western Australia.

INTRODUCTION

Anthropogenic habitat destruction, fragmentation and the threats associated with climate change have increased the likelihood of extinction for many taxa (Groom, Meffe & Carroll, 2006). With increased focus on the conservation of rare and threatened taxa has come an appreciation that effective conservation management must recognize and target appropriate conservation units (Ryder, 1986). The ultimate goal of species conservation should be to protect genetic and ecological diversity and evolutionary processes and potential. Therefore, it is important to identify and conserve entities that are evolutionarily and genetically divergent and occur across heterogeneous environments (Ryder, 1986; Moritz, 1994, 1995; Luck, Daily & Ehrlich, 2003), thus maintaining the genetic diversity and environmental context necessary for selection (Moritz, 2002). However, the existence of evolutionarily and genetically divergent taxa or population groups is often not recognized. For example, while most taxa are delineated using morphological characters, advances in molecular genetics techniques

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have revealed that a surprising number of nominal species contain two or more morphologically cryptic species, some of which appear to show ancient divergences (Bickford *et al*., 2007). A failure to identify cryptic complexes can have significant conservation implications, because individual taxa may be much rarer or more threatened than the nominal taxon and because each might require different management strategies (Schönrogge *et al*., 2002).

As cryptic species research has focused predominately on animals, with a comparative lack of studies on plants (Bickford *et al*., 2007), the existence of morphologically cryptic species may be under-appreciated in plant conservation. This is likely to be even more so for evolutionarily divergent lineages and genetically distinct population units occurring within biological species (Allendorf, Luikart & Aitken, 2013). Defining diversity at species and population levels is fundamental for developing effective conservation strategies that not only aim to preserve current levels of species diversity, but also consider intraspecific variation and the evolutionary and ecological processes associated with its generation and maintenance (Moritz, 2002; Allendorf *et al*., 2013).

The concept of evolutionarily significant units (ESUs) was developed to address the problem of evolutionarily and genetically divergent lineages that are highly distinctive at both genetic and ecological levels (Ryder, 1986). There has been significant controversy over the definition of what constitutes an ESU, but they are typically geographically discrete and functionally independent with little or no connectivity among them (Crandall *et al*., 2000), and are divergent in organellar and/or nuclear DNA, but may show little or no morphological differentiation (Ryder, 1986; Moritz, 1994). They may represent deep evolutionary lineages or strong adaptive variation (Allendorf *et al*., 2013). Using ESUs to delineate conservation units enables prioritization of populations for conservation and ensures appropriate implementation of conservation management strategies, such as germplasm storage, population augmentation or translocation, and may facilitate recognition of important ecological or biological differences such as pollinator species, breeding systems or chromosome differences (Coates, 2000; Crandall *et al*., 2000; Allendorf *et al*., 2013).

It has been suggested that cryptic species or subspecific units such as ESUs may be more prevalent in certain habitats or landscapes, such as those with exceptionally high species richness (Bickford *et al*., 2007). Landscapes in which geological or climatic history has resulted in a high proportion of species with naturally disjunct population distributions may also be particularly likely to harbour cryptic species or subspecific units. Historically isolated populations are generally expected to display high levels of genetic differentiation due to limited or absent gene flow, fluctuations in population size, genetic drift, selection or local adaptation, with the degree of differentiation expected to increase with the time elapsed following isolation and the spatial distance among populations (Loveless & Hamrick, 1984).

The highly diverse and endemic flora of the south-western Australian biodiversity hotspot includes a large number of rare species with geographically restricted and disjunct distributions (Hopper *et al*., 1996; Hopper & Gioia, 2004), offering an excellent opportunity to explore divergence among disjunct populations of restricted species. This ancient, geologically stable but deeply weathered landscape is characterized by low topography and complex mosaics of nutrient-deficient soils that appear to have caused naturally fragmented species distributions (Hopper *et al*., 1996). South-western Australia became increasingly arid from the Miocene, and the Pleistocene was characterized by increasingly large climatic oscillations that led to repeated expansion and contraction of the arid and mesic zones (Hopper, 1979; Bowler, 1982; Hopper *et al*., 1996). For many species, these climatic changes would have led to increased fragmentation of their ranges and more restricted distributions (Hopper *et al*., 1996; Byrne, 2007), with long-term isolation of populations.

Most genetic studies that have assessed plant species with disjunct distributions in south-western Australia have found unusually high levels of genetic divergence (Coates, 2000; Byrne, Macdonald & Coates, 2002; Coates, Carstairs & Hamley, 2003; although see Millar & Byrne, 2013 for an exception) and some have found divergent genetic systems, such as clonality (Coates, 1988; Kennington & James, 1997; Millar, Byrne & Coates, 2010), or chromosomal rearrangements including complex heterozygosity and changes in karyotype and chromosome number (Coates, 2000; James, 2000). Further investigations have sometimes revealed morphological differences among the disjunct populations (Wege & Coates, 2007). These studies indicate that disjunct populations probably represent separate ESUs and that the current taxonomy may not necessarily reflect all the variation that may be important when determining conservation units and developing appropriate conservation strategies to maximize evolutionary potential and minimize extinction risk. Added to this, there has been extensive recent vegetation fragmentation in south-western Australia following agricultural clearing during the 20th century. This may both obstruct interpretation of pre-existing distributional patterns and increase the rarity, isolation and threat status of already disjunct populations, thus heightening the imperative to apply appropriate conservation assessments and strategies.

Eremophila R.Br. (Scrophulariaceae: Myoporeae) is a large genus of woody shrubs and small trees mainly restricted to semi-arid and arid regions of Australia. Of the 104 taxa recognized in Western Australia, 18 are listed as threatened under the Western Australian *Wildlife Conservation Act 1950* and a further 86 are classified as rare or poorly known (Smith, 2012).We investigated genetic differentiation across significant disjunct distributions for two rare and geographically restricted species endemic to south-western Australia.

Eremophila microtheca F.Muell. ex Benth. is a shrub occurring in only four populations on the west coast of Western Australia, with two small populations in a discrete southern group and two large populations in a second group, 265 km to the north. The intervening area contains very few occurrences of the preferred soil type of the species (that have now been cleared for agriculture), and only one known (now extinct) population, which suggests long-term isolation. *Eremophila microtheca* is classified at the State level as rare but not currently threatened. It was previously listed as Threatened under the State *Wildlife Conservation Act* and Vulnerable under the Commonwealth *Environment Protection and Biodiversity Conservation Act 1999* (*EPBC Act*), but was delisted following the discovery of the two large northern populations in the early 1990s.

Eremophila rostrata Chinnock is a shrub occurring in four small populations distributed among two discrete population groups 280 km apart. The regional occurrences were recently described as two subspecies, *E. rostrata* Chinnock subsp. *rostrata* and *E. rostrata* subsp. *trifida* Chinnock, based on differences in leaf and flower morphology (Chinnock, 2007). However, this taxonomy is not fully recognized at the Federal level. Although each subspecies is listed separately as Threatened (Critically Endangered) under the *Wildlife Conservation Act*, the *EPBC Act* lists *E. rostrata* as a single Critically Endangered taxon containing four subpopulations.

Here, we hypothesized that disjunct population groups in *E. microtheca* and *E. rostrata* are historically isolated and that we would find a large degree of genetic differentiation between them, possibly equivalent to that found among recognized species. To test our hypothesis we used microsatellite markers and compared levels of differentiation with that observed between allied species.

MATERIALS AND METHODS

Study species

Eremophila is a variable genus, showing a range of growth forms and occurring in a wide variety of soils and habitats. Approximately 81% of *Eremophila* species are adapted for pollination by insects and the remainder are adapted for bird pollination (Chinnock, 2007). Many species are disturbance opportunists, plants regenerating through root suckering or mass germination from a long-lived soil-stored seed bank. Mature fruits are usually dry with a papery exocarp and fall to the ground, where they may remain dormant for many years. Water and wind are thought to be the main seed dispersal mechanisms, with some bird dispersal in the few species with succulent fruits (Chinnock, 2007). Polyploidy is common in the genus, with approximately one-third of species studied by Barlow (1969, 1971) showing variation in ploidy.

Eremophila microtheca (section *Australophilae* Chinnock) is an erect shrub up to 1.5 m high with lilac/white flowers that are probably pollinated by a range of insects (Chinnock, 2007). Fruits are small (2 × 3 mm) and compact (Chinnock, 2007), with dispersal probably restricted to local surface water flows. The two northern populations are 17 km apart and occur in sandy clay and gravel soils along drainage lines in low mallee woodland on sand plains (Patrick, 2001; Chinnock, 2007). Both northern populations were most recently recorded to contain 10 000 + plants following a flush of post-fire germination. The two southern populations are only 1 km apart and were probably connected until the mid- to late 20th century. They occur in a wetland system on winter-wet sandy clay soils in open woodland. One population occurs on a narrow road reserve and contains approximately 65 mature plants. The second population is on private property and formerly contained over 10 000 plants, but was severely affected by a prolonged severe flood in 1999 accompanied by agricultural land clearing and grazing, leaving only 17 plants. Leaf morphology varies between the two regions, with plants having linear-subterete leaves in southern populations and flattened leaves in northern populations (Chinnock, 2007).

Eremophila rostrata (section *Amphichilus* (A.DC.) L.S.Sm.) is a small tree up to 3.5 m high with deep pink flowers that are probably pollinated by some of the many honeyeater (Meliphagidae) species that occur within its range (Chinnock, 2007). Fruits are relatively large (7 × 15 mm) with papery wings that may facilitate wind dispersal. The two subsp. *rostrata* populations are restricted to saline quartzite loam soils on quartzite hills and flats near the town of Cue, where they grow in open shrubland (Chinnock, 2007) *c*. 0.5 km apart. The populations occur on a mining lease and have been subject to past land clearing and continuing mining activities. The two subsp. *trifida* populations are restricted to a small area near the town of Perenjori and occur on hard sandy light brown loams under open mallee/*Acacia* Mill. shrubland (Chinnock, 2007). One occurs on a narrow road

verge and the other 2.5 km away in an isolated remnant on a private farm, with both subject to threats including road maintenance, grazing and lack of habitat. Census population sizes are small for both subsp. *rostrata* (< 100 individuals) and subsp. *trifida* (< 30 individuals).

In the absence of a phylogenetic tree, we selected the most closely allied species (as determined by Chinnock, 2007) from the same sections of *Eremophila* with which to compare genetic variation found in *E. microtheca* and *E. rostrata*. For *E. microtheca*, we selected *E. lehmanniana* (Sond. ex Lehm.) Chinnock, a widespread species in the Southwest Australian Floristic Region (SWAFR), where it occurs in open woodlands and overlaps with the southern distribution of *E. microtheca*. For *E. rostrata*, we selected *E. laanii* F.Muell. and *E. longifolia* (R.Br.) F.Muell. for comparison. *Eremophila laanii* is restricted to two river systems where it is abundant on river beds and adjacent flats, whereas *E. longifolia* is common and widespread across Australia, and overlaps with the distribution of *E. rostrata*. All comparison species show the same pollination syndrome as the focal species they are allied with, and none appears to possess specialized adaptations for seed dispersal (Chinnock, 2007). Ploidy information is only available for *E. longifolia*, which is diploid in the area sampled (Barlow, 1971).

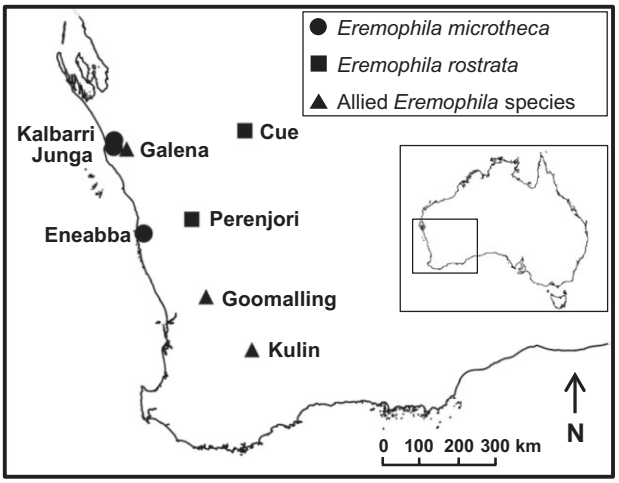

Figure 1. Map showing the locations of the sampled populations of *Eremophila microtheca*, *E. rostrata* and three allied species (*E. lehmanniana*, *E. laanii* and *E. longifolia*) in Western Australia.

Population sampling

Leaf samples were collected from adult plants in three populations of *E. microtheca* (one from its southern and two from its northern distribution) and two populations each of *E. rostrata* subsp. *rostrata* and subsp. *trifida* (Fig. 1). The smallest *E. microtheca* population was not collected due to access issues. Leaf samples were also collected from two populations of *E. lehmanniana* *c*. 200 km apart at Goomalling and Kulin (similar to the 265 km among *E. microtheca* groups), and one population each of *E. laanii* and *E. longifolia*, both collected from banks of the Murchison River at Galena Bridge (Fig. 1). Between 10 and 30 individuals were sampled per population. Sample sizes were limited by population size in many cases and are given in Table 1. In the larger populations, samples were collected representatively throughout the population. Herbarium accession codes for voucher specimens are listed in Table 1.

Cytology

Variation in ploidy within a species may provide evidence that evolutionary divergence has occurred among populations. As polyploidy is common in *Eremophila* (Barlow, 1971), we conducted chromosome counts for each population group of the focal species to determine whether ploidy changes had occurred within the species. Chromosomes were also counted for a population of *E. laanii*, but no seeds were available for *E. lehmanniana*. Chromosome counts were performed on the root tips of at least four individuals from each collection site. Previously collected seeds of *E. microtheca* (Eneabba and Junga), *E. rostrata* subsp. *rostrata* (Cue1 and Perenjori1) and *E. laanii* (Ballinyoo Bridge, 110 km ENE of Galena Bridge) were germinated in a temperature-controlled room, and roots were harvested after 20 days. Roots were treated with 0.1% colchicine for 2–3 h, fixed overnight in 3:1 100% ethanol/glacial acetic acid and stored at 4 °C in 70% alcohol. The roots were stained using the Feulgen technique (Darlington & La Cour, 1970) with 3 min hydrolysis in 1 M HCl at 60 °C and stained with Leuco-Basic Fuchsin in the dark for 1 h. Stained tips were squashed in aceto-orcein and viewed at 1000× under oil immersion. Chromosomes were counted in cells with suitable mitotic spreads.

DNA extraction and genotyping

Total genomic DNA was extracted from 100–200 mg of frozen (–80 °C) leaf material using Qiagen DNeasy plant mini extraction kits. DNA samples were assayed for nine microsatellite loci, EG129, EG149, EG167, EG236, EG239, EG244, EG282, EG402 and EG475, using primer pairs described by Elliott (2009). Polymerase chain reaction (PCR) amplifications were performed in 15-μL reactions containing 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 0.2 mM each dNTP, 1.75–4 mM $MgCl_2$, 0.08 μM fluorescent labelled M13

Table 1. Genetic diversity of *Eremophila* populations: (A) *Eremophila microtheca* and allied species *E. lehmanniana*; (B) *Eremophila rostrata* and allied species *E. laanii* and *E. longifolia*

Population	Specimen code	Population code	Population size	N	P	N_a	N_p	H_o	H_e	F_{IS}
(A)										
E. microtheca South										
Eneabba	PERTH 08490910	ENE	65	29	0.89	3.25 (0.70)	2.08 (0.49)	0.359 (0.108)	0.376 (0.109)	0.038 (0.036)
E. microtheca North										
Junga	PERTH 06118836	JUN	>10000	30	1.00	5.07 (1.25)	3.40 (1.33)	0.519 (0.085)	0.577 (0.089)	0.032 (0.039)
Kalbarri	PERTH 08523215	KAL	>15000	30	0.78	3.61 (0.79)	2.54 (0.93)	0.343 (0.083)	0.408 (0.099)	0.035 (0.036)
E. lehmanniana										
Kulin	PERTH 06223214	KUL	>100	20	1.00	5.44 (0.88)	3.81 (0.73)	0.464 (0.085)	0.604 (0.08)	0.034 (0.033)
Goomalling	PERTH 05844738	GOO	>100	20	0.89	3.33 (0.60)	1.90 (0.55)	0.467 (0.107)	0.473 (0.087)	0.032 (0.039)
(B)										
E. rostrata subsp. *rostrata*										
Cue 1	PERTH 05705010	CUE1	26	20	0.88	4.00 (0.74)	2.71 (0.67)	0.452 (0.108)	0.461 (0.105)	0.022 (0.025)
Cue 2	AD 98209039	CUE2	81	22	0.88	3.63 (0.73)	2.29 (0.63)	0.415 (0.099)	0.466 (0.103)	0.032 (0.033)
E. rostrata subsp. *trifida*										
Perenjori 1	PERTH 05541700	PRJ1	25	25	0.63	2.09 (0.39)	1.01 (0.38)	0.343 (0.105)	0.328 (0.101)	0.021 (0.023)
Perenjori 2	PERTH 08008280	PRJ2	10	10	0.63	2.13 (0.44)	1.17 (0.45)	0.338 (0.107)	0.363 (0.108)	0.041 (0.045)
E. laanii										
Galena	PERTH 04316835	LAA	17	17	0.63	3.61 (0.95)	2.96 (0.72)	0.336 (0.104)	0.416 (0.129)	0.052 (0.051)
E. longifolia										
Galena	PERTH 05414822	LON	25	16	0.00	1.00 (0.00)	0.44 (0.17)	0.000 (0.000)	0.000 (0.000)	0.053 (0.064)

Standard errors are in parentheses. Specimen code of herbarium record for each population; population code used in tables and figures; N, number of sampled adults; P, proportion of loci polymorphic; N_a, mean number of alleles per locus, estimated using rarefaction; N_p, mean number of private alleles per locus, estimated using rarefaction; H_o, observed heterozygosity; H_e, unbiased expected heterozygosity; F_{IS}, inbreeding coefficient.

primer, 0.016 μM forward primer, 0.08 μM reverse primer, 0.75 U *Taq* DNA polymerase and 20 ng template DNA. Loci were amplified using the thermocycler conditions described by Elliott (2009), with the addition of a final step of one cycle at 72 °C for 8 min. Amplification products were separated on an Applied Biosystems 3730 capillary sequencer and genotypes were scored manually using GENEMAPPER v4.0 (Applied Biosystems). Samples were re-amplified if they did not amplify clearly.

Data analyses

To confirm the reproducibility of microsatellite genotypes, some clearly amplifying samples from each region were re-amplified and scored blindly. Amplification products for EG167 were not interpretable for *E. rostrata*, *E. laanii* or *E. longifolia*, so the locus was omitted from analyses involving these species. We used MICRO-CHECKER (van Oosterhout *et al*., 2004) to test for occurrence of large allele dropout and to estimate frequencies of null alleles. To determine whether the presence of nulls was likely to have a significant effect on estimates of population differentiation, we applied the ENA method of Chapuis & Estoup (2007) using FreeNA. Estimates of differentiation were not affected by null alleles, so all loci were retained for analysis.

As sample sizes varied among populations, the mean number of alleles per locus (N_a) and the mean number of private alleles per locus (N_p) were estimated using rarefaction in HP-RARE 1.0 (Kalinowski, 2005). Observed heterozygosity (H_o) and unbiased expected heterozygosity (H_e) were estimated using GENALEX 6.5 (Peakall & Smouse, 2012). We calculated the inbreeding coefficient F_{IS} using INEst (Chybicki & Burczyk, 2009), which takes account of null alleles within populations. INEst was run using the individual inbreeding model with 10 000 iterations.

Pairwise genetic differentiation among populations was estimated using F_{ST} and D_{est} in GENALEX, with significance tested using 9999 permutations. Genetic variation was partitioned hierarchically among and within taxa, populations and regions with analyses of molecular variance (AMOVAs) in GENALEX using 999 permutations. F_{ST} and the standardized F'_{ST} were calculated for higher levels of the hierarchies. F_{ST} is dependent upon within-population diversity, which causes difficulties in interpretation when comparisons are conducted among populations or species that differ in diversity (Meirmans & Hedrick, 2011). The newer measures D_{est} (which partitions diversity based on the effective number of alleles rather than expected heterozygosity) and F'_{ST} (F_{ST} standardized by its maximum possible value) are unaffected by within-population diversity and therefore provide better estimates of differentiation (Meirmans & Hedrick, 2011). As suggested by Meirmans & Hedrick (2011), F_{ST} is presented for comparison with older studies. Relationships among populations were also examined with principal coordinate analysis (PCoA) conducted in GENALEX using a standardized covariance matrix of pairwise genetic distances.

The genetic distance among populations was estimated using D_A distance (Nei, Tajima & Tateno, 1983), which does not make assumptions about evolutionary models. We used POWERMARKER 3.25 (Liu & Muse, 2005) to estimate D_A and produce 1000 bootstrapped phylogenetic neighbour-joining (NJ) trees, then constructed a consensus NJ tree using CONSENSE in the software PHYLIP 3.69 (Felsenstein, 1989).

Genetic structure was inferred using Bayesian model-based clustering in STRUCTURE 2.3.3 (Pritchard, Stephens & Donnelly, 2000; Falush, Stephens & Pritchard, 2007), which estimates the optimal number of clusters (K) in the data and the distribution of individuals among them. The program was run using a model with admixture and correlated allele frequencies. Analyses used Markov chain Monte Carlo (MCMC) parameters with a burn-in period of 10^5 and 10^5 iterations, with 20 replicate runs for each value of K from 1 to 8. The optimal K was determined from ΔK, as described by Evanno, Regnaut & Goudet (2005) and implemented in STRUCTURE HARVESTER 0.6.92 (Earl & vonHoldt, 2012). The optimal alignment of 20 replicates at the optimal K was determined using CLUMPP 1.1.2 (Jakobsson & Rosenberg, 2007), which also calculates a similarity coefficient h' that assesses the similarity of individual replicates. The proportion of the genome (q) of each individual that originated in each cluster was determined, and populations were assigned to clusters based on the proportion of membership (Q) of the population to each cluster. Clusters were visualized in DISTRUCT 1.1 (Rosenberg, 2004).

RESULTS

Nine loci were scored for *E. microtheca* and *E. lehmanniana* and eight loci for *E. rostrata*, *E. laanii* and *E. longifolia*. Rates of genotyping error were negligible. There were seven cases in which loci showed evidence of null alleles (EG129 in *E. laanii*, EG167 in *E. microtheca* at Kalbarri and *E. lehmanniana* at Kulin and Goomalling, EG402 in *E. lehmanniana* at Kulin and EG475 in *E. microtheca* at Eneabba and Junga; see Table S1), but no evidence for the occurrence of large allele dropout.

Eremophila microtheca

Genetic diversity was low to moderate for the three *E. microtheca* populations. Mean values for all meas-

Table 2. Matrices of pairwise genetic differentiation among populations of *Eremophila* species: (A) *E. microtheca* and allied species *E. lehmanniana*; (B) *E. rostrata* and allied species *E. laanii* and *E. longifolia*

(A)

Taxon	Population	ENE	JUN	KAL	KUL	GOO
E. microtheca S	ENE	–	0.301	0.383	0.325	0.394
E. microtheca N	JUN	0.756	–	0.129	0.207	0.236
E. microtheca N	KAL	0.774	0.264	–	0.300	0.345
E. lehmanniana	KUL	0.880	0.700	0.833	–	0.092
E. lehmanniana	GOO	0.919	0.645	0.793	0.196	–

(B)

Taxon	Population	CUE1	CUE2	PRJ1	PRJ2	LAA	LON
E. rostrata subsp. *rostrata*	CUE1	–	0.026	0.346	0.339	0.401	0.583
E. rostrata subsp. *rostrata*	CUE2	0.024	–	0.334	0.325	0.397	0.577
E. rostrata subsp. *trifida*	PRJ1	0.657	0.630	–	0.082	0.458	0.649
E. rostrata subsp. *trifida*	PRJ2	0.660	0.628	0.071	–	0.447	0.661
E. laanii	LAA	0.994	0.989	0.960	0.959	–	0.661
E. longifolia	LON	0.809	0.803	0.706	0.802	0.983	–

The upper and lower halves of each matrix contain values for F_{ST} and D_{est}, respectively. $P < 0.001$ in all cases, based on 9999 permutations.

ures of genetic diversity were highest at Junga and most were lowest at Eneabba, but no among-population differences were statistically significant (Table 1A). The *E. lehmanniana* populations displayed a similar range of values to those found for *E. microtheca*, with a non-significant trend for higher diversity at Kulin. Estimated values for F_{IS} were low and not significantly different from zero for all populations.

There was a high incidence of private alleles in all *E. microtheca* populations, with considerably more observed in between- than within-region comparisons. Of the alleles observed at Eneabba, 74.2% were not found at either Junga or Kalbarri, while 87.5% of alleles observed in the northern populations were not found at Eneabba (data not shown). When compared with each other, Junga had 58% private alleles and Kalbarri had 40%. For six of the nine loci, the most common allele at Eneabba was not observed in either Junga or Kalbarri, whereas at Junga the most common allele at seven of nine loci was also present at Kalbarri. Comparison with *E. lehmanniana* showed that 83.0% of alleles at Eneabba were not found in *E. lehmanniana* populations, whereas 74.1% of alleles in the northern *E. microtheca* populations were not found in *E. lehmanniana* populations.

We found highly significant differentiation among the three *E. microtheca* populations, with high values for overall F_{ST} and D_{est} (0.338 and 0.610, respectively; $P < 0.001$). The two northern *E. microtheca* populations were moderately differentiated from each other ($F_{ST} = 0.129$; $D_{est} = 0.264$) but were extremely differentiated from Eneabba ($F_{ST} = 0.301$–0.383; $D_{est} = 0.756$–0.774; Table 2A). Notably, the among-region values were similar to those obtained for interspecific comparisons between *E. microtheca* and *E. lehmanniana* populations ($F_{ST} = 0.207$–0.394; $D_{est} = 0.645$–0.919). Differentiation among *E. lehmanniana* populations was much lower ($F_{ST} = 0.092$; $D_{est} = 0.196$), even though they were sampled over a similar distance to the disjunct *E. microtheca* populations. Estimates of pairwise population differentiation were significant at $P < 0.001$ for all population pairs tested.

The AMOVAs revealed that 41% of genetic variation was found among *E. microtheca* populations, and a hierarchical AMOVA with the populations partitioned among regions estimated there was more than twice as much variation among regions (33%) as among populations within regions (15%) (Table 3A). The last two values remained almost identical when *E. lehmanniana* populations were included in the hierarchical comparison and treated as an additional region.

The PCoA of *E. microtheca* and *E. lehmanniana* individuals showed distinct groupings that were consistent with the previous analyses (Fig. 2A). The first two axes together explained 44.85% of the variation and separated individuals into three clusters: a fairly

Table 3. Analysis of molecular variance (AMOVA) for *Eremophila* populations and taxa: (A) *Eremophila microtheca* and allied species *E. lehmanniana*; (B) *Eremophila rostrata* and allied species *E. laanii* and *E. longifolia*

Grouping	Source of variation	d.f.	SS	Variance component	Variance (%)	Fixation indices*	Standardized *F*-statistics
(A)							
E. microtheca populations	Among populations	2	177.486	1.456	41	$F_{ST} = 0.412$	$F'_{ST} = 0.767$
	Within populations	175	364.255	2.081	59		
E. microtheca regions	Among regions (N/S)	1	139.728	1.314	33	$F_{RT} = 0.329$	$F'_{RT} = 0.791$
	Among populations within regions	1	37.758	0.595	15	$F_{SR} = 0.222$	$F'_{SR} = 0.427$
	Within populations	175	364.255	2.081	52		
E. microtheca regions and *E. lehmanniana*	Among regions	2	278.058	1.342	33	$F_{RT} = 0.332$	$F'_{RT} = 0.825$
	Among populations within regions	2	55.138	0.507	13	$F_{SR} = 0.188$	$F'_{SR} = 0.377$
	Within populations	253	55.537	2.196	54		
(B)							
E. rostrata populations	Among populations	3	126.503	1.083	40	$F_{ST} = 0.397$	$F'_{ST} = 0.675$
	Within populations	150	246.886	1.646	60		
E. rostrata subspp.	Among subspecies	1	114.299	1.406	44	$F_{RT} = 0.442$	$F'_{RT} = 0.791$
	Within subspecies	2	12.204	0.126	4	$F_{SR} = 0.071$	$F'_{SR} = 0.122$
	Within populations	150	246.886	1.646	52		
E. rostrata subspp., *E. laanii* and *E. longifolia*	Among taxa	3	326.386	1.974	56	$F_{RT} = 0.559$	$F'_{RT} = 0.909$
	Within taxa	2	12.204	0.133	4	$F_{SR} = 0.085$	$F'_{SR} = 0.137$
	Within populations	214	305.555	0.428	40		

*$P < 0.001$ in all cases.

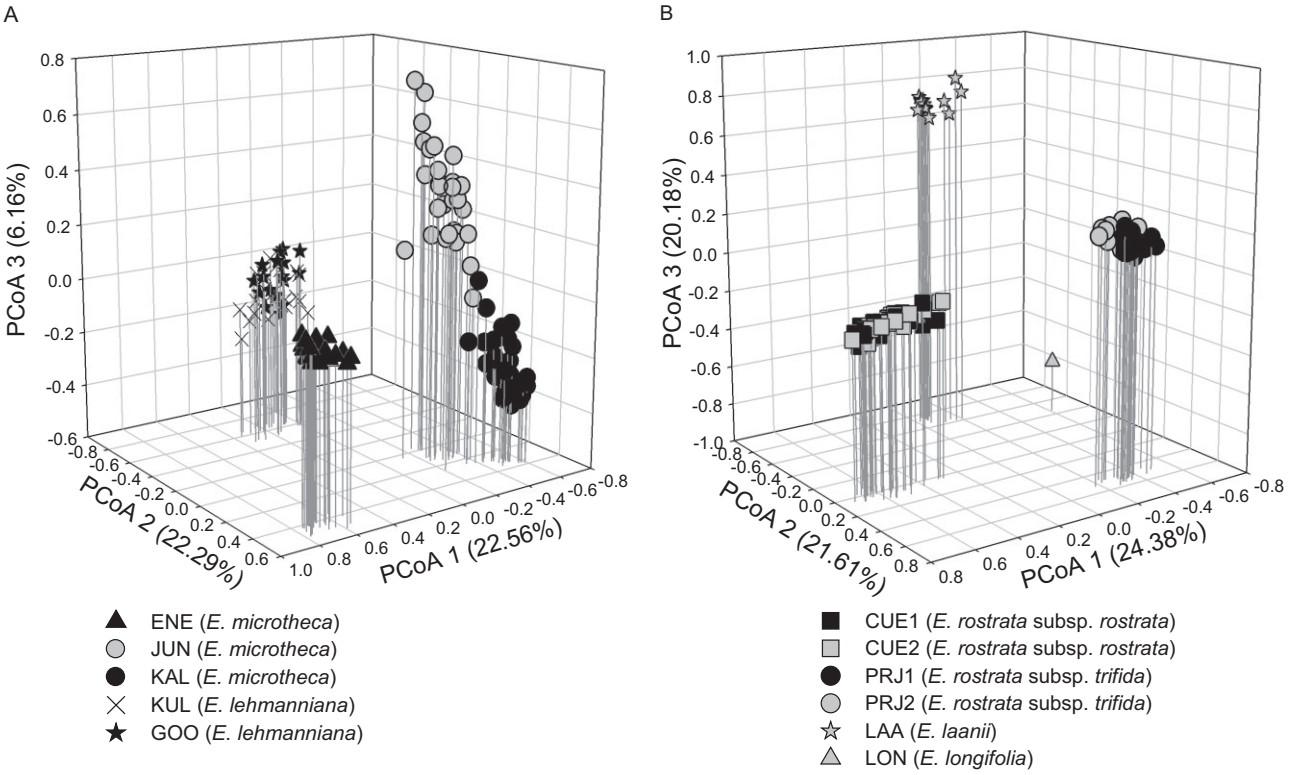

Figure 2. Principal coordinate analysis (PCoA) of pairwise genetic distances between individuals from populations of (A) *Eremophila microtheca* and *E. lehmanniana*, and (B) *Eremophila rostrata* subsp. *rostrata*, *E. rostrata* subsp. *trifida*, *E. laanii* and *E. longifolia*. The percentage of variance explained by each axis is shown in parentheses. See Table 1 for population codes.

tight group of individuals from Eneabba, a more dispersed group from Junga and Kalbarri in which there was little overlap among the two populations, and a group of *E. lehmanniana* individuals with considerable intermixing among populations. The three clusters were approximately equidistant apart. When the PCoA was produced without *E. lehmanniana* there was no change in relationships among the *E. microtheca* populations (results not shown).

The NJ consensus trees based on D_A genetic distance indicated clear divergence of the northern and southern populations of *E. microtheca* (Fig. 3A). There was almost 100% bootstrap support for the divergence of Eneabba from both the grouping of Junga and Kalbarri and the grouping of the *E. lehmanniana* populations.

Bayesian STRUCTURE analysis clearly supported the differentiation of Eneabba from the other two *E. microtheca* populations. Analysis of the *E. microtheca* and *E. lehmanniana* populations produced an unambiguous peak at $K = 3$ and the three clusters were supported by very high similarity among 20 replicates ($h' = 0.999$). The first cluster consisted of

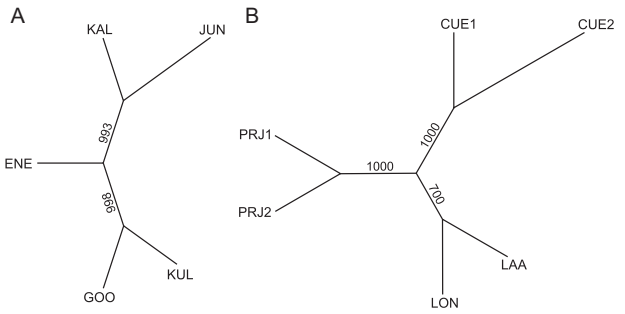

Figure 3. Neighbour-joining trees of Nei's D_A genetic distance between populations sampled from (A) *Eremophila microtheca* and *E. lehmanniana*, and (B) *E. rostrata* subsp. *rostrata*, *E. rostrata* subsp. *trifida*, *E. laanii* and *E. longifolia*. Bootstrap support is shown on branches as number of bootstraps/1000. See Table 1 for population codes.

E. microtheca individuals from Eneabba, the second contained the two northern *E. microtheca* populations and the third contained the two *E. lehmanniana* populations (Fig. 4A). There was virtually no admixture between the clusters.

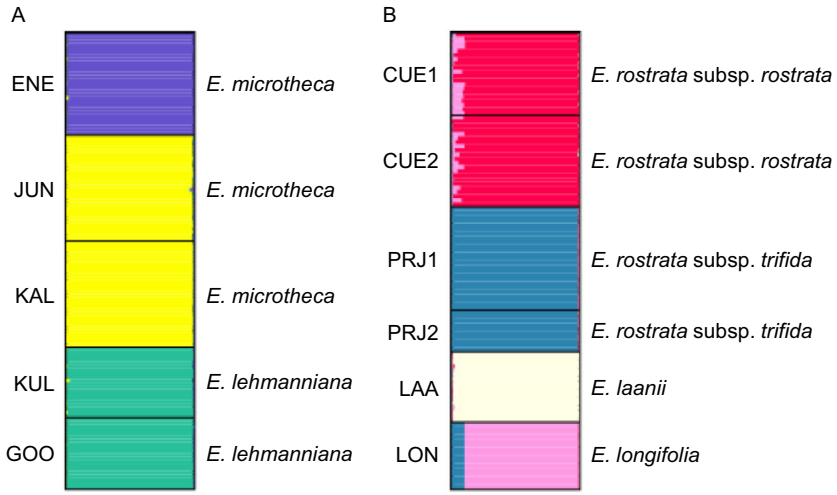

Figure 4. Bar plot illustrating the genetic ancestry of individuals sampled from populations of (A) *Eremophila microtheca* and *E. lehmanniana*, and (B) *E. rostrata* subsp. *rostrata*, *E. rostrata* subsp. *trifida*, *E. laanii* and *E. longifolia*, estimated using STRUCTURE analysis of microsatellite data. Individuals were assigned to (A) three and (B) four optimal clusters using Bayesian assignment. The genome of each individual is represented by a line partitioned into segments proportional to the estimated membership of each cluster. The clusters are the optimal alignment of 20 replicates. Bar plots are shown with population (left) and taxon (right) names. See Table 1 for population codes.

EREMOPHILA ROSTRATA

Genetic diversity was relatively low for the four *E. rostrata* populations. Mean values were up to twice as high in the subsp. *rostrata* populations than in the subsp. *trifida* populations, but the differences were not significant (Table 1B). Most measures of diversity for *E. laanii* were within the range found for *E. rostrata*, whereas all *E. longifolia* individuals were genetically identical and homozygous at all loci. Estimated values for F_{IS} were low and not significantly different from zero for all populations and species.

There was a high incidence of private alleles for both *E. rostrata* subspecies. Comparing the Cue and Perenjori populations, 83.3% of alleles at Cue were private alleles, whereas 61.1% of alleles at Perenjori were not found at Cue (data not shown). For six of the eight loci, the most common allele at Cue was not detected at all at Perenjori, and the reverse was true for four loci. Comparison with *E. laanii* showed that 84.4% of alleles at Cue and 96.9% of alleles at Perenjori were not found in the *E. laanii* population sampled.

Differentiation among the four *E. rostrata* populations was highly significant, with high values for overall F_{ST} and D_{est} (0.347 and 0.445, respectively; $P < 0.001$). Differentiation was quite low between populations within subspecies (*E. rostrata* subsp. *rostrata* $F_{ST} = 0.026$, $D_{est} = 0.024$; *E. rostrata* subsp. *trifida* $F_{ST} = 0.082$, $D_{est} = 0.071$), but it was high between subspecies ($F_{ST} = 0.325$–0.346; $D_{est} = 0.628$–0.660; Table 2B). Differentiation was higher again for interspecific comparisons among *E. rostrata*, *E. laanii* and *E. longifolia* populations ($F_{ST} = 0.397$–0.661; $D_{est} = 0.706$–0.994). Estimates of pairwise population differentiation were significant at $P < 0.001$ for all population pairs tested.

The AMOVAs revealed that 40% of genetic variation occurred among the four *E. rostrata* populations, which was almost entirely attributable to differences among subspecies: a hierarchical analysis partitioned 44% of the variation among and only 4% within subspecies (Table 3B). A second hierarchical AMOVA that treated the *E. rostrata* subspecies on the same hierarchical level as *E. laanii* and *E. longifolia* similarly estimated most variation among taxa (56%) and little within subspecies (4%).

The PCoA of *E. rostrata* and the two allied species revealed four distinct, tight but widely separated groupings of individuals, each cluster corresponding to an *E. rostrata* subspecies or allied species (Fig. 2B). The first axis, which explained 24.38% of the variation, separated the *E. rostrata* subsp. *rostrata* individuals and the *E. longifolia* individuals from all other populations, and the second and third axes (21.61 and 20.18%, respectively) completely separated all four taxa. The four clusters were approximately equidistant from each other. There was approximately 50% intermixing among subsp. *trifida* populations and complete intermixing among individuals from subsp. *rostrata* populations. When the PCoA was produced without the allied species there was no change in relationships among the *E. rostrata* populations (data not shown).

The NJ consensus trees based on D_A genetic distance indicated clear divergence of the two *E. rostrata* subspecies with 100% bootstrap support (Fig. 3B). *Eremophila laanii* and *E. longifolia* formed a third grouping with 70% bootstrap support.

The differentiation among the *E. rostrata* subspecies was clearly supported by STRUCTURE (Fig. 4B). Analysis of the *E. rostrata*, *E. laanii* and *E. longifolia* populations produced a peak at $K = 2$, but similarity among the replicates was low ($h' = 0.555$). Examination of the distribution of mean $L(K)$ values showed a distinct plateau for values of $K > 4$ (indicating $K = 4$); this was supported by the high similarity among the 20 replicates ($h' = 0.898$). One cluster consisted of the subsp. *rostrata* populations, the second of the subsp. *trifida* populations, and *E. laanii* and *E. longifolia* individuals formed the third and fourth clusters, respectively. There was little admixture, with all individuals having most of their ancestry assigned to the same cluster ($q > 0.89$).

Cytology

All individuals of *E. microtheca*, *E. rostrata* and *E. laanii* on which root tip squashes were performed had a diploid number of $2n = 36$ chromosomes. There were no obvious karyotype differences among the species or populations tested.

DISCUSSION

Species with historically fragmented natural distributions may show high levels of genetic divergence across the range disjunctions (Allendorf *et al*., 2013). Our investigation of relationships among highly disjunct populations of *E. microtheca* and *E. rostrata* revealed that each was characterized by a large degree of genetic differentiation, which was either equivalent or close to that found among reference species. These results support our hypothesis that the disjunct distributions represent historically isolated populations. Our findings indicate that a reassessment of the taxonomy of *E. microtheca* and of the conservation management of both species is required.

Divergent lineages in *E. microtheca* and *E. rostrata*

Both *E. microtheca* and *E. rostrata* showed high levels of genetic differentiation among regions. The observed level of divergence was approximately equivalent for both *E. microtheca* and *E. rostrata*, with each species displaying similar values for F_{ST}, the variance partitioned among populations by AMOVA and the incidence of private alleles. A review of microsatellite-based studies found mean F_{ST} values among populations of 0.19 for long-lived perennials and 0.13 for species with wind and/or water dispersal of seeds (Nybom, 2004). The values obtained here for disjunct populations of *E. microtheca* and *E. rostrata* were considerably higher than these. A wide range of F_{ST} or equivalent values has been recorded among plant subspecies by other studies using microsatellites (e.g. Clarke *et al*., 2012), but only the highest of these values (Besnard *et al*., 2007; Millar *et al*., 2010) approach the level of genetic divergence measured here for *E. microtheca* and *E. rostrata*. The validity of making among-taxon comparisons of F_{ST} values is debatable (Meirmans & Hedrick, 2011) and few studies report values that have been standardized for within-population diversity. Nevertheless, the standardized F'_{ST} and D_{est} estimates indicate that *E. microtheca* regions and *E. rostrata* subspecies share few alleles.

Our study provides strong support for taxonomic differentiation or consideration of separate ESUs in both *E. microtheca* and *E. rostrata*. For *E. microtheca*, the southern population at Eneabba and the two northern populations at Kalbarri and Junga were as genetically divergent from each other as they were from populations of the closely allied *E. lehmanniana*, suggesting that the two regions may represent highly divergent lineages, the taxonomic status of which warrants reconsideration. For *E. rostrata*, our results support the recent taxonomic split. Although genetic divergence between the *E. rostrata* subspecies was lower than that between *E. rostrata* and the two allied species, this result is not surprising: the relationship of *E. rostrata* with *E. laanii* and *E. longifolia* may not be as close as that between *E. microtheca* and *E. lehmanniana* (Chinnock, 2007).

A growing body of research is revealing highly genetically divergent, geographically structured population groupings in other nominal plant species in south-western Australia, suggesting that historical divergence is an unusually prevalent characteristic of the flora of the region. This pattern has been found in a range of families and for rare and geographically restricted species (Byrne, Macdonald & Coates, 1999; Byrne & Hopper, 2008; Millar *et al*., 2010; Millar & Byrne, 2013) and common, widespread species (Byrne *et al*., 2002; Byrne, Macdonald & Brand, 2003; Coates *et al*., 2003; Byrne & Hines, 2004) and does not correspond to topographic or biogeographical features. Some studies have taken a phylogeographical approach using plastid variation, showing that the observed patterns are probably due to past population isolation with estimated divergence of lineages ~0.7–1 Mya (see Byrne, 2007), corresponding with the mid-Pleistocene Transition, when climatic oscillations increased in cycle length and amplitude (Bowen, 1978; Dodson, 1994). However, there is also evidence

that some speciation processes in this landscape began prior to the Pleistocene (Byrne *et al*., 2002).

The occurrence of such similar phylogeographical patterns across a range of species in south-western Australia suggests that the genetic divergences observed within *E. microtheca* and *E. rostrata* may have occurred on a similar timescale and that the disjunct populations may represent distinct evolutionary lineages. Population groups probably became increasingly isolated over time as a result of historically increasing aridity and Pleistocene climatic fluctuations. Sea-level rises may also have contributed to the fragmentation of the near-coastal distribution of *E. microtheca* populations. These processes probably exacerbated population distribution patterns that may have already been significantly fragmented due to the edaphic complexity of the landscape. The notion of long-term separation is also supported by the apparent lack of suitable habitat between isolated population groups in this geologically stable landscape and by the lack of adaptations for long-distance dispersal, particularly in *E. microtheca*.

This situation presents an interesting comparison with the flora of the Mediterranean basin, which has a similar climate to south-western Australia and also contains a large number of species with disjunct geographical distributions. Many of the fragmented Mediterranean distributions probably resulted from geological complexity, tectonic movement and isolation of microplates in the Tertiary and/or more recent sea-level changes and may also show the genetic signature of historical isolation processes (Thompson, 1999, 2005). In the California Floristic Province, phylogeographical analysis indicated that aridification, Pleistocene climatic fluctuations and spatial climatic gradients have been major factors driving genetic divergence in plant species, thus contributing to the high plant diversity in the region (Calsbeek, Thompson & Richardson, 2003).

A combination of different evolutionary processes may have been involved in the genetic divergence of the disjunct *Eremophila* population groups, including genetic drift (in the absence of gene flow) and natural selection. Divergence due to drift is expected to be greater when populations are small or experience large fluctuations in size, whereas selection is more effective in large populations (Wright, 1931). Although *E. microtheca* and *E. rostrata* populations probably naturally experience large fluctuations in size due to variation in fire frequencies and other natural events, such as flooding and periods of varying aridity, the impact of this on evolutionary processes would depend on how long population sizes remained large or small, which is unknown. The long-lived soil seed bank of these species may mitigate against the loss of diversity during periods when adult numbers are low. Nevertheless, it is possible for high divergence at nuclear loci to occur among relatively recently isolated populations, for example due to founder effects or other bottlenecks (McCauley, Raveill & Antonovics, 1995).

If a species was characterized by isolation by distance (IBD) prior to a fragmentation event, then population differentiation should have been apparent immediately following fragmentation. The surprisingly high divergence we observed among populations of the northern *E. microtheca* lineage, which are separated by 17 km but linked by continuous natural vegetation, suggests that even relatively local populations have experienced restricted gene flow and subsequent genetic drift, and that gene flow throughout the pre-European distribution of *E. microtheca* may always have been low. By contrast, population divergence within *E. rostrata* subspecies was fairly minimal, consistent with the much smaller geographical isolation. It is possible that gene flow may historically have been a little more extensive in *E. rostrata* than in *E. microtheca* due to differences in fruit morphology and because the avian pollinators of *E. rostrata* may potentially have dispersed pollen more widely than the insect pollinators of *E. microtheca*.

The trend for higher genetic diversity at Junga compared with either Kalbarri or Eneabba suggests that any differences in levels of genetic diversity within *E. microtheca* are not related to the north–south divergence among lineages, but to demographic or other ecological factors. The population at Junga occurs at a site that is more water-gaining than that at Kalbarri (D. Coates, pers. observ.) and is therefore likely to have supported larger population sizes during past dry periods and to have maintained a higher effective population size over time. At Eneabba, the relatively low diversity is likely to be a result of the recent severe reduction in population size and extent and can be considered a sampling effect, as the pre-agricultural population was probably more genetically diverse.

Selective adaptation to local climatic or soil conditions may have also been a factor in the divergence of lineages. The two *E. rostrata* subspecies occur in distinctly different soil types and habitats (Chinnock, 2007). Contemporary rainfall data show average annual rainfall to be 39% higher at Eneabba than at Kalbarri (488.6 vs. 350.4 mm; 1971–2012), and 43% higher at Perenjori than at Cue (335.6 vs. 235.0 mm; 1918–2000) (Bureau of Meteorology, 2013). Additionally, there are strong seasonal differences among *E. rostrata* locations: at Cue the wettest months are January to July inclusive, whereas at Perenjori rainfall peaks from May to August. Concomitantly, peak flowering tends to occur earlier at Cue (Western

Australian Herbarium, 1998–2014), although flowering times can overlap and flowering can also occur sporadically at other times of the year (Chinnock, 2007). Such phenological changes may have created some degree of reproductive separation among the population groups.

Some species have responded to historically fragmented population systems by evolving divergent genetic systems, such as clonality or chromosome change (Coates, 2000; James, 2000), that often serve to maintain locally adaptive variants and may increase genetic divergence and reinforce geographical barriers to gene flow. Indeed, one of the reference species used in this study, *E. longifolia*, shows both variation in chromosome number (Barlow, 1971) and clonality (Chinnock, 2007); all *E. longifolia* plants sampled for this study were genetically identical, indicating that the sampled population was clonal. This does not appear to be the case for *E. microtheca* or *E. rostrata*, as we found no evidence for variation in reproductive systems and chromosome counts indicated that all studied populations were diploid ($2n = 36$) with no obvious karyotypic differences between populations within or between taxa. Evidence from other *Eremophila* species and several other unrelated arid zone genera showing variation in ploidy suggests that diploid populations in this landscape represent old evolutionary lineages, whereas polyploid populations are recently derived adaptive variants with less morphological variation (Carolin, 1958; Randell, 1970; Barlow, 1971; Stewart & Barlow, 1976). This supports our conclusion that the populations we sampled are the remnants of old lineages rather than more recent variants that have moved into new environments.

Implications for conservation

The identification of multiple evolutionary lineages within a single taxon should trigger a reassessment of the conservation status of the taxon and its component lineages (Moritz, 2002; Bickford *et al.*, 2007). The large genetic divergences among population clusters within *E. microtheca* and *E. rostrata* and the apparent lack of any recent connection among them, together with differences in leaf and/or flower morphology noted by Chinnock (2007) and ecological and climatic differences, indicate a strong case for each cluster to be considered as an ESU. Appropriate conservation management strategies can be implemented only by managing each ESU as a separate conservation unit.

In some jurisdictions, both internationally and in Australia, genetically distinct populations or phylogenetic groups within species are recognized in formal legislation, enabling their separate listing for conservation protection (Allendorf *et al.*, 2013). However, in the State of Western Australia, the *Wildlife Conservation Act* requires that only taxa formally recognized by the Western Australian Herbarium are eligible for listing as threatened flora and hence afforded protection and high priority for conservation. Therefore, recognition of appropriate subspecific conservation units is often dependent upon the outcome of a taxonomic study. *Eremophila microtheca* is currently undergoing taxonomic review based on our genetic data, likely long-term population isolation and differences in leaf morphology noted by Chinnock (2007), so that the southern *E. microtheca* lineage can be afforded appropriate conservation protection, if appropriate. A notable precedent for this situation occurred with *Lambertia orbifolia* C.A.Gardner (Proteaceae), an endangered shrub occurring in two disjunct population groups. The two groups were afforded subspecies status on the basis of their distribution and high differentiation at allozyme loci (Coates & Hamley, 1999), and the deep evolutionary divergence among the lineages was later confirmed using plastid markers (Byrne *et al.*, 1999).

The southern populations of *E. microtheca* would meet the IUCN criteria for listing as Critically Endangered if they were treated as a separate taxon, and therefore as a separate conservation unit. The main threats to the southern populations are lack of habitat, flooding, grazing and road works, and immediate conservation strategies to alleviate some of these threats may include the establishment of new populations on conservation estate adjacent to the current populations. In contrast, the relatively large northern populations are well conserved within a National Park, where there are no significant threats; these populations therefore require a quite different conservation ranking and management approach.

For *E. rostrata*, recently recognized morphological differences have already resulted in both subspecies being separately listed as Declared Rare Flora (Critically Endangered) under State legislation, facilitating appropriate conservation measures such as translocation of plants to secure sites. However, our study emphasizes the large divergence among the subspecies and the need for revision of the Federal listing under the *EPBC Act* of *E. rostrata* as a single taxon with four subpopulations. This situation highlights the perceptions and conservation issues which may be associated with subspecific taxonomic levels (Ryder, 1986; Haig *et al.*, 2006).

For many fragmented species it is desirable to manage larger conservation units to encourage connectivity among disjunct populations and therefore reduce some of the negative consequences associated with reduced population size and increased isolation (Groom *et al.*, 2006; Allendorf *et al.*, 2013). However,

such an approach may not be appropriate for these *Eremophila* species, which are characterized by long-standing genetic, ecological and climatic differences, may have experienced selective adaptation to their current environments, and each is likely to consist of two morphologically cryptic species. Managing the genetically divergent population clusters within *E. microtheca* and *E. rostrata* as separate conservation units will enable the preservation of these differences and hence maintain different evolutionary potential within each ESU. However, careful conservation management will be required to alleviate the multiple threats that small population size poses to the viability of most of these populations. Alternatively, it may be desirable to test experimentally for the possibility of hybrid vigour in among-ESU hybrids: introduction of additional genetic diversity into the smallest populations may increase resilience to changing environmental conditions and provide additional evolutionary potential.

CONCLUSIONS

Our study has provided further evidence that the south-western Australian flora contains an unusually high prevalence of evolutionarily divergent lineages within nominal taxa due to historical population fragmentation that occurred despite geological stability. The accumulating body of research suggests many evolutionary lineages in this landscape may currently be unrecognized as cryptic taxa, posing significant challenges for the effective conservation of a diverse flora. Similar challenges are likely to exist in other parts of the world where high plant diversity, cryptic species and historically disjunct population distributions are prevalent, such as the four other Mediterranean bioregions (Thompson, 1999; Calsbeek *et al*., 2003).

As it is not possible to conduct genetic studies on every species, adopting a precautionary approach to recognizing the potential for cryptic evolutionary lineages may greatly improve conservation outcomes in landscapes such as these. For rare species that have wide and obvious geographical disjunctions that are likely to be historical, it seems reasonable to assume they represent different lineages, and to treat them as separate conservation units. However, for some species the delimitation of each lineage may not be obvious from its geographical distribution, particularly if recent human activity has obscured historical disjunctions (e.g. Millar & Byrne, 2013).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Estimates of null allele frequencies for nine microsatellite markers in populations of *Eremophila*, estimated using Micro-Checker. Figures in bold denote significant estimates of null allele frequencies ($P < 0.05$). Values of zero indicate the locus was invariant.