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Abstract

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Keywords

populations, banksia, spinulosa, dwarf, coastal, genetic, rare, tests, isolation

Disciplines

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Genetic tests of the isolation of rare coastal dwarf populations of *Banksia spinulosa*

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Abstract. In southern New South Wales, a suite of widespread plant species exhibit short-statured ‘dwarf’ growth forms on coastal headlands. It is unclear whether such populations are genetically distinct or whether dwarfism is a plastic response to the environment. We used four microsatellite markers to assess genetic differentiation among populations from coastal and inland sites for *Banksia spinulosa* var. *spinulosa*. We sampled plants from six locations, including from three ‘dwarf’ and three ‘normal’ populations. Mean levels of genetic diversity were slightly higher in the forest ($N_a = 7.07 \pm 0.25$; $H_e = 0.80 \pm 0.09$) than on the coast ($N_a = 5.92 \pm 0.70$; $H_e = 0.72 \pm 0.10$). In general, populations displayed genotypic diversity expected for outcrossed sexual reproduction, with 161 of 172 individuals displaying unique genotypes and mean values of F_{IS} close to zero. However, we found evidence of at least limited clonal replication in each of four populations and, within one coastal population, 11 of 27 individuals displayed one of three replicated genotypes, implying that the effective population size may be considerably smaller than would be inferred from the number of plants at this site. Relative to studies with other Proteaceae, this set of populations showed low, although significant, levels of differentiation (global $F_{ST} = 0.061$; $P < 0.001$), with extremely low, although significant, divergence of forest and coastal populations ($F_{RT} = 0.009$; $P < 0.001$). There was no evidence of isolation by distance. These data imply that coastal dwarf populations are genetically similar to more extensive inland populations but in at least one case, may be at a greater risk of extinction because of low effective population size.

Introduction

Many plant species exhibit phenotypic variation among populations for traits such as growth form, life history and flower and leaf morphology, often in association with different habitat types (e.g. Turesson 1922; Heaton *et al.* 1999; Fang *et al.* 2006; Foster *et al.* 2007). Morphologically divergent populations of widespread species are often considered to have special conservation significance (e.g. Millar and Libby 1991; Lesica and Allendorf 1995), reflecting the assumption that such populations harbour unique genetic variation, which may enhance the species’ evolutionary potential and facilitate persistence in the face of future environmental change (Etterson and Shaw 2001). However, the significance of such variation is often unclear; although it may indicate the presence of multiple species or subspecies (e.g. Gottlieb *et al.* 1985; Hogbin and Crisp 2003), substantial variation can also occur within species because of phenotypic plasticity or local adaptation driven by divergent natural selection (Linhart and Grant 1996; Fang *et al.* 2006; Foster *et al.* 2007). Establishing the genetic relationship between morphologically divergent populations is therefore critical for appropriate conservation

and management, especially where particular morphological types have highly restricted distributions, or are at risk from human disturbance.

In south-eastern Australia, dwarf populations of widely distributed species commonly occur on coastal sites and the cause of this morphological variation has only rarely been subject to careful scrutiny. In most cases, coastal dwarfs are assumed to be environmental variants of more widespread species. However, recent taxonomic investigations have concluded that in some cases coastal populations warrant classification as separate species (de Kok and West 2002; Hogbin and Crisp 2003), whereas other studies have suggested that species are simply morphologically variable (Hogbin and Crisp 2003; Foster *et al.* 2007). For example, RAPD data support recognition of *Zieria prostrata*, a prostrate plant restricted to coastal headlands in northern New South Wales (NSW), Australia, as a separate species from *Z. smithii*, which is an erect shrub found on inland sites (Hogbin and Crisp 2003). However, *Z. smithii* does produce both short-statured plants, on some coastal headlands, and larger plants within genetically similar inland populations (Hogbin and Crisp 2003).

We used *Banksia spinulosa*, one of the plant species present in coastal headland 'dwarf' communities in NSW to assess genetic differentiation between morphologically divergent forest and coastal populations. *B. spinulosa* is a potentially important case study because it occurs on coastal headlands in southern NSW that have largely been cleared for agriculture or urban developments, and herbarium records reveal that its distribution was previously much more extensive (Australian Virtual Herbarium: <http://plantnet.rbgsyd.nsw.gov.au> last accessed October 2010). Unlike many of the plant species that exhibit dwarfism in this region, morphologically divergent *B. spinulosa* populations do not occur parapatrically. Rather, there is generally separation of at least 1 km between 'dwarf' coastal and 'normal' forest plants (E. O'Brien, pers. obs.), which may inhibit gene flow between the two sets of populations. However, the scale over which gene flow by seeds and pollen occurs in this species is not known and it is possible that pollen transfer by highly mobile bird and mammal pollinators may occur over greater distances.

Previous studies of the mating system of *B. spinulosa* have demonstrated that there is substantial variation among varieties in levels of self-compatibility (Carthew *et al.* 1988; Vaughton 1988; Vaughton and Carthew 1993) and in their capacity to resprout after fire (George 1999). If similar variation in these characters exists among morphologically divergent populations within varieties, it may have significant implications for genetic diversity and genotypic structure within populations and connectivity among populations. There have not been any studies of the distribution of genetic variation within and among populations of this species.

By using highly polymorphic microsatellite markers, we examined partitioning of genetic variation within and among populations of *B. spinulosa* to assess the importance of conserving coastal populations. We addressed the following questions:

- (1) what is the magnitude and distribution of genetic variation among populations of *B. spinulosa*;
- (2) does genetic diversity differ among forest and coastal regions;
- (3) is regional variation in growth form associated with significant genetic divergence at neutral loci; and
- (4) is there evidence of variation in the breeding system or the extent of clonality between forest and coastal populations of this species?

Materials and methods

Study species and region

Banksia spinulosa is a long-lived, perennial woody shrub, with a broad distribution along Australia's eastern coast. It exhibits substantial morphological variation throughout its distribution, which has resulted in the identification of up to four distinct varieties (George 1999; Harden 2000). In southern New South Wales, where the present study was conducted, plants are *B. spinulosa* var. *spinulosa*. Reproductively mature *B. spinulosa* var. *spinulosa* (hereafter *B. spinulosa*) plants growing on sandy soils on coastal headlands are of small stature (<1 m), broad and with densely packed leaves. By contrast, *B. spinulosa* further inland, where it occurs in dry sclerophyll forest, typically grows up to 2–3 m, with longer

branches and a sparser canopy. *B. spinulosa* produces large inflorescences composed of ~800 flowers (Vaughton and Carthew 1993). Previous studies of the breeding system of *B. spinulosa* have revealed that a suite of potential pollinators, including birds, marsupials and insects, visits inflorescences. Flowers are hermaphroditic and self-fertilisation is possible; however, experimental hand-pollinations and genetic analyses of open-pollinated seed confirm a strong preference for outcrossed seed, and very high realised outcrossing rates (Carthew *et al.* 1988; Carthew *et al.* 1996). *B. spinulosa* plants from forest populations in this region possess an underground lignotuber, which enables resprouting following a fire. However, this is known to vary among varieties of this species (George 1999) and has not been examined in coastal dwarf plants.

In forested areas of southern NSW, *B. spinulosa* is common and has a fairly continuous distribution, although there has been some fragmentation of habitat as a result of clearing for agriculture, roads and forestry. On the coast, *B. spinulosa* is restricted to just a few headlands, resulting in populations that are smaller and more isolated than those in the forest.

Sample collection

We sampled leaves of *B. spinulosa* for microsatellite genotyping from six locations in southern NSW, three being from coastal headland populations, which contained plants of the 'dwarf' growth form, and three from forest populations of 'normal'-stature plants (Fig. 1). The three coastal headlands were selected because they were the only accessible headlands that we were able to survey that supported dwarf *B. spinulosa* populations. Because of the differing distribution of *B. spinulosa* on the coast and in the forest, forest populations sampled in the present study potentially contained many tens of thousands of plants, whereas coastal populations consisted of at most a few hundred. At each site, we sampled plants within a 1-km radius to ensure consistency between sites in the different environments. To control for the effect of geographic separation on genetic divergence, we chose to sample forest locations separated by approximately the same mean distance as that separating coastal headlands (Fig. 1).

Leaves were collected from 20–35 adult plants per location. Because of the potential for *B. spinulosa* to resprout, care was taken to ensure that samples were taken from plants that did not share a lignotuber.

DNA extraction and microsatellite genotyping

DNA was isolated from freeze-dried leaf material by using the cetyltrimethyl ammonium bromide (CTAB) procedure of Doyle and Doyle (1987). Individuals were genotyped at four nuclear microsatellite loci: Bo22, Bo3, Bo8 and Br13. These had previously been developed for the related species *B. oblongifolia* and *B. robur* (Usher *et al.* 2005), except for Bo8, for which primers were redesigned to optimise amplification in *B. spinulosa* (K. Ottewell, unpubl. data). Details of each of these loci are provided in Table 1.

Polymerase chain reactions (PCR) were carried out in 15- μ L volumes. For Bo22, Bo3 and Bo8, reaction mixtures comprised 1 \times PCR buffer (Applied Biosystems, Foster City, CA, US), 0.25 mM each dNTP, 2 mM MgCl₂, 0.25 μ M each forward and

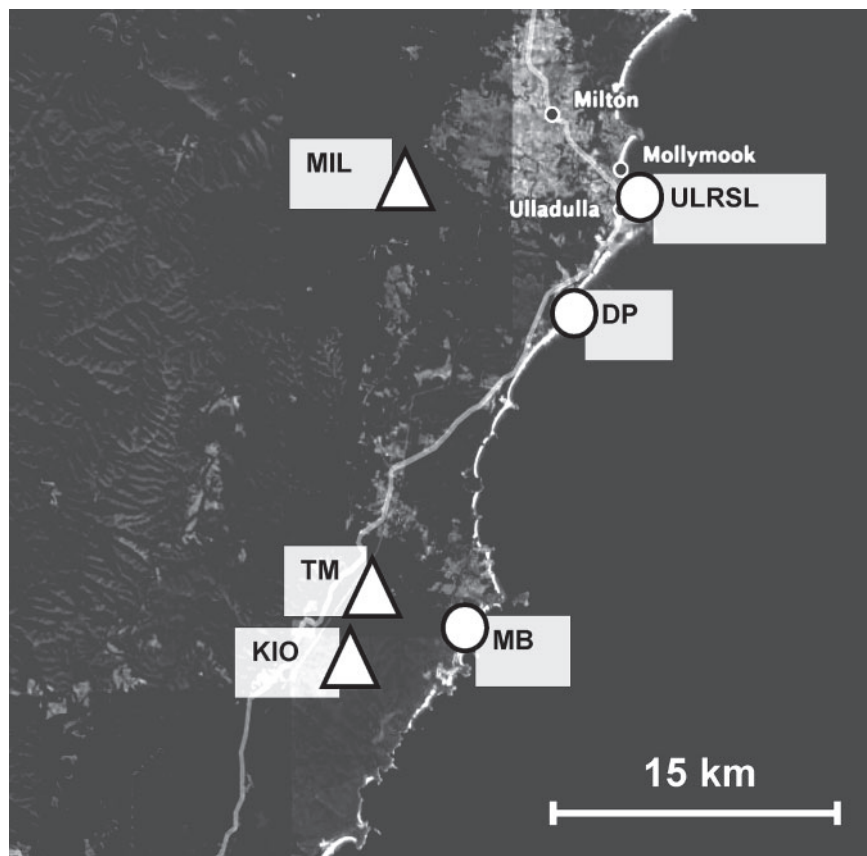


Fig. 1. Location of *Banksia spinulosa* var. *spinulosa* populations sampled for microsatellite genotyping. Circles indicate ‘dwarf’ populations on coastal headlands, triangles indicate ‘normal’ populations in the forest. Map courtesy of Google Earth.

Table 1. Details of microsatellite loci used to genotype *Banksia spinulosa*

Primer sequences, total number of alleles, allele size range and N_a (allelic richness, corrected for sample size) for each of the four microsatellite loci. For N_a , numbers shown are means across populations, with standard errors in parentheses

Locus	GenBank accession ID	Repeat motif	Primer sequences (5′–3′)	No. of alleles	Annealing temperature (°C)	Allele size range (bp)	N_a
Bo22	AY953301	(AG) ₁₀	F: GCTCGAGTATTCGACCCAAA R: TTTGAATGCCTCCATTCCCTC	5	52	213–225	3.32 (0.30)
Bo3	AY953304	(AG) ₁₁	F: AGATGGAGGTGGATGGTCTG R: GTTAACTTGCGCCGCTTTAG	17	52	143–179	9.35 (0.57)
Bo8	AY953307	(GA) ₉	F: GTAAACSGGTGCAACTGAG R: TTGTGGTATGTTCTTGCATC	19	52	163–200	10.74 (0.79)
Br13	AY953309	(AG) ₁₃	F: TAGCCCAATCAAACCGAAC R: CTTTTGCTGAATCCCCTCAG	14	55	218–246	8.23 (0.49)

reverse primer, 0.45 U AmpliTaq Gold (Applied Biosystems) and deionised H₂O. PCR was carried out on an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) gradient thermocycler under the following conditions: denaturing at 95°C for 9 min, then 30 cycles of denaturing at 94°C for 30 s, annealing at 52°C for 30 s and extension at 72°C for 45 s, followed by a final extension step of 72°C for 10 min. The reaction used to amplify the Br13 locus was identical to that described above, except that it included 0.4 μM of each primer and an annealing temperature of 55°C was used. For each sample, 0.5 μL of the product from each PCR reaction was combined with 9.5 μL of a

mixture containing formamide and 1% LIZ size standard (Applied Biosystems) and denatured at 95°C for 3 min. PCR products were separated by electrophoresis using a 3130 automated capillary sequencer (Applied Biosystems). Data were collected and genotypes assigned using GeneMapper v3.7 (Applied Biosystems), with alleles sized by comparison of electrophoretic migration against fragments in the LIZ size standard.

For each locus, several individuals did not amplify cleanly and could not be scored even after repeat extractions and independent amplifications. This did not reflect null alleles because the product

was clearly present but could not be scored unambiguously. Consequently, the genotypes utilised in our analyses were based on either three or four loci.

Data analysis

We tested for linkage disequilibria among all pairs of loci in all populations to verify that loci assort independently and tested for departures from Hardy–Weinberg equilibria within each population to test for evidence of inter-population variation in levels of outcrossing or clonality (potentially reflecting the occurrence of shared lignotubers). Within a population, biparental inbreeding or self-fertilisation is expected to produce consistent heterozygous deficits, whereas the asexual replication of multi-locus genotypes may produce both deficits and excesses of heterozygotes because genotypes become fixed (Balloux *et al.* 2003). Each of these analyses was carried out in GENEPOP v. 4.0 (Raymond and Rousset 1995). In each case, sequential Bonferroni corrections were used to adjust P -values to account for the number of tests (n). For tests of linkage disequilibria, $n=36$ and for tests for departure from Hardy–Weinberg equilibria, $n=24$.

Allelic richness (the mean number of alleles per locus), the number of private alleles, observed and expected heterozygosity, and the inbreeding coefficient F_{IS} were calculated for each location and region, to provide measures of genetic diversity. Because of the potential effect of sample size on estimates of allelic diversity, we used the rarefaction approach to calculate allelic richness, controlling for the number of individuals sampled, as implemented in FSTAT (Goudet 1995). Other measures of allelic diversity were calculated with GENALEX v6.1 (Peakall and Smouse 2006).

We calculated hierarchical F statistics with TFPGA (Miller 1997) and tested the significance of genetic variation among populations (F_{ST}) or regions (F_{RT}) by determining whether zero lay outside their 95% CI (determined by 1000 bootstrap replications across loci). We calculated values for F_{ST} among all pairs of populations with GENALEX v6.1 (Peakall and Smouse 2006) and tested for isolation by distance across the set of six sampled populations, by comparing the matrix of $F_{ST}/(1 - F_{ST})$ with the matrix of linear geographic distances among pairs of locations using a Mantel test. The significance of the association between genetic and geographic distance was evaluated using 999 random permutations of the data. We used the matrix of genetic distances among individuals to conduct a principal coordinates analysis, which was then plotted to provide a visual representation of the genetic relationships among the sampled populations and among the two ecotypes.

We also used Bayesian analysis implemented in the program STRUCTURE v. 2.2 (Pritchard *et al.* 2000) to identify the most likely number of genetic ‘clusters’ (K) within our dataset and to determine the probability of assignment of individuals to each cluster, providing a further test of the genetic relationships among our sampled populations. We used an admixture model with correlated allele frequencies to estimate the probability of the data containing K clusters, for values of K between 1 and 6. We used a burn-in period of 10 000 and a Markov Chain Monte Carlo (MCMC) of 50 000, which was sufficient to result in stable estimates of the log-likelihood of a given value of K across replicate runs. We carried out five runs for each value of K .

We identified the number of unique multilocus genotypes present within each population sample and calculated the probability of identity PI (an estimate of the average probability that two unrelated individuals, drawn from the same randomly mating population, will by chance have the same multilocus genotype) using GENALEX v6.1 (Peakall and Smouse 2006). When identical multilocus genotypes were represented by two or more individuals within a population, we calculated the probability that the observed level of genotypic replication (Pr) could have occurred via sexual reproduction, by using the formula (Willis and Ayre 1985):

$$Pr = n^{-1} C_{r-1} \times P^{(r-1)} \times q^{(n-r)},$$

where n = the number of plants sampled, r = the number of replicates, P = the probability of occurrence of a single copy of the multilocus genotype within a randomly mating population (calculated as the product of expected single-locus genotype frequencies) and $q = 1 - P$.

Results

All four microsatellite loci were polymorphic within each population, with an average of 7.91 ± 1.61 alleles per locus (Table 1), suggesting that they were suitable to estimate allelic divergence among populations. Genotyping yielded an overall four-locus PI of 8.5×10^{-6} , providing sufficient power to test for evidence of clonal replication within populations.

Our genotyping of 172 *B. spinulosa* plants yielded 161 unique multilocus genotypes and within each population, values of F_{IS} , averaged across loci, ranged from -0.02 at Merry Beach to 0.11 at Termeil, and were never significantly different from zero ($P > 0.05$) (Table 2).

All populations were genetically diverse as indicated by high levels of allelic richness (N_a) and expected heterozygosity (H_e) (Table 2). These measures showed little variation between forest (N_a : mean = 7.07, s.e. = 0.25; H_e : mean = 0.80, s.e. = 0.09) and coastal sites ($N_a = 5.92 \pm 0.70$; $H_e = 0.72 \pm 0.10$). N_a was highest at Kioloa forest site (7.40 ± 1.63) and lowest within the coastal Merry Beach population ($N_a = 4.80 \pm 0.57$), which contained three replicated genotypes. H_e was highest at the coastal Dolphin Point site ($H_e = 0.78 \pm 0.06$) and was again lowest at Merry Beach ($H_e = 0.62 \pm 0.09$) (Table 2). In total, forest populations displayed a greater number of private alleles (11, compared with five in coastal populations) (Table 2). However, there was greater variation among forest populations, with seven private alleles detected at Kioloa and none at Termeil, whereas each of the coastal populations exhibited one or two private alleles (Table 2).

Only two cases of heterozygous deficiency (Bo8 and Br13 at Kioloa) were significant after sequential Bonferroni corrections for multiple tests. The absence of consistent heterozygote deficits at any locus also suggests that null alleles are not a significant problem at any of these loci. However, we also tested for null alleles by using the program FreeNA (Chapuis and Estoup 2007), which produced negligible (<1%) null allele-frequency estimates in all cases.

The outcome of 12 of the possible 36 tests for linkage disequilibria, carried out for the six pairs of loci within each of the six populations, were statistically significant ($P < 0.05$).

Table 2. Summary of genetic-diversity measures of *Banksia spinulosa* populations and regions

N , number of individuals genotyped; N_a , mean number of alleles per locus (allelic richness, corrected for sample size); PA, number of private alleles (alleles unique to a particular population/environment); H_o , observed heterozygosity; H_e , expected heterozygosity; and F_{IS} , the fixation index ($F_{IS} = 1 - (H_o/H_e)$). Numbers shown in parentheses are standard errors. Note that the negative F_{IS} value for Merry Beach is a result of a H_o higher than that expected under Hardy–Weinberg equilibrium. In no case was the F_{IS} for a population significantly different from zero

Region	Population	N	N_a	PA	H_o	H_e	F_{IS}
Forest	Milton (MIL)	28	7.21 (1.26)	4	0.70 (0.05)	0.77 (0.07)	0.08 (0.08)
	Termeil (TM)	20	6.57 (1.24)	0	0.71 (0.09)	0.74 (0.07)	0.11 (0.08)
	Kioloa (KIO)	35	7.40 (1.63)	7	0.70 (0.05)	0.77 (0.07)	0.07 (0.13)
	Total (forest)	85	7.07 (0.25)	11	0.70 (0.06)	0.80 (0.09)	0.09 (0.00)
Coast	Dolphin Pt (DP)	33	7.20 (1.27)	2	0.73 (0.11)	0.78 (0.06)	0.08 (0.07)
	Ulladulla RSL (ULRSL)	29	5.77 (1.43)	1	0.65 (0.12)	0.67 (0.13)	0.00 (0.09)
	Merry Beach (MB)	27	4.80 (0.57)	2	0.65 (0.13)	0.62 (0.09)	-0.02 (0.07)
	Total (coast)	89	5.92 (0.70)	5	0.68 (0.12)	0.72 (0.10)	0.02 (0.00)
Total		172	6.49 (0.42)		0.69 (0.08)	0.77 (0.09)	0.05 (0.03)

However, although significant disequilibria was detected for all pairs of loci in at least one population, significant disequilibria for any pair of loci was never detected in more than three populations, and only two outcomes remained significant after sequential Bonferroni correction (Bo22 and Bo8 at Milton; Bo3 and Br13 at Merry Beach). Therefore, all loci were considered independent and all were used in subsequent analyses.

Our hierarchical analysis of genetic variation revealed little differentiation of the six surveyed populations ($F_{ST} = 0.06 \pm 0.01$) and strikingly little variation between the groups of forest and coastal populations ($F_{RT} = 0.009 \pm 0.004$), although in both cases these values were significantly different to zero (Table 3). Similarly, estimates of F_{ST} for all pairs of populations revealed low–moderate, although highly significant ($P < 0.001$, exact tests), genetic differentiation. However, a Mantel test did not reveal any evidence of isolation by distance ($r^2 = 0.007$, $P = 0.40$). The first two axes in our principal coordinates analysis explained 50.6% of the variation, and did not reveal any separation of populations or ecotypes (Fig. 2).

In our STRUCTURE analysis, the log probability was highest for $K = 4$. All populations displayed an estimated membership of one of the four clusters of over 50%. Two of the coastal populations (ULRSL and MB) showed the highest proportion of membership for the same cluster. The third coastal population

(DP) showed the highest proportion of membership in the same cluster as did the forest site TM, although DP showed almost as high a membership with the same cluster as the other coastal populations. The remaining forest populations (MIL and KIO) each had the highest proportion of membership within their own individual cluster. A representative example of the proportion of ancestry of each population assigned to each genetic cluster with $K = 4$ is provided in Table 4.

The number of individuals with replicate multilocus genotypes varied slightly among populations, ranging from 0 to 11 (Table 5). Although the majority of individuals within each population displayed unique genotypes and were presumably sexually derived, in each case where a genotype was displayed by two or more individuals, this was judged significantly ($P < 0.02$) unlikely to have occurred as a result of sexual reproduction with random mating (Table 5). Our sample of 27 genotyped individuals from Merry Beach appeared to display quite a high level of clonality, with three groups of replicate genotypes identified, one represented by six individuals, one by three and another by two. With the exception of two plants from Kioloa, all replicate multilocus genotypes were detected within coastal populations (Table 5).

Discussion

Our data indicated that a set of three coastal dwarf populations and three forest populations of *B. spinulosa* separated by up to 28 km show little evidence of differentiation and no significant isolation by distance. Although we found some evidence of clonal replication, predominantly within one coastal site, our results in general are as expected for a single preferentially outcrossing species (Carthew *et al.* 1996) that forms large, continuous populations and has a diverse suite of potentially highly mobile pollinators (Carthew 1993). Our data indicated that most populations are genetically diverse and, at least historically, have been strongly interconnected by pollinators or seed dispersers.

Genetic structuring among populations of *B. spinulosa* examined in the present study was, although significant, weaker than levels typically reported for other Australian Proteaceae species that have been subject to genetic investigation. A comparison of genetic structuring detected

Table 3. Partitioning of genetic structure among populations and regions

F -statistics assessing genetic differentiation among regions and among populations within regions and P -values obtained using 1000 bootstrap replications across loci. Genetic structuring among regions and among populations within regions was highly significant ($P < 0.001$)

Source of variation	F -value	95% CI	P -value
Overall			
Among regions (F_{RT})	0.0086	0.0023–0.0177	<0.001
Among populations (F_{ST})	0.0614	0.0426–0.0843	<0.001
Coast			
Among populations (F_{ST})	0.0354	0.0268–0.0569	<0.001
Forest			
Among populations (F_{ST})	0.0636	0.0333–0.0979	<0.001

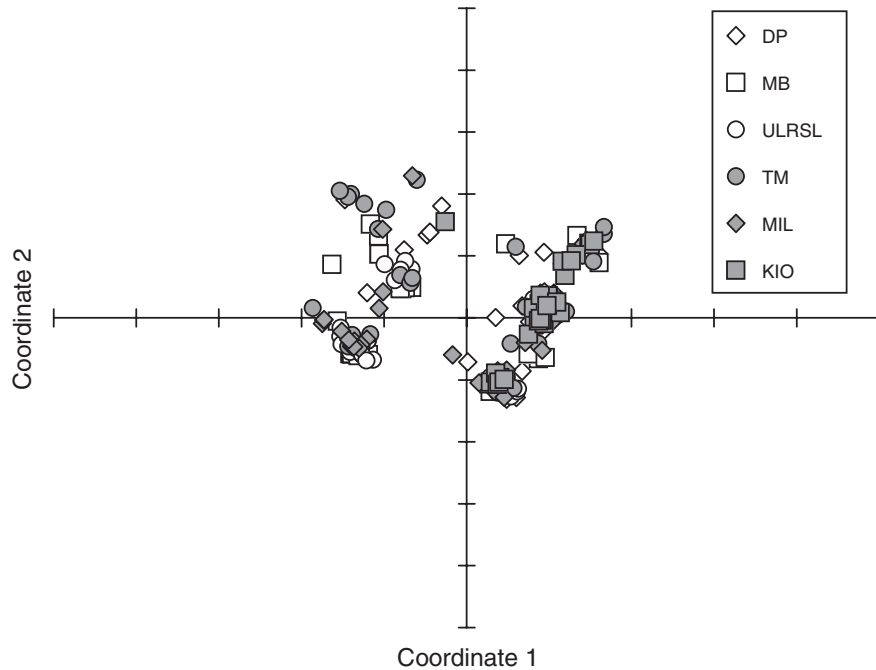


Fig. 2. Principal coordinates analysis. The first two principal coordinates explained 29.6% and 21.0% of the variation in the data, respectively. Open symbols have been used to represent coastal populations and closed symbols to represent forest populations of *Banksia spinulosa*.

Table 4. Inferred genetic clustering using Bayesian analysis

The proportion of membership of each population within each cluster inferred from a STRUCTURE run, assuming $K=4$ genetic clusters. For each population, the proportion of membership that was the highest across the four inferred clusters is highlighted in bold

Population	Cluster			
	1	2	3	4
MIL	0.037	0.152	0.103	0.709
TM	0.018	0.642	0.322	0.018
KIO	0.830	0.032	0.071	0.068
DP	0.024	0.511	0.453	0.012
ULRSL	0.125	0.314	0.510	0.051
MB	0.013	0.070	0.896	0.022

in studies of 10 Australian Proteaceae species (including the present study) is presented in Table 6. Intraspecific genetic structuring within this family tends to vary in association with species life histories and distributions, similar to the pattern observed across plant species in general (e.g. Hamrick and Godt 1989, 1996; Nybom and Bartish 2000). For example, strong genetic structuring has been observed in *Grevillea caleyi* ($F_{ST}=0.66$ using AFLP, and $F_{ST}=0.46$ using microsatellites; Llorens 2004; Llorens et al. 2004), *G. longifolia* ($F_{ST}=0.33$; Llorens 2004), *G. macleayana* ($F_{ST}=0.218$; England et al. 2002) and *G. repens* ($F_{ST}=0.272$; Holmes et al. 2009), all of which have highly disjunct distributions and most of which are self-compatible. Outcrossing Proteaceae species tend to exhibit weaker genetic structuring, e.g. *Grevillea iaspiculata* ($F_{ST}=0.204$;

Table 5. Locations of putative clones within the sampled *Banksia spinulosa* populations

Locations of replicated multilocus genotypes detected in microsatellite analyses of *B. spinulosa* from the six sampled populations, along with the probability of identity (PI) within each population and the probability of obtaining the observed number of replicates of the individual genotype under random mating (Pr). Because in some cases, pairs of individuals shared identical three-locus genotypes but could not be scored for the fourth locus, PI and Pr values were calculated conservatively on the basis of the minimum number of loci scored

Population	No. of individuals with replicate multilocus genotypes	No. of loci genotyped	PI	Pr
ULRSL	2	3	0.002	1.045×10^{-2}
MB	6	3	0.012	7.847×10^{-8}
MB	3	3	0.012	2.187×10^{-2}
MB	2	3	0.012	1.433×10^{-3}
KIO	2	4	0.000019	3.053×10^{-4}
DP	2	4	0.000019	6.454×10^{-6}

Table 6. Comparison of genetic structuring (F_{ST}) detected in studies of 10 Australian Proteaceae species displaying a range of distributions and mating patterns

Species	Species distribution	Mating system	Scale of sampling (km)	Genetic marker	F_{ST}	Reference
<i>Banksia spinulosa</i>	Widespread, but more fragmented near the coast	Predominantly outcrossing (Carthew <i>et al.</i> 1988, 1996)	28	Microsatellites	0.06	Current study
<i>Grevillea caleyi</i>	Fragmented, but previously widespread	Self-compatible	10	Microsatellites	0.46	Llorens (2004)
<i>G. iaspicula</i>	Fragmented, but previously more widespread	Predominantly outcrossing	20	Allozymes	0.20	Hoebee and Young (2001)
<i>G. longifolia</i>	Fragmented	Self-compatible	20	Microsatellites	0.33	Llorens (2004)
<i>G. macleayana</i>	Fragmented	Self-compatible	20	Microsatellites	0.22	England <i>et al.</i> (2002)
<i>G. repens</i>	Fragmented	Obligate outcrosser	100	Microsatellites	0.27	Holmes <i>et al.</i> (2009)
<i>G. robusta</i>	Fragmented	Predominantly outcrossing	500	Allozymes	0.18	Harwood <i>et al.</i> (1997)
<i>Persoonia glaucescens</i>	Fragmented	Obligate outcrosser	27	AFLP	0.16	Rymer and Ayre (2006)
<i>P. lanceolata</i>	Widespread	Obligate outcrosser	380	AFLP	0.20	Rymer and Ayre (2006)
<i>P. mollis</i>	Fragmented	Obligate outcrosser	200	Allozymes	0.18	Krauss (1997)

Hoebee and Young 2001), *G. robusta* ($F_{ST}=0.179$; Harwood *et al.* 1997), *Persoonia mollis* ($F_{ST}=0.179$; Krauss 1997), *P. glaucescens* ($F_{ST}=0.158$; Rymer and Ayre 2006) and *P. lanceolata* ($F_{ST}=0.206$; Rymer and Ayre 2006), although the genetic structuring observed in our study ($F_{ST}=0.061$) is much weaker than has been observed even for other Proteaceae species with similar life histories and distributions, measured over a comparable scale (Table 6). This result implies that *B. spinulosa* populations are well connected, at least historically, probably because of the diverse suite of potential pollinators (including birds, mammals and insects) that visit *B. spinulosa* inflorescences and can facilitate long-distance pollen dispersal. The potential for pollinator-mediated gene flow at this scale has been demonstrated for the bird-pollinated *Grevillea macleayana*, where Roberts *et al.* (2007) used paternity analysis to detect pollen movement of up to 2 km.

Although the population-genetic effects of resprouting by *Banksia* have been largely ignored, the ability to resprout from below-ground lignotubers provides the potential for genotypes to persist for long periods as individual plants and to undergo localised clonal proliferation. This aspect of *B. spinulosa*'s life-history has several implications for interpretation of its population structure. For example, although the low levels of population differentiation that we observed imply that populations have been well connected by gene flow, it is important to realise that the present day population structure may reflect potentially ancient patterns of dispersal and colonisation and prolonged genotype \times environment interactions. Indeed, because individual plants resprout from lignotubers after disturbance such as fire or tree falls, genotypes are potentially immortal and allele frequencies within populations are unlikely to have been affected either by the isolating effects of relatively recent anthropogenic habitat fragmentation or even the potential disruption of mating systems as a result of the introduction of the European honeybee in the early 1800s (Paton 1996). Importantly, the ability of *B. spinulosa* genotypes to replicate by resprouting could provide a simple explanation of the morphological differentiation of coastal and forest sites if each coastal population was dominated by one or a few morphologically unusual clones.

The incidence of dwarfism in coastal regions has been documented in numerous plant species worldwide (e.g. Clausen *et al.* 1948; Hogbin and Crisp 2003; Foster *et al.* 2007). Such a growth habit is often assumed to be adaptive to the high wind speeds and salt spray typical of coastal sites (Gottlieb 1984; Griffiths and Orians 2003); however, the relative importance of local adaptation versus phenotypic plasticity in driving this morphological divergence is generally unknown. The evolution of adaptive morphological divergence through natural selection requires that selection pressures are sufficient to overcome the homogenising effect of gene flow (Slatkin 1987). This is therefore only possible when (i) gene flow is limited or (ii) selection coefficients are very strong. The maintenance of morphological divergence in *B. spinulosa* despite very weak genetic differentiation among populations implies that morphological variation is driven either by phenotypic plasticity, or by selection strong enough to counter high rates of gene flow.

Molecular markers such as microsatellites are assumed to be selectively neutral and to therefore largely reflect past and present gene flow. If morphological divergence is driven by selection on a small number of genes, with high underlying levels of gene flow among populations, genetic differentiation may not be detected by using neutral markers (Reed and Frankham 2001). Our results suggest there are likely to be strong genetic connections among populations in the area covered by the present study; however, we used only four, putatively neutral, microsatellite markers. Although these markers were highly variable and provided considerable power to distinguish among individuals, their capacity to detect differentiation driven by divergent selection at a small number of loci may be limited. Therefore, we are not able to rule out adaptive genetic divergence as the cause of growth form variation in this species. Disentangling the effects of genetic and environmental factors in driving morphological divergence requires reciprocal transplant trials, to assess whether morphologies are maintained when plants are transplanted to the opposite environment, or analysis of genetic differentiation at a much larger number of loci, where it is likely that at least some are closely associated with genes under selection.

Conclusion

Despite morphological divergence and geographic isolation of coastal headland *B. spinulosa* populations in southern New South Wales, the weak genetic structuring we detected by using microsatellite markers implies strong connections among populations, from which we could conclude that coastal dwarf plants of this species do not require targeted conservation measures. However, the higher incidence of clonality we detected in coastal dwarf populations, in particular the Merry Beach population, suggests that the effective population size is likely to be considerably lower than would be inferred from the number of plants. This may reduce the resilience of this population to environmental perturbations and render it more susceptible to extinction. Further studies are needed to determine whether increased clonality is characteristic of coastal dwarf populations of this and other species on coastal headlands in this region. It would also be beneficial to undertake population genetic studies of the other plant species exhibiting dwarfism in populations on coastal headlands in this region, to assess the broader significance of this phenomenon.

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