

Genetic variation in *Banksia saxicola* (Proteaceae), a rare Australian plant with a markedly disjunct distribution

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Abstract. *Banksia saxicola* A.S. George (Proteaceae) is a rare Australian endemic, found in only two locations in Victoria that are separated by approximately 500 kms: the Grampians and Wilson's Promontory National Parks. The organisation of genetic variation between and within populations at the two locations was assessed using the Amplified Fragment Length Polymorphism (AFLP) technique. Two populations were sampled in the Grampians National Park and one population was sampled at Wilson's Promontory. The three populations were genetically divergent from each other, in particular the Grampians and Wilson's Promontory populations, and this relates to the ancient geographic isolation of these two regions. The Wilson's Promontory population had lower genetic diversity than either Grampians populations, consistent with its smaller population size. The findings are important for strategies to conserve *B. saxicola*.

Key words: *Banksia saxicola* A.S. George, rock banksia, AFLP, genetic variation, population differentiation, genetic diversity, biogeography, conservation.

Banksia saxicola A.S. George (Proteaceae), the rock banksia, is a rare endemic found in only two locations in Victoria, Australia that are separated by approximately 500 km: the Grampians (a) and Wilson's Promontory (b) National

Parks (Fig. 1). At the Grampians, *B. saxicola* is relatively widespread and locally common (Taylor and Hopper 1988), and grows predominantly on rocky mountain summits as a shrub or small tree up to six metres in height (Middleton et al. 1996). At Wilson's Promontory, it is less abundant and occurs as an understory tree, of up to 15 metres in open *Eucalyptus* forest (Middleton et al. 1996). *Banksia saxicola* is distinguished from *B. integrifolia* L. f. and *B. canei* J.H. Willis, to which it is closely related, by the absence of a lignotuber, grey-yellow coloured inflorescences, flowering time (January to March) and whorls of relatively large (40–100 × 10–35 mm) leaves (George 1981).

Wilson's Promontory National Park, situated approximately 230 km south-east of Melbourne, is the most southerly part of the Australian mainland and includes Devonian granitic mountains in the south and Quaternary sands on the Yanakie Isthmus in the north. During the last glacial maximum, 18–20,000 years ago, sea level was approximately 150 m lower than at present and Wilson's Promontory formed part of a land bridge to Tasmania; sea-level probably reached its present height about 7,000 years ago (Costermans 1998). There is a wide range



Fig. 1. Location of the Grampians (a) and Wilson's Promontory (b) National Parks within Victoria, Australia

of vegetation types at Wilson's Promontory and at least 668 native plant species have been recorded (Wescott 1998). Floristic links between Wilson's Promontory and Tasmania are evident (Costermans 1998).

The Grampians Ranges in western Victoria, 260 km north-west of Melbourne, are a group of isolated mountains consisting of quartzose sandstone of late Silurian-early Devonian age (Calder 1987). Today, the closest sea coast to the Grampians is more than 150 km to the south. However, about 14 million years ago, the Grampians were probably part of the coastline (Calder 1987). The Grampians are one of Australia's most botanically rich regions, containing over 1,000 plant species. They include at least 20 species that are endemic (Elliot 1984, Newnham et al. 1986, Whiffin and Ladiges 1992, Ladiges and Whiffin 1993) and several that are limited to a single range, for example *Eucalyptus victoriana* Ladiges and Whiffin (Ladiges and Whiffin 1993). A number of species, in addition to *B. saxicola*, have unexpectedly disjunct distributions, including links with Tasmania (e.g. *Leptospermum turbinatum* J.H. Willis) and the Blue Mountains, New South Wales (e.g. *Hibbertia cistiflora* N.A. Wakef.; Calder 1987).

Middleton et al. (1996) studied the population ecology of *B. saxicola* at the Grampians

and Wilson's Promontory and concluded from morphological measurements that there was some degree of differentiation between Grampians and Wilson's Promontory populations. However, because these population differences could have been at least partly due to differences in the environments from which the samples were collected, and also because the differences were not clear-cut, recognition of distinct taxonomic units was not warranted.

This paper describes research that aimed to resolve more fully the degree of genetic differentiation between disjunct populations of *B. saxicola*, and to investigate differences in the genetic diversity of populations. The data collected are used to assess the conservation status of *B. saxicola*.

Materials and methods

Plant material. Young leaf tissue was collected from 59 plants from three populations of *B. saxicola*: 20 plants were from each of two populations at the Grampians National Park (Mt William and Victoria Range), and 19 plants were from a population at Wilson's Promontory National Park. Mt William is the highest point in the Grampians (1,167 m) and lies on the eastern side of the Park (37°18'S, 142°36'E; 950–1,167 m a.s.l.). The Victoria Range lies approximately 25 km west

of the Mt William population (37°18'S, 142°19'E; 820 m a.s.l.). At Wilson's Promontory, *B. saxicola* was collected in the south of the Park along the Sealers Cove walking track (39°2'S, 146°23'E; 150–300 m a.s.l.). Park rangers report that there is an additional population at Wilson's Promontory, accessible only by helicopter, approximately seven kilometres to the north-west of the Sealers Cove population (Elaine Thomas *pers. comm.*).

At the Grampians sites, plants were sampled at approximately ten metre intervals. At Wilson's Promontory, where *B. saxicola* is less common and sampling was more difficult due to the height of the trees, samples were taken wherever possible. Leaves were immediately wrapped in aluminium foil and placed on ice. Upon returning to the laboratory, leaves were stored at –70 °C until processed.

DNA extraction. Genomic DNA was isolated from 0.5–1.0 g of leaf tissue using the CTAB method described by Doyle and Doyle (1990), with the modifications suggested by Maguire et al. (1994). DNA samples were stored at –20 °C in Tris-EDTA buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA).

AFLP analysis. Approximately 250 ng of DNA were used for AFLP analysis, which was performed using the AFLP Analysis System I Kit and the protocol recommended by the manufacturer (Life Technologies). Briefly, genomic DNA was digested to completion with *EcoRI* and *MseI* and ligated to kit-supplied *EcoRI* and *MseI* adapters. Two rounds of PCR were then performed. Non-selective pre-amplification used primers provided by the manufacturer. During selective amplification, only some of the available restriction fragments are amplified. Selectivity was achieved using primers that annealed to either the *EcoRI* or *MseI* end of a restriction fragment and had three additional 3' nucleotides. These primers are referred to as *Eco*+3 and *Mse*+3, respectively.

The sequences of the *Eco*+3 primers were 5'-GACTGCGTACCAATTCNNN-3' (where NNN is either ACC or ACA). The sequences of the *Mse*+3 primers were 5'-GATGAGTCCTGAGTAANNN-3' (where NNN is either CTA or CTT). Table 1 lists the four primer combinations used in this study. The *Eco*+3 primers were end-labelled using [γ -³³P]-ATP (Amersham) and T4 polynucleotide kinase (Promega).

PCR products were mixed with 10 μ L of sequencing loading buffer (98% (v/v) formamide, 10 mM EDTA, 0.025% (w/v) xylene cyanol, 0.025% (w/v) bromophenol blue). Mixtures were heated for 3 min at 90 °C and then quickly cooled on ice. Four microlitres of each sample were electrophoresed in a 5.5% denaturing polyacrylamide sequencing gel at 65 W for approximately 2 h. After electrophoresis, gels were dried on filter paper and exposed to X-ray film (Kodak or Fuji) for 1–2 days at room temperature.

Classification and ordination. Bands on the autoradiographs were scored as present (1) or absent (0) for each individual plant and the resultant data matrices analysed using the multivariate analysis programme NTSYSpc 2.02i (Rohlf 1998). Similarity matrices were constructed using the Jaccard coefficient (Sneath and Sokal 1973). Two different pattern analysis techniques were used: hierarchical classification and ordination. The fusion strategy used for cluster analysis was the Unweighted Pair Group Method of arithmetic Averages (UPGMA). The accuracy of fit of data to the dendrogram was assessed by calculating the correlation between the phenetic matrix (derived from the dendrogram) and the original similarity matrix. The ordination method used was non-metric multidimensional scaling (NMDS). NMDS is an iterative process using a steepest descent algorithm to minimise stress, the deviation between the final similarity matrix and the original one (Kruskal 1964).

Table 1. Primer sequences used in AFLP selective amplification

Primer combination	<i>Eco</i> +3 primer (5'–3')	<i>Mse</i> +3 primer (5'–3')
1	GACTGCGTACCAATTC/ACC	GATGAGTCCTGAGTAA/CTA
2	GACTGCGTACCAATTC/ACC	GATGAGTCCTGAGTAA/CTT
3	GACTGCGTACCAATTC/ACA	GATGAGTCCTGAGTAA/CTA
4	GACTGCGTACCAATTC/ACA	GATGAGTCCTGAGTAA/CTT

Population genetic analyses. The population genetics software package TFPGA 1.3 (Miller 1997) was used to estimate genetic parameters. This package uses the method of Lynch and Milligan (1994) to estimate the frequency of the null recessive allele. Although estimating allele frequencies from dominant markers presents some statistical difficulties (Lynch and Milligan 1994), Krauss (2000) showed that accurate gene diversity estimates can be obtained with dominant markers from another outcrossing member of the Proteaceae, *Persoonia mollis* R. Br. From estimates of allele frequency, a number of polymorphism and population genetic measures were derived.

Genetic polymorphism. Two measures of genetic polymorphism were used: percentage of polymorphic loci and expected heterozygosity. Although the percentage of polymorphic loci is sensitive to sample size and the presence of rare alleles, it is still a useful relative measure of genetic polymorphism between populations. A more appropriate measure of genetic variation, which takes into account the systematic bias that a small sample size can cause, is Nei's (1978) unbiased measure of expected heterozygosity (H): the expected proportion of heterozygotes per locus in a randomly mating population. It is also equal to the expected proportion of heterozygous loci in a randomly chosen individual (Nei 1987).

Estimates of population divergence. Nei's (1978) unbiased estimate of genetic distance, D , is based on the probability that a randomly chosen allele from two populations will be different, relative to the probability that two randomly chosen alleles from the same population will be different. Under the neutral hypothesis, it enables a good genetic interpretation of the time elapsed since the populations became isolated. Although monomorphic loci should be included in the

analysis to provide an unbiased estimate, it is still a useful comparative measure.

Population genetic structure. The multivariate analysis programme NTSYSpc 2.02i (Rohlf 1998) was used to conduct randomisation tests to test significance of genetic differentiation between populations. Mantel tests (Sokal 1979) were used to estimate the association between the Jaccard similarity matrices and a population design matrix (Livshits et al. 1991). P -values indicate the probability of obtaining a more extreme estimate by chance alone. Ten thousand permutations were used in each comparison to obtain sufficient accuracy on the final probability.

Results

AFLP analysis. Although large numbers of amplified fragments were generated by each primer combination, only fragments that were unambiguous and polymorphic within at least one of the populations were scored. Those that were poorly amplified and thus difficult to score were excluded. Each primer pair generated a different pattern of bands and number of scorable polymorphisms. The total number of scorable bands, the number of polymorphic bands, and the percentage of polymorphic bands per primer pair for *B. saxicola* are shown in Table 2.

From a total of 123 fragments, 71 (58%) were polymorphic. Four of the 71 polymorphic markers were unique to the Wilson's Promontory population; three were unique to the Mt William population; five were unique to the Victoria Range population; and 15 were unique to both Grampians populations com-

Table 2. The total number of scorable bands, the number of polymorphic bands and the percentage of polymorphic bands per primer pair for *B. saxicola* individuals across all three populations sampled

Primer combination	No. scorable bands	No. polymorphic bands	Polymorphism (%)
1	28	17	61
2	33	16	48
3	32	20	63
4	30	18	60
Total	123	71	58

bined. Wilson's Promontory individuals, WP4 and WP5 had almost identical band patterns, differing at only two of the 71 loci scored. All other individuals in the Wilson's Promontory population differed by at least three bands. All individuals in the Grampians differed by at least five bands.

Classification and ordination. Cluster analysis of all the AFLP data by UPGMA resulted in a dendrogram that very closely conformed to the three different populations: Wilson's Promontory, Mt William and Victoria Range (Fig. 2). The co-phenetic correlation coefficient between the similarity matrix and the dendrogram was 0.824, indicating that the information presented in the dendrogram is a good representation of the original similarity data. In particular, the Wilson's Promontory population clustered separately from both Grampians populations. The two Grampians populations also clustered separately, although there was some overlap. The Mt William individuals, MW6, MW7, MW10 and MW18 clustered with the Victoria Range samples, and MW5 and MW8 were distinct outliers of the Mt William population.

NMDS in two dimensions converged after 213 iterations with a stress 1 value of 0.169. Figure 3 shows that all the Wilson's Promontory individuals group together and are clearly distinct from the Grampians individuals. The two Grampians populations are also distinct, with only one Mt William individual, MW10, grouping with the Victoria Range population, and two Victoria Range individuals, VR11 and VR17, grouping with the Mt William population. MW5 and MW8 again appear as clear outliers of the Mt William population.

Genetic polymorphism. The relative degree of genetic diversity, as measured by the percentage of polymorphic alleles and Nei's (1978) unbiased measure of expected average heterozygosity, was calculated for each population, the Grampians populations combined, and all three populations combined (Table 3).

The percentage of polymorphic loci present in each of the three populations differed markedly: in particular the value was much

lower for the Wilson's Promontory population than for both Grampians populations. In the Wilson's Promontory population, 46.5% of the 71 loci scored were polymorphic compared with 71.8% in the Mt William population and 66.2% in the Victoria Range population. Even when the two outliers MW5 and MW8 from the Mount William population were excluded from the analysis, the percentage of polymorphic loci present in the Mount William population fell to only 63.4%, with the total number of polymorphic loci in the analysis being 69. When the two Grampians populations were combined, 90.1% of loci were polymorphic.

Average expected heterozygosity also differed between populations. The mean expected heterozygosity for all three populations was 0.31. The two Grampians populations had higher levels of expected heterozygosity: $H=0.26$ for Mt William and $H=0.22$ for Victoria Range, than the Wilson's Promontory population, $H=0.19$.

Estimates of population divergence. Nei's (1978) unbiased genetic distance (D) was estimated for all three populations separately as well as the two Grampians populations combined as a single group. The genetic distances were high between the Wilson's Promontory population and Mt William population ($D=0.21$), between the Wilson's Promontory population and Victoria Range population ($D=0.18$), and between the Wilson's Promontory population and the Grampians populations combined ($D=0.18$). By contrast, the distance separating the Mt William and Victoria Range populations was low: $D=0.08$.

Population Genetic Structure. Mantel statistics were calculated to test the association between the Jaccard similarity matrix and a population design matrix. The design matrices were constructed of zero (0) and one (1) elements. Zero was used to indicate that the two samples in the Jaccard matrix were from the same group, and one was used to indicate that the two samples were from different groups. Three Mantel tests were performed.

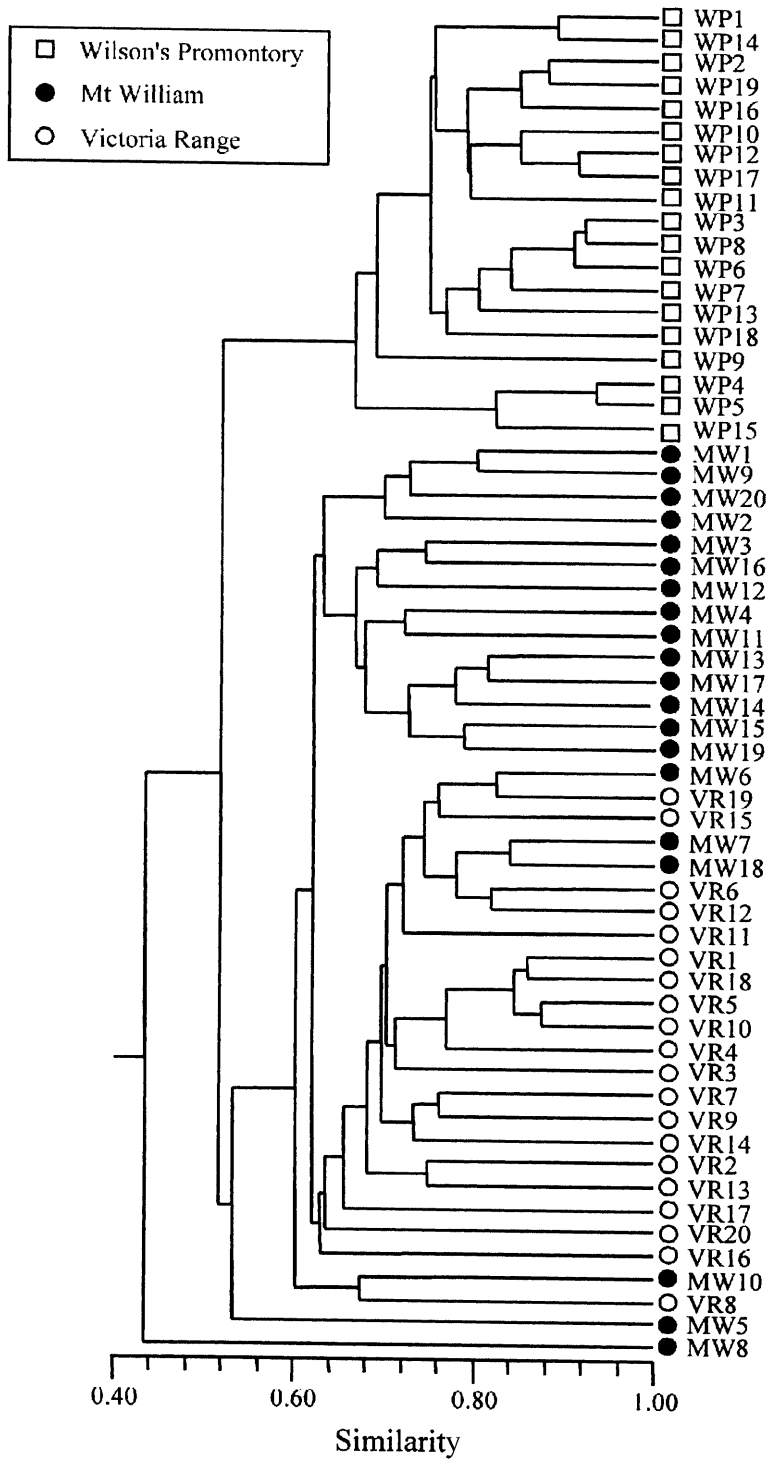


Fig. 2. UPGMA dendrogram of 59 plants from three populations of *B. saxicola* generated from a Jaccard similarity matrix using 71 polymorphic AFLP markers

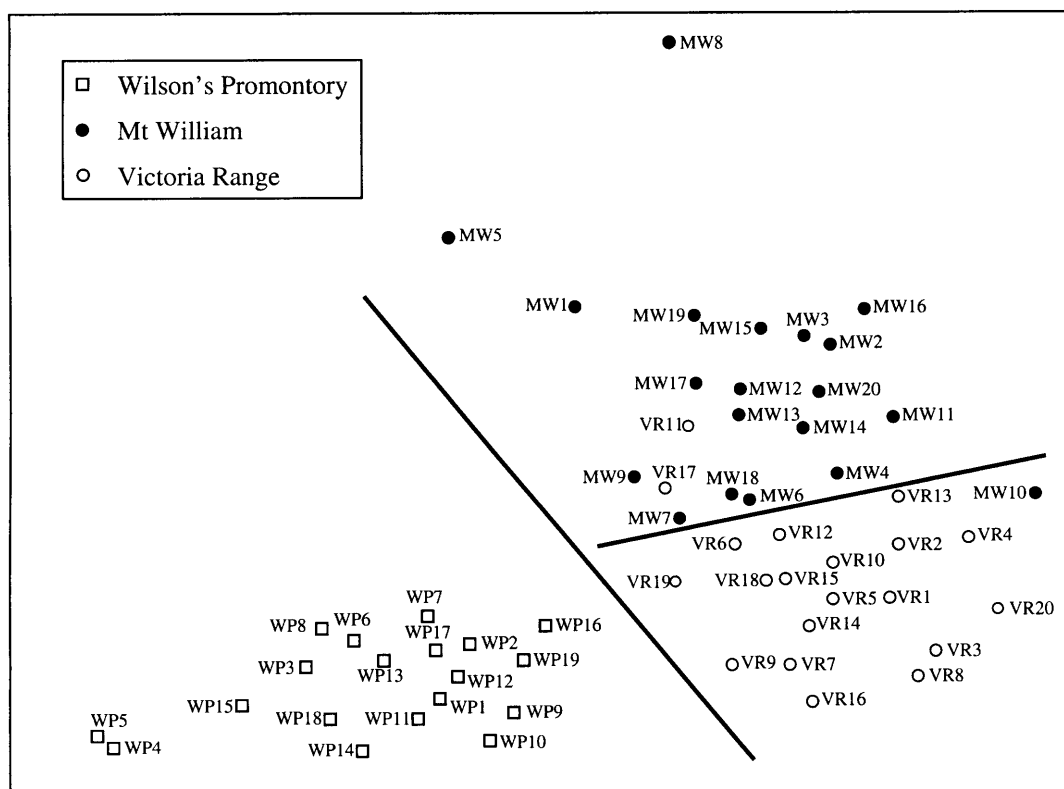


Fig. 3. NMDS in two dimensions of 59 plants from three populations of *B. saxicola* generated from a Jaccard similarity matrix using 71 polymorphic AFLP markers

Table 3. Genetic diversity estimates for the three *B. saxicola* populations, considered separately and with the two Grampians populations considered as a single group

	Wilson's Promontory	Mt William	Victoria Range	Grampians populations	All populations
Polymorphic loci (%)	46.5	71.8	66.2	90.1	100
Nei's average heterozygosity (<i>H</i>)	0.19	0.26	0.22	0.28	0.31

The first was an overall test of differentiation between populations, where all three populations were included. The second was to test whether the Wilson's Promontory population was differentiated from the two Grampians populations combined, i.e. two groups but all three populations included. The third was used to test whether the Mt William population was differentiated from the Victoria Range population, i.e. only the two Grampians populations were included.

The results were highly significant ($p \leq 0.0001$) for all three tests. The correlation coefficient for the comparison between the Wilson's Promontory and Grampians populations was the highest: $r = 0.63$. The matrix correlation was lower for the test of differentiation between the two Grampians populations ($r = 0.33$), suggesting weaker differentiation between the Grampians populations than between the Grampians and Wilson's Promontory populations. This was responsible for the

slightly lower matrix correlation when all three populations were compared: $r = 0.60$. The measures of population genetic divergence and population genetic structure are consistent with the results of the cluster analysis and ordination.

Discussion

Genetic divergence of populations. It was found that there was significant genetic differentiation between all three populations of *B. saxicola*, with the differences most pronounced between the Wilson's Promontory population and the combined Grampians populations. The three populations also had different levels of genetic diversity, with the smallest population, Wilson's Promontory, having lower genetic diversity than either Grampians population.

The significant genetic differentiation between the two Grampians populations is consistent with their geographic isolation, but more recent or less complete than between the Grampians and Wilson's Promontory. The geological nature of the Grampians, high mountain ranges separated by wide intervening valleys with unsuitable habitat, has resulted in genetic isolation of populations and contributed to a high level of endemism. The degree of genetic differentiation, however, is perhaps surprising considering the probable mating system of *B. saxicola*. As suggested by their floral morphology, the majority of banksias are preferential or obligate outcrossers (Scott 1980, Carthew et al. 1988, Carthew et al. 1996, Maguire and Sedgley 1997). Therefore it is likely that *B. saxicola*, which has conspicuous inflorescences and large stigma to nectary distances, is also predominantly an outcrosser. Although relatively little is known about the potential pollinators of *B. saxicola* (Taylor and Hopper 1988), it is likely that the large inflorescences attract a range of nectarivorous birds, which then disperse pollen over relatively large distances.

The disjoint distribution of *B. saxicola* is most likely due to localised extinction and

fragmentation of populations by increased aridity, climatic instability, volcanic flows and changes in sea-level during the Pleistocene. It is not known whether other populations of *B. saxicola* have become extinct since European settlement. In the absence of gene flow between populations of *B. saxicola* and given the small sizes of the populations involved, genetic drift and/or natural selection would lead to the large degree of genetic differentiation demonstrated in this study and the divergence of morphological traits described by Middleton et al. (1996). Similar genetic divergence between populations, associated with clear geographic separation, is a feature of several species elsewhere in Australia, where large contiguous distributions have been fragmented by climatic changes, for example in members of the Mimosaceae (Coates 1988), Myrtaceae (Moran and Hopper 1983), and Proteaceae (Coates and Sokolowski 1992, Coates and Hamley 1999) in south-west Western Australia.

Certain individuals in this study were noticeably different at the genetic level from others in the same population, for example MW5 and MW8. These outliers contributed to the high estimate of diversity in that population and could have resulted from introgression from another species. However, hybridisation does not seem to be a common occurrence between *B. saxicola* and other *Banksia* species either at the Grampians or at Wilson's Promontory. The only reported hybrids, based on morphology, are a small cluster of about six individuals in the Grampians that are thought to be hybrids of *B. saxicola* and *B. marginata* Cavanilles (Middleton et al. 1996). None of these individuals was sampled. Other explanations include gene flow from other populations within the Grampians and, due to the small number of individuals sampled, there may be an under-representation of rare alleles.

The lower level of genetic diversity (as measured by H) for the Wilson's Promontory population suggests that geographic isolation and small population size have led to loss of alleles and lower heterozygosity through genetic

drift and inbreeding. There is also the possibility that the Wilson's Promontory population has undergone adaptation to grow in this location, and that this has influenced levels of genetic diversity. The decline in diversity, depending upon how rapidly it has occurred, may have direct effects on individual fitness or depress reproductive success (Ellstrand and Elam 1993). Although less diverse than the two Grampians populations, the Wilson's Promontory population has maintained a degree of genetic variation, most likely because of the mating system of *B. saxicola*, which promotes outcrossing.

In addition, it is possible that gene flow between the sampled population and other *B. saxicola* individuals at Wilson's Promontory also contributes to genetic diversity. There are reports of an isolated individual at Refuge Cove, approximately five kilometres south-east of the Sealers Cove track population, and a population at Five Mile Peak (450 m a.s.l.), approximately seven kilometres to the north-west (Elaine Thomas *pers. comm.*).

Conservation of *B. saxicola*. In the Grampians, populations of *B. saxicola* are comparatively large and regeneration occurs in the absence of fire. There appears to be no apparent or immediate threat to the survival of the species at the Grampians. At Wilson's Promontory, *B. saxicola* is less abundant and no regeneration in the Sealers Cove track population has taken place for at least twenty years, there being no reports of fire in the area since 1951 (Middleton et al. 1996). Middleton et al. (1996) reported 166 *B. saxicola* individuals in close proximity to the walking track, but during this study, problems were experienced finding just nineteen individuals from which leaf samples could be taken. There was evidence that some individuals alongside the walking track had recently fallen, presumably blown over in their weakened and senescent state.

Banksia saxicola is thought to be killed by fire and to rely on post-fire seedling recruitment for regeneration (George 1981). Evidence for this includes the thin bark of old trees (2–4 mm), and the absence of a lignotuber. Since *B. saxicola* predominantly flowers in late

summer and seeds mature after one year, late summer and autumn fires would maximise the release of seed from the woody follicles (Middleton et al. 1996).

For the Grampians National Park, Tolhurst (1996) identified *B. saxicola* as a key fire response species in scrub communities (key fire response species are those in a community that are likely to be lost from a site, either through too frequent or too infrequent burning). Tolhurst concluded that the minimum interfire period to maintain both structural diversity and species richness is ten years, and the maximum interfire period is 50 years.

The *B. saxicola* population at Wilson's Promontory therefore appears vulnerable to extinction or loss of diversity in the medium term unless active management is undertaken in the near future. *Banksia saxicola* at Wilson's Promontory is a remnant population in 'natural' decline, and it has to be decided whether it deserves to be maintained here by active management. This population is genetically distinct from the Grampians populations and, as a result, it would be desirable to preserve it in order to maintain maximum genetic diversity within this rare species.

A burn combined with suitable conditions for post-fire seedling recruitment would be the most obvious management option available. Results from this study suggest that transfer of material between the Grampians and Wilson's Promontory would have to be considered very carefully, due to the high degree of genetic differentiation between *B. saxicola* at these two sites, especially if some of this differentiation is a result of adaptation to different environments. Transfer of seed between populations at Wilson's Promontory is, however, an option worth considering.

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