Genetic structure and diversity in the soil-stored seed bank of the endangered *Grevillea caleyi*

Tanya Llorens

Institute for Conservation Biology
University of Wollongong

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Tanya Llorens
Institute for Conservation Biology
Department of Biological Sciences
University of Wollongong
Wollongong NSW 2522 Australia

Post-fire *Grevillea caleyi* seedling
(Photo: S. Humphrey)

Grevillea caleyi inflorescence
(Photo: T. Llorens)

Cover illustration: young adult *Grevillea caleyi* plants (Photo: S. Humphrey)

Summary

Grevillea caleyi is an endangered, fire-sensitive and self-compatible shrub that has been severely affected by recent habitat fragmentation. Small population size appears to have a negative impact on plant fecundity, plant survivorship, the mating system, and pollinator visitation in *G. caleyi*. These effects, as well as the random processes that occur in small populations, render small remnants of formerly more extensive *G. caleyi* populations vulnerable to genetic problems such as the loss of genetic diversity and increased inbreeding, which may in turn cause reduced population viability. However, *G. caleyi* possesses a long-lived soil-stored seed bank that, theoretically, might buffer some of these genetic changes by storing genetic diversity from previous generations. In this study, I investigated whether the seed bank of *G. caleyi* possesses genetic characteristics that may allow it to slow the rate of genetic changes in populations that have suffered various effects of habitat fragmentation.

I used seven microsatellite loci to genotype both the adult plants at three *G. caleyi* populations, and also the seedlings that emerged following fire. One population, DF, was of moderate size (several hundred plants), while two (RA and JJM) had very small pre-fire adult populations (5 and 14 adults) but over 100 post-fire seedlings. DF had no genetic diversity among the adults or seedlings, which were genetically identical for the markers I used, indicating historical isolation and/or the effects of past population bottlenecks. At the other two populations, post-fire seedlings had similar allele frequencies to pre-fire adults but greater genetic diversity, as indicated by the percent of loci that were polymorphic and the levels of heterozygosity. In addition, the seed bank contained 20% (RA) and 12.5% (JJM) more alleles than were found among the adults. Parentage analyses revealed that, for some seedlings, neither parent was found among the pre-fire adults.

These results indicate that the seed bank was highly effective at preserving both the genetic composition and frequencies of alleles present in pre-fire adults, despite the severe bottleneck that occurred in the pre-fire adult population. They also indicate the effectiveness of the seed bank in storing alleles and diversity that has been lost from, or not expressed in, the adult population, although these results may be partly due to some pre-fire adults having died prior to my sampling. The major mechanisms by which the seed bank has apparently buffered the loss of genetic diversity from these small populations is by increasing their effective population size to well above that of the adult plants, as well as the presence of multiple generations of seed in the seed bank, due to some seed remaining dormant following a fire.

Despite the above results, post-fire seedlings showed evidence of greater inbreeding than pre-fire adults at RA and JJM, and greater spatial genetic structure at JJM, indicating a possible effect of habitat fragmentation on the current genetics of these populations. When fresh seed collected from JJM were genotyped, they revealed higher estimates of inbreeding than seedlings and adults, large variation among plants in outcrossing rate and in male reproductive success, and showed that most outcrossing is between very near neighbours. In addition, parentage analyses of seedlings at JJM revealed that most seedlings were likely to have been the selfed progeny of a very small proportion of the pre-fire adults. These results suggest that genetic changes are occurring within these populations and are likely to continue occurring, particularly at JJM, where each subsequent adult generation is certain to be very small, due to the limited habitat patch size.

While the results of this study indicate that the seed bank of *G. caleyi* possesses properties that should enable populations to slow the rate of loss of genetic diversity and changes in allele frequencies following habitat fragmentation, they also suggest that the ability of the

seed bank to buffer such changes is limited and short-term, and likely to be exhausted after several generations of continuing small population size and reduced pollinator visitation.

Introduction

Grevillea caleyi R. Br. (Proteaceae) is an endangered, fire-sensitive perennial shrub that occurs in the northern suburbs of Sydney, Australia. It occurs in open forest and is naturally restricted to the laterite soils of four ridges within an area of approximately only 8 x 8 km. *Grevillea caleyi* probably historically consisted of four large and essentially continuous populations, with one occurring on each ridge (Scott *et al*. 1995). However, following urbanisation in the area following European settlement, an estimated 85% of the species' habitat has been lost (Scott *et al*. 1995). Each ridge now contains several *G. caleyi* populations in remnant patches of bush, that presumably represent the only remaining fragments of a single, much larger, former population (Scott *et al.* 1995). There are currently more than 20 known *G. caleyi* populations (NSW NPWS 2001), all of which are undoubtedly much smaller in size than they would have been prior to the last century (T. Auld pers. comm.). The remaining populations vary dramatically in size, from less than ten to several thousand plants, and degree of isolation from the nearest population (from 300 m to 2.2 km). Only one population, which is located wholly within the Ku-ring-gai Chase National Park, is protected from the urban development that continues in the area (NSW NPWS 2001). The species is listed as endangered in NSW (*NSW TSC Act* 1995) and federally (*EPBC Act* 1999).

Recent experience has shown that extinction rates increase and species diversity decreases with increasing habitat fragmentation (Soulé *et al.* 1992). Plant populations occurring in fragmented habitats tend to be smaller, more isolated and less dense, and may experience changes to their physical environment, disturbance regimes, and ecological processes. Consequently, fragmented populations are much more susceptible to a range of environmental, demographic and genetic processes that may reduce population viability and ultimately lead to population extinction (Barrett and Kohn 1991; Lacy 2000). For example, population genetic theory predicts that small, isolated populations should experience increased random genetic drift and inbreeding and reduced inter-population gene flow (Wright 1969; Barrett and Kohn 1991). This, in turn, may result in a depletion of genetic diversity within populations, increased genetic divergence among populations, inbreeding or outbreeding depression, and ultimately, reduced fitness (Barrett and Kohn 1991, Young *et al.* 1996). In the long term, inbreeding depression and reductions in genetic diversity may be the most important contributors to extinction in small populations (Ellstrand and Elam 1993).

Previous research has indicated that *Grevillea caleyi* is at risk from some of these processes. Small *G. caleyi* populations have smaller plant size, produce fewer flowers and fruit, and have fewer pollinator visits and a less diverse pollinator assemblage than large populations (Llorens in prep.). These results suggest that small populations are at risk demographically due to low fecundity and mate availability. However, they also suggest that small populations may experience greater inbreeding; *G. caleyi* is highly self-compatible (Llorens in prep.), so reduced pollinator visitation may result in increased rates of selfing, and a paucity of mates may mean that any outcrossing is more likely to be with relatives. In conjunction with the increased genetic drift that occurs in small populations, an increase in inbreeding may lead to the loss of genetic diversity within populations, increased genetic divergence among populations, and possibly inbreeding depression if inbred plants are less fit. Indeed, small *G. caleyi* populations do appear to have lower current rates of outcrossing compared with large populations (Llorens in prep.). However, several generations after fragmentation, small populations do not appear to have lost genetic diversity when compared with large populations, despite the smallest populations containing less than ten adult plants (Llorens *et al.* submitted; Llorens in prep.).

One of the major reasons for the maintenance of genetic diversity within small populations of *G. caleyi* may be the species' long-lived soil-stored seed bank. A census of adult plants or seedlings may significantly underestimate actual population size. At any one time, the seed bank may contain a considerable proportion of a population's real abundance of *G. caleyi*, and can buffer large demographic changes to the adult population (Llorens in prep.; Auld and Scott submitted). Therefore, the seed bank may also have the potential to buffer the loss of genetic diversity, altered allele frequencies and increased inbreeding that might occur in populations following habitat fragmentation. Persistent seed banks are thought to act as a genetic "memory", storing seed genotypes produced over many seasons, often over several generations, and potentially under different selective regimes (Templeton and Levin 1979). Thus, seeds that germinate from a persistent seed bank represent genetic migration from the past, dampening genetic and fitness fluctuations and slowing evolutionary change (Templeton and Levin 1979; Hairston and De Stasio 1988). In addition, a dormant seed bank should increase the effective population size (N_E) of a population to beyond that of the adult generation, and may contain a valuable store of genetic diversity that is not present in the current adult generation. Therefore, seed banks are often predicted to buffer small populations from the loss of genetic diversity (*e.g.* Falk and Holsinger 1991; Ellstrand and Elam 1993), and may be important in slowing the genetic, and potentially fitness, changes that may occur following alterations to population and habitat characteristics, such as reductions in population size and loss of pollinators.

No empirical studies have directly tested the ability of the seed bank to buffer genetic changes to a population. However, several recent studies have investigated the genetic characteristics of the seed banks of various, mostly common, plants. Of these, one of the greatest insights was provided by Del Castillo (1994), who found indirect evidence that the seed bank of *Phacelia dubia* maintained the genetic diversity of its populations despite wide fluctuations in population size. Several studies have reported greater genetic diversity in the seed bank than in the current crop of adult plants (Alvarez-Buylla and Garay 1994; McCue and Holtsford 1998; Morris *et al.* 2002), while others found no difference in genetic diversity between the seed bank and adult plants (*e.g.* Peroni and Todd 2001). This provides support for the idea that seed banks can maintain genetic diversity in populations over time, and re-introduce diversity from past generations that has been lost from the adult population. However, most studies have found allele frequencies to vary significantly among the seed bank and adult plants (Tonsor *et al.* 1993; Cabin 1996). Among the plants studied so far, there appears to be a general phenomenon for seed banks to be more homozygous and have less spatial genetic structure than adult plants (*e.g.* Tonsor *et al.* 1993; Cabin *et al.* 1998; McCue and Holtsford 1998). Unfortunately, this may make it difficult to detect the effects of any changes to the mating system or gene flow that may follow recent anthropogenic disturbances. In addition, as nearly every study has examined the seed bank and adult plants over only a single generation, it is difficult to draw conclusions about the potential for seed banks to retard genetic changes over a longer time frame.

The apparent longevity of *G. caleyi* seeds in the seed bank (Auld *et al.* 1993), as well as the sizeable residual seed bank that remains following a fire (Auld 1994; Scott *et al.* 1995), provide its seed bank with the means to store and re-introduce diversity from previous generations that has been recently lost from adult populations. However, a lack of empirical data makes it difficult to make informed predictions about the genetic characteristics of the seed bank of *G. caleyi*. No other studies have investigated the genetics of *Grevillea* seed banks, and I failed to find any studies that have investigated the genetics of seed banks in other Proteaceous species or fire-sensitive Australian plants. In addition, I could only find three studies of the seed bank genetics of a rare plant (McCue and Holtsford 1998; Aparicio *et al.* 2002; Morris *et al.* 2002).

In this study, I investigate the genetic characteristics of the seed bank of *G. caleyi* to determine the degree to which it reflects the genetic composition, structure and inferred mating system of adult populations. My aim was to both characterise the genetics of the seed bank, and to interpret the results in terms of the species' current and potential future response to habitat fragmentation. I address the following questions:

(1) Do seed bank and adult populations of *G. caleyi* display similar genetic diversity, allele frequencies and fine-scale spatial genetic structure?

(2) Do adult plants, freshly-produced seed and soil-stored seed appear to have been generated by similar mating systems, and is there spatial variation in the mating system?

(3) Are the likely parents of seeds in the seed bank found among the currently extant adult plants?

Methods

Biology of *G. caleyi*

Grevillea caleyi occurs within an area of approximately 8 x 8 km, centred around Terrey Hills, in the northern suburbs of Sydney (33°42'S, 151°13'E). It has a very narrow habitat preference, occurring in open eucalypt forest on the laterite soils of four ridges with elevations between 170 to 240 m above sea level (NSW NPWS 2001). Plants grow to approximately 3 m tall and 4 m wide. They are typically distributed in a series of discrete patches within their habitat, and may occur very densely within patches (up to 70 adult plants in a 10 x 10 m area (T. Llorens pers. obs.)). In the absence of fire, adult plants senesce rapidly at about 12-15 years of age (Scott *et al.* 1995). Adult plants are killed by fire, and there is negligible germination between fires, so populations are typically even-aged. Therefore, the time elapsed since the most recent fire (= population 'age') determines, to some extent, plant abundance.

Plants flower sporadically throughout the year, with a definite peak from late winter to spring (McGillivray 1993), but do not mature and become reproductive until 2-5 years of age (Scott *et al.* 1995). They have large, red, toothbrush-shaped inflorescences that produce large quantities of nectar and are mainly pollinated by birds; the introduced honeybee *Apis mellifera* is also a frequent flower visitor (T. Llorens pers. obs.). The species is self-compatible, and readily sets seed autogamously and when mediated by pollinators (Llorens in prep.). Fruits mature two to three months after fertilisation, depending on season, and contain a single woody seed that is dehisced as soon as maturation is complete. Seed viability is high (69-95%; Auld *et al.* 1993; NSW NPWS 2001). The seeds are large (15-20 mm long) and heavy (300-400 mg), and are dispersed by gravity alone; there is no apparent mechanism for secondary seed dispersal (Auld & Denham, 1999). The majority of seeds are eaten by rodents and macropods shortly after they fall to the ground (Auld and Denham 1999). The remaining seeds become buried in the soil, probably facilitated by the activities of soil-dwelling invertebrates and foraging vertebrates as well as heavy rainfall events. The seeds are relatively long lived - they have an estimate half-life of 7.6 years (Auld *et al.* 2000) – and may remain stored in the soil seed bank for many years after the death of adult plants (T. Auld pers. comm.). However, the magnitude of the seed bank should decline when most adult plants are senescing, due to a reduction in seed production and decay of older seeds in the soil (Auld *et al.* 1993). Fire stimulates mass germination and rapid emergence of seedlings (Auld and Scott 1997). There is some polymorphism among seed in their responses to germination cues, but smoke is the major stimulant for germination (Llorens in prep.). However, a large proportion of the seed bank may remain dormant following a fire. Auld (1994) used soil sieving to show that between 55% and 83% of the available seed bank of *G. caleyi* emerged following a fire, and field observations of germination following two fires separated by a short time interval (Scott *et al.* 1995) also indicated that many seed must have remained dormant but viable following the first fire.

Population sampling

Seed bank

I accessed the soil seed bank indirectly by sampling seedlings that appeared post-fire. This was necessitated by very low densities of seed in the seed bank; previous work showed that soil sieving gave a very low reward per unit effort (Llorens in prep.). This approach ensures that only viable seed are sampled but inevitably fails to sample the viable seed that may remain dormant after a single fire event (Auld 1994). Sampling only seedlings may therefore

underestimate the genetic diversity of the entire germinable seed bank, but it provides the most direct comparison between the genetic composition of adults and of the first cohort of post-fire seedlings. Stimulating the germination of the residual seed bank for sampling was not an option, as this would probably have completely exhausted the seed bank (Auld 1994) and therefore greatly increased the risk of local extinction (Auld and Scott 1996).

Post-fire seedlings were sampled from each of three *G. caleyi* sites (DF, JJM and RA; Table 1). The selection of sites was limited to those that, during this project: (a) experienced a fire that burned all or most of the site, and (b) had previously been adequately sampled for the genetic diversity of pre-fire adult plants (Llorens in prep.). The sampling methodologies and sample sizes varied among sites, due to differences in seedling and adult distribution and abundance (Table 1).

Table 1. Abundance of pre-fire adults and post-fire seedlings, and sampling for genetic analysis, in three *Grevillea caleyi* populations

For JJM, details are given for the population as a whole and for each individual patch of plants. For RA, only seedlings from the same patch as the adult plants were considered. Scott *et al.* (1995) and NPWS (2001) had estimated adult population size 3 years earlier; the largest of these estimates is shown in brackets if it exceeded population size at the time of sampling.

Population	No. pre-fire adults	No. adults sampled	No. post-fire seedlings	No. seedlings sampled
DF	~100 (~500)	15	707	30
RA	5 (6)	5	866	20
JJM	11* (17)	11	160	60
JJM1	5	5	55	20
JJM3	3	3	60	20
JJM4	3	3	45	20

* At JJM, there were 11 pre-fire adult plants in the three burned patches, but 14 in the whole site counting the unburned patch not included in this study.

At DF, the entire adult *Grevillea* population was burned in October 1998. At this site, 30 seedlings were sampled using a stratified random approach that was designed to maximize the amount of the population's genetic diversity sampled, and had been previously used to sample the pre-fire adult population. The distribution of patches of *G. caleyi* plants and lone individuals throughout the population was roughly mapped, and the number of plants in each patch was directly counted or, for very large patches, visually estimated. The number of plants sampled from each patch was proportional to the abundance of adult plants in each patch relative to the whole population. Therefore, sampling reflected the pattern of distribution of plants within the population and covered the full geographic extent of the population. Plants to be sampled from each patch were selected randomly. Adults were sampled in 1997, approximately 9 to 12 years following the previous fire (Scott *et al.* 1995).

At JJM, there were four discrete patches of remnant bushland containing *G. caleyi* (JJM1 - JJM4), but only three of these patches were burned in September 2001 (JJM1, JJM3 and JJM4). From each of the three burnt patches, 20 seedlings were sampled using a stratified random approach. The unburnt patch (JJM2) was not dealt with in this study. JJM3 and JJM4 were separated by a distance of only 9 m, while JJM1 was separated from JJM3 and JJM4 by 48 m and 58 m respectively. All adults present in these patches were sampled in 1997,

approximately 10 years after the previous fire (Scott *et al.* 1995). The three adult plants from the unburnt patch (JJM2) were not included in comparisons between seedlings and adults.

At RA, post-fire seedlings emerged in seven discrete patches distributed over a wide area following a fire in 1999. However, only five adults from one of these patches were alive at the time of pre-fire sampling, as the site had been long unburned prior to the most recent fire (NSW NPWS 2001). As the pre-fire samples had not adequately sampled the whole site, I only sampled seedlings from same patch as the pre-fire adults, and did not sample the other patches. Although this probably resulted in an underestimate of seed bank genetic diversity within the population, there was no valid way to compare this with the whole population's pre-fire adult diversity. Within the sampled patch, seedlings were distributed among three fairly distinct but closely positioned clumps (RA1, RA2 and RA3). I sampled seven, eight and five seedlings respectively from each clump, but treated the 20 seedlings as a single sample (RA) for most of the analyses. The whole patch was contained within an area of approximately 18 x 28 m, with each clump separated by less than 10 m. The site appears to have been created by movement of soil during nearby excavations about 30 years ago (T. Auld pers. comm.). There is evidence that *G. caleyi* plants had been more widely spread over the site in the past, probably having germinated following soil disturbance (NSW NPWS 2001). Prior to the recent fire, the site did not appear to have been burned since its creation.

A single leaf was collected from each seedling sampled, and dried for several days using crystalline silica desiccant prior to DNA extraction.

Fresh seed

Genotyping adults and seedlings can produce indirect estimates of the mating systems that produced these cohorts. However, these estimates may deviate considerably from the true mating system due to selection, or to the temporal or spatial mixing of mating groups that differed in their mating system. To obtain a more direct estimate of mating systems, it is necessary to genotype cohorts of seed with known maternal parentage. This was done for adult plants at one *G. caleyi* site, JJM, therefore providing three separate estimates of the mating system at this site.

Freshly-produced seed were collected from *G. caleyi* plants at JJM between three and four years before the fire. Seed were collected from maternal plants by bagging mature fruit shortly before fruit dehiscence. Ten seed were collected from each of two plants at JJM1, and two plants at JJM4.

Genotyping

Preliminary trials had shown allozyme loci to be almost invariant in *G. caleyi* (A. Hunt and T. Llorens, unpublished data), so microsatellite loci were used to conduct the genetic analyses.

DNA extraction

I extracted genomic DNA from plant tissue using a CTAB procedure slightly modified from Doyle and Doyle (1987), with 1% w/v PVP (polyvinylpyrrolidone MW 40 000) added to the extraction buffer. For leaves, I ground 15-20 mg of fresh or frozen (-20^{o}C) tissue in liquid nitrogen before the addition of extraction buffer. For seeds, I ground a small piece of embryo (approx. 3 mm^3) directly in CTAB buffer. Following extraction, I resuspended the DNA in 50 µl TE buffer, and stored it at –20°C.

Microsatellite markers - transfer from G. macleayana *and* G. iaspicula

I successfully transferred seven microsatellite primers to *G. caleyi* from two congeneric species; these primers had previously been developed for *G. macleayana* (England *et al.* 1999; England *et al.* unpublished) and *G. iaspicula* (Hoebee 2002). I screened 16 *G. macleayana* and 10 *G. iaspicula* primers for transferability to *G. caleyi*.

To determine whether each primer could produce amplification products from *G. caleyi* DNA, PCR was performed with an annealing temperature of 50°C in 20 µl reaction mixtures containing ≈ 20 ng of genomic DNA, 2.5 mM $MgCl_2$ and 10 pM of primer (see section 3.2.3 for remaining ingredients and PCR procedure). Where amplification was successful, the salt concentration of the PCR mix and the annealing temperature were optimised for band brightness and sharpness with the aid of a gradient thermal cycler. All PCR products were visualised on 1.8% agarose gels containing ethidium bromide. Each successfully amplifying primer was then screened for polymorphism in 6 or 8 plants per species (2 plants from each of 3 or 4 geographically distant populations). Primers were used in this study only if their amplification products were polymorphic and could be produced reliably, scored unambiguously and produced repeatably. Seven microsatellite primers were selected for use with *G. caleyi*, the details of which are shown in Table 2.

Table 2. Microsatellite loci used to genotype *Grevillea caleyi* plants

Details are given for the repeat motif, quantity and size range of alleles identified in the species, and the annealing temperature used in PCR reactions. Loci that were confirmed to not be true microsatellites are indicated by *.

Locus	Repeat motif	Number of alleles	Allele size range	Annealing temp (°C)
Gm10	$(CT)_n$	2	156-158	53
Gm13	$(CT)_n$	4	129-149	55
Gm37	$(CT)_n$	3	134-140	50
GmD	$(GA)_n$*	11	159-173	50
GmI	$(GA)_n$*	4	168-171	65
Gi7	$(TG)_n$	2	220-224	50
Gi9	$(GA)_n$	11	187-215	53

Microsatellite procedure

PCR reactions were performed in a total volume of 20 µl, containing 1 Unit Taq Polymerase, 200 µM of each dNTP, 2.5 mM $MgCl_2$, 10x buffer (all purchased from Promega), 10 pM of each primer (synthesized by Sigma Genosys), 250 nM of fluorescent dCTP (R110, R6G or TAMRA; Applied Biosystems) and ≈ 20 ng of genomic DNA. An initial denaturation at 94°C for 4.5 min was followed by 30 cycles of: 30 s at 94°C, 30 s annealing then 1 min at 72°C; ending with one cycle of 5 min at 72°C. The only modification to this program was that *GmD* reactions proceeded for 36 rather than 30 cycles due to the faintness of the amplification products. All reactions were performed using either a PTC-100 thermal cycler (MJ Research) or an Eppendorf Mastercycler gradient thermal cycler. Reactions were multiplexed, where possible, and annealing temperature and salt concentration optimised. Salt concentration was

optimised at 2.5 mM $MgCl_2$ for all reactions. *Gm10* and *Gi9* were multiplexed, as were *Gi7* and *Gm37*; the remaining primers were amplified individually. Annealing temperatures for each primer are shown in Table 2. Most seed extracts contained a white starchy substance that inhibited PCR, but this problem was overcome by centrifuging the DNA solution for two minutes immediately prior to the removal of DNA for addition to the PCR solution.

I electrophoresed PCR products on 5% polyacrylamide gels and visualised them using an ABI Prism 377 automated DNA sequencer. Prior to electrophoresis, I combined denatured PCR products with loading mix containing GeneScan Rox 500 internal size standard (Applied Biosystems). I determined allele sizes using ABI Prism GeneScan Analysis (PE Applied Biosystems, Version 3.1) and ABI Prism Genotyper (Perkin-Elmer, Version 1.1.1). I ran at least one previously-scored individual on each gel to ensure between-run consistency in assignment of allele sizes. If samples amplified poorly (*i.e.* alleles had low peak intensities), I re-ran PCR products or repeated the PCR amplification.

Data analysis

Genetic diversity and allele frequencies in seedlings and adults

Several measures of genetic diversity were calculated for pre-fire adults and post-fire seedlings at each site. The number of alleles per locus (A_O), proportion of loci that were polymorphic (P_L) and expected heterozygosity (H_E: a measure of allelic diversity) were calculated using POPGENE 1.32 (Yeh *et al.* 1997). At JJM, genetic diversity was calculated both for the population as a whole, and separately for the three burnt patches of plants. Paired *t*-tests were used to test for differences in A_O among adults and seedlings, with each locus representing a replicate data point.

To test for genetic differentiation among adults and seedlings, F_{ST} was calculated among adults and seedlings for each site, and for each patch at JJM, using FSTAT (Goudet 2001). F_{ST} was estimated as θ (Weir and Cockerham 1984), and is a measure of genetic subdivision among populations that can range from 0 to 1, where 0 indicates no subdivision and 1 complete population differentiation. Means were jackknifed over loci, and their statistical significance tested using bootstrapped confidence intervals.

Fine-scale spatial genetic structure in the seed bank

To test for fine-scale genetic differences among *G. caleyi* seedlings within sites, F_{ST} was calculated among (a) three patches of seedlings at JJM, and (b) three clumps of seedlings within the seedling patch at RA. At JJM, F_{ST} was also calculated among pre-fire adults from the three patches, to determine whether seedlings and adults demonstrated the same degree of genetic differentiation among patches. In addition, principal coordinate analyses (PCoA) were used to provide visual representations of the genetic relationships among seedlings within and among clumps at RA and patches at JJM. PCA was performed on an NxN matrix of pairwise genetic distances among seedlings in GenAlEx V5 (Peakall and Smouse 2001).

Genetic diversity (A_O, P_L and H_E) and allele frequencies were contrasted among patches of seedlings at JJM and clumps of seedlings at RA.

Spatial and temporal variation in the mating system

Wright's fixation index, f (Weir and Cockerham 1984), was used to produce indirect estimates of the mating system that had generated (a) the adult population, and (b) the seedling

population, at each site. Values of *f* can range from –1 to 1, where negative and positive values represent excesses and deficits of heterozygotes, respectively, compared with expectations under conditions of Hardy-Weinberg equilibrium (random mating). At one *G. caleyi* site, JJM, *f* was also determined for fresh seed. Exact tests were used to test for heterozygote deficiency. For the reasons outlined by Ryman and Jorde (2001), Hurlbert and Lombardi (2003) and Moran (2003), no adjustments were made for multiple testing within populations. All calculations were performed using GENEPOP 3.3 (Raymond and Rousset 1995). Direct estimates of the mating system at JJM were obtained by calculating the outcrossing rate (*t*) among fresh seed using MLTR (Ritland 2001).

To detect temporal variation in the mating system, single-locus *f* values were compared among the adult and seedling populations at each site using paired *t*-tests, with the assumption that the seedlings provided a more recent estimate of the mating system than did the adults. As each generation of adult plants continues to produce seed over several to many years, however, mating systems may change even during a generation. With the residual seed bank allowing some overlap of generations, it is clear that each adult and seedling population may have been produced by more than one mating system. These possibilities were acknowledged during interpretation of the results.

At JJM, direct estimates of the current mating system were obtained by calculating the multi-locus outcrossing rate (*t*) among fresh seed collected from pre-fire adult plants. The outcrossing rate provided an estimate of the mating system over a short period of time (1 or 2 seasons), and represented a much more recent mating system than that which produced the adult plants. It is likely that at least some of the seedlings were produced by the same mating system as that which produced the fresh seed, thereby assisting in the interpretation of the seedlings' mating system. *t* was calculated using the program MLTR version 2.2 (Ritland 2001), using the expectation-maximization (EM) method to calculate the maximum likelihood estimates, and standard errors based on 1000 bootstraps with resampling among maternal plants. The microsatellite loci were previously shown to be unlinked in *G. caleyi* (Llorens in prep.), thus satisfying an important assumption of this parameter.

To compare *f* in fresh seed with that for seedlings and adults at JJM, the "direct estimate" of the fixation index ($f = [1-t]/[1+t]$; Brown 1979) was calculated for fresh seed, with *t* the estimated outcrossing rate.

To detect spatial variation in the mating system, *f* was compared among the three clumps of seedlings at RA and among the three patches of seedlings and adults at JJM. The significance of differences in *f* among clumps or patches were tested with single-factor ANOVAs.

Parentage of seedlings and paternity of seed

Parentage analyses were performed for seedlings at each site, and for fresh seed at JJM, to determine whether any of the pre-fire adults were likely parents of each seed or seedling. CERVUS 2.0 (Marshall 1998; http://helios.bto.ed.ac.uk/evolgen) was used to determine all potential parents of each seed or seedling; all adults with a LOD score greater than zero were considered equally likely parents. The parentage analyses were further refined using visual inspection of genotypes to determine whether, for each seed or seedling with potential parents among the adults, both parents or only one parent may be among the adults. At JJM, the three adults from the unburnt patch (JJM2) were also included in the analyses as candidate parents of seeds and seedlings; these three adults had been genotyped previously.

Results

Previous studies have revealed that adult populations of *G. caleyi* generally have low levels of genetic diversity, high levels of fine-scale genetic structuring, and evidence for large amounts of inbreeding (Llorens in prep.). This study demonstrated that the seed bank of *G. caleyi* essentially reflects the genetic characteristics of the adult populations, although with several minor but potentially important differences.

Genetic diversity and composition of the seed bank

Genetic diversity in the seed bank

The seed bank of *G. caleyi* contained a fairly low level of microsatellite variation at every population and for all loci surveyed (Table 3). No more than four alleles were detected at a single locus within any population, all populations were monomorphic for at least one locus, and expected heterozygosity (H_E) was low in all populations (Table 3).

One population (DF) completely lacked genetic diversity in the seedlings. For the remaining populations and patches, the proportion of polymorphic loci (P_L) ranged from 0.71 to 0.86 (Table 3). However, the polymorphism of some loci within patches at JJM was due to the occurrence of a rare allele in only one or two individuals. The mean number of alleles per locus ranged from 1.7 to 2.6, and mean H_E ranged from 0.128 to 0.358.

Comparison of seed bank and adults

The seed bank at each *G. caleyi* site was, overall, genetically very similar to the pre-fire adult population. There were some differences, however, in allelic composition and frequency, and in levels of genetic diversity.

The allelic composition of seedlings largely reflected that of the pre-fire adults, but showed some differences at two of the three sites. At DF, the adults and seedlings contained an identical set of alleles. Every allele found in pre-fire adults at RA and JJM was also present in post-fire seedlings, but seedlings contained two alleles not found in adults. At RA, these additional alleles were fairly common among the seedlings, with frequencies of 0.275 and 0.263, respectively, for $Gm37^{138}$ and $Gi9^{205}$. At JJM, the additional alleles were rare: $Gm13^{131}$ and GmD^{170} only had frequencies of 0.025 and 0.033, respectively, in the seedlings. Each individual patch at JJM also showed differences in allelic composition between adults and seedlings. Additional alleles were found among seedlings in every patch: two alleles at JJM1, six at JJM3 and one at JJM4. Seedlings contained 20% more alleles than pre-fire adults at RA, 13% at JJM, 17% at JJM1, 75% at JJM3 and 9% at JJM4. There was one case in which seedlings lacked an allele found in adults - at JJM4, GmD^{164} was found within adults at a frequency of 0.167, but was absent from the seedlings in this patch. However, this allele only appeared in one adult individual, as a heterozygote.

Table 3. Genetic diversity measures for pre-fire adults and post-fire seedlings of *Grevillea caleyi*

Seven microsatellite loci were used to genotype three populations, and three patches within the JJM population. A_O = number of alleles, H_E = expected heterozygosity, f = Wright's inbreeding coefficient estimated according to Weir and Cockerham (1984), and P_L = proportion of loci polymorphic. Standard errors of means are shown in brackets. See Table 1 for sample sizes. Significance of departure from Hardy-Weinberg equilibria is indicated by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

		Locus								
		Gm10	Gm13	Gm37	GmD	GmI	Gi7	Gi9	Mean (SE)	P_L
DF										
A_o	seedlings	1	1	1	1	1	1	1	1 (0)	0
	adults	1	1	1	1	1	1	1	1 (0)	0
H_E	seedlings	0	0	0	0	0	0	0	0	
	adults	0	0	0	0	0	0	0	0	
f	seedlings	-	-	-	-	-	-	-	-	
	adults	-	-	-	-	-	-	-	-	
RA										
A_o	seedlings	2	1	2	2	2	1	2	1.7 (0.2)	0.71
	adults	2	1	1	2	2	1	1	1.4 (0.2)	0.43
H_E	seedlings	0.358	0	0.409	0.467	0.431	0	0.398	0.295 (0.077)	
	adults	0.200	0	0.000	0.556	0.467	0	0.000	0.175 (0.092)	
f	seedlings	0.022	-	0.639**	-0.073	0.542*	-	-0.059	0.221*	
	adults	0.000	-	-	-0.091	-0.333	-	-	-0.167	
JJM										
A_o	seedlings	1	2	2	4	3	2	4	2.6 (0.4)	0.86
	adults	1	1	2	3	3	2	4	2.3 (0.4)	0.71
H_E	seedlings	0	0.049	0.361	0.496	0.663	0.195	0.739	0.358 (0.110)	
	adults	0	0.000	0.368	0.437	0.658	0.091	0.680	0.319 (0.111)	
f	seedlings	-	-0.017	0.262*	0.933***	0.575***	0.403*	0.641***	0.596***	
	adults	-	-	0.268	0.596*	0.459*	0.000	0.477**	0.442***	
JJM1										
A_o	seedlings	1	1	2	3	3	2	2	2.0 (0.3)	0.71
	adults	1	1	2	2	2	2	2	1.7 (0.2)	0.71
H_E	seedlings	0	0	0.328	0.099	0.497	0.385	0.409	0.245 (0.078)	
	adults	0	0	0.533	0.200	0.533	0.200	0.467	0.276 (0.089)	
f	seedlings	-	-	0.088	0.500*	0.098	0.224	0.147	0.159	
	adults	-	-	0.273	0.000	0.273	0.000	-0.333	0.077	
JJM3										
A_o	seedlings	1	2	1	2	2	2	4	2.0 (0.4)	0.71
	adults	1	1	1	1	1	1	2	1.1 (0.1)	0.14
H_E	seedlings	0	0.097	0	0.097	0.508	0.142	0.581	0.204 (0.090)	
	adults	0	0.000	0	0.000	0.000	0.000	0.600	0.086 (0.086)	
f	seedlings	-	-0.027	-	1.000*	0.415	0.655	0.230**	0.375**	
	adults	-	-	-	-	-	-	0.500	0.500	
JJM4										
A_o	seedlings	1	2	2	2	2	1	2	1.7 (0.2)	0.71
	adults	1	1	2	2	2	1	2	1.6 (0.2)	0.57
H_E	seedlings	0	0.050	0.513	0.050	0.097	0	0.185	0.128 (0.069)	
	adults	0	0.000	0.333	0.333	0.533	0	0.000	0.248 (0.093)	
f	seedlings	-	0.000	0.026	0.000	-0.027	-	1.000**	0.222*	
	adults	-	-	0.000	0.000	-0.333	-	1.000	0.273	

Seedlings and adults were generally not very genetically differentiated. The values obtained for F_{ST} tests indicated that only at JJM4 was there significant genetic differentiation among adults and seedlings (Mean F_{ST} = 0.30 ± 0.17; P < 0.01; Table 4). The jackknifed mean F_{ST} ranged from –0.008 to 0.05 at the other sites. Single-locus F_{ST} values were low at JJM (F_{ST} < 0.05) and did not exceed 0.1 at JJM1, but were occasionally much higher at JJM3 and JJM4, where they reached as high as 0.23 and 0.54, respectively.

Table 4. F_{ST} values for comparisons of pre-fire adults with post-fire seedlings in three *Grevillea caleyi* populations

At JJM, analyses were performed for the population as a whole and separately for each discrete patch of plants. F_{ST} was estimated using θ (Weir and Cockerham 1984). Means were jackknifed over loci, and their statistical significance tested using bootstrapped confidence intervals. * P < 0.05; ** P < 0.01.

	Locus							
Comparison	Gm10	Gm13	Gm37	GmD	GmI	Gi7	Gi9	Mean (SE)
DF								
Adults vs seedlings	-	-	-	-	-	-	-	-
RA								
Adults vs all seedlings	-0.017	-	0.106	-0.011	-0.094	-	0.135	0.020 (0.043)
JJM								
All adults vs all seedlings	-	-0.012	-0.035	-0.030	-0.016	-0.015	0.023	-0.008 (0.015)
JJ1 - adults vs seedlings	-	-	0.036	-0.049	0.076	-0.012	-0.069	0.014 (0.035)
JJ3 - adults vs seedlings	-	-0.071	-	-0.196	0.233	-0.132	-0.128	0.052 (0.169)
JJ4 - adults vs seedlings	-	-0.091	0.112	0.171	0.321	-	0.540	0.299 (0.168)**

In most cases, there was little variation in genetic diversity among adults and seedlings, either at individual loci or over all loci (Table 3; Fig. 1). However, some trends were clearly discernible for A_O and P_L, which were always either greater in seedlings than in adults, or did not differ (Fig. 1). At no site did the adults have a greater value for A_O or P_L than the seedlings. At DF, all adults and seedlings were monomorphic for the same allele at each locus, and hence did not vary for any measure of genetic diversity. A_O was greater in seedlings than adults at RA, JJM and the three JJM patches. At JJM3, this difference was significant (t = 3.29, P = 0.02). P_L did not differ among adults and seedlings at JJM1, but was greater in seedlings at RA, JJM, JJM3 and JJM4. Mean H_E was low in both adults and seedlings, and showed little variation among adults and seedlings at most sites. However, there was a trend for slightly higher H_E in seedlings than in adults, with the exception of two patches at JJM (Fig. 1). There were several instances in which adults and seedlings showed large differences in H_E at an individual locus, but the direction of the variation was inconsistent among loci.

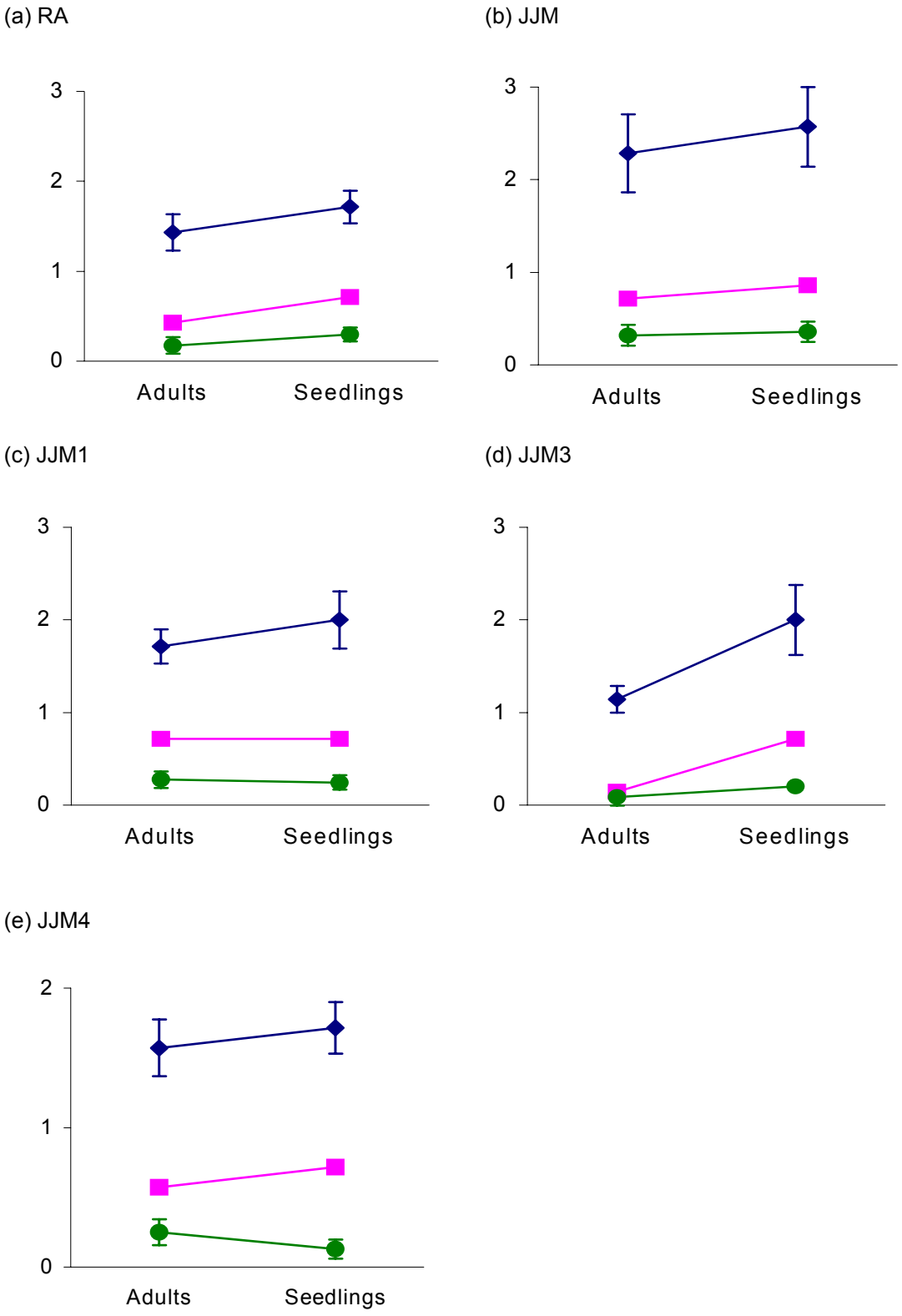

Figure 1. Three measures of genetic diversity in pre-fire adults and post-fire seedlings of *Grevillea caleyi* at RA, JJM and three patches at JJM

◆ = mean number of alleles per locus (A_O), ■ = proportion of loci polymorphic (P_L), ● = mean expected heterozygosity (H_E). A_O and H_E are presented with standard error bars.

Fine-scale spatial genetic structure in the seed bank

There was a very high degree of structuring among clumps of *G. caleyi* seedlings at RA (F_{ST} = 0.33 ± 0.14, $P < 0.01$) and among patches of seedlings at JJM (F_{ST} = 0.57 ± 0.10, $P < 0.01$). This can be clearly seen from the principal coordinate analyses of the genetic relationships among seedlings (Fig. 2); seedlings from each clump or patch clustered into almost discrete groups. At JJM, the amount of structuring among patches was greater for seedlings than for adults (adult F_{ST} = 0.39 ± 0.11; $P < 0.01$).

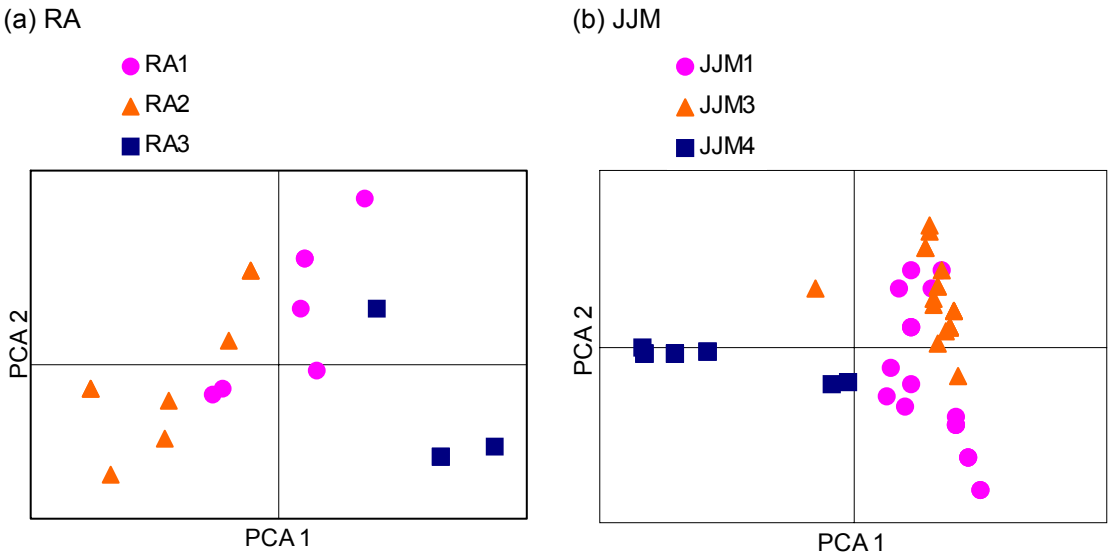

Figure 2. Principal coordinate analyses of the genetic relationships among post-fire seedlings within two *G. caleyi* populations, (a) RA and (b) JJM

Seedlings were sampled from each of three clumps at RA and three patches at JJM.

The fine-scale structuring in the seed bank is illustrated by the dramatic variation in allelic frequencies among clumps at RA and patches at JJM (Fig. 3). For example, RA3 was almost fixed for allele $Gm37^{138}$, while RA1 and RA2 were almost fixed for allele $Gm37^{140}$. Similarly, the alleles GmD^{161}, GmI^{169} and $Gi9^{209}$ showed large differences in frequency among clumps of seedlings. The same trends were clearly apparent at JJM, where allele frequencies varied dramatically among patches at *GmD*, *GmI* and *Gi9*, and moderately at *Gi7*. This was particularly striking at *GmD*, where JJM4 was almost fixed for allele 169 (freq. = 0.975), while JJM1 and JJM3 were almost fixed for allele 164 (freq. = 0.950). However, the direction of variation in allele frequencies was not consistent among loci at either RA or JJM; each clump or patch differed from the other two clumps or patches at some loci but not at others. There was additional striking variation in allelic composition among patches at JJM that cannot be seen from Fig. 3. This was most obvious at *Gi9*, for which JJM3 and JJM4 both had very low frequencies of the allele $Gi9^{203}$ (Fig. 3), but had very different allelic compositions. While seedlings at JJM3 mostly contained alleles $Gi9^{205}$ (freq. = 0.425) and $Gi9^{207}$ (freq. = 0.500), neither of these alleles was present in seedlings at JJM4.

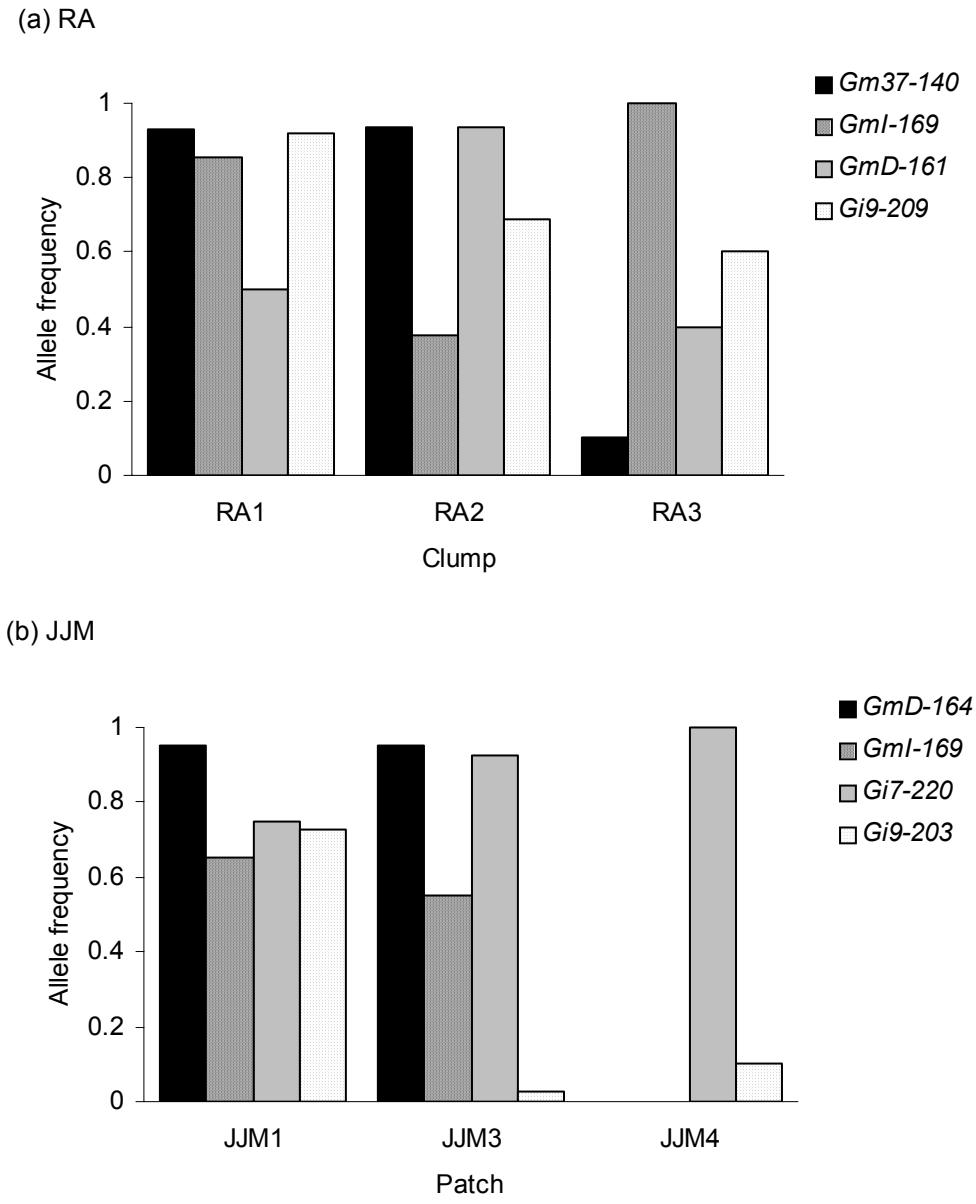

Figure 3. Allelic frequencies at four microsatellite loci in (a) three clumps of post-fire *Grevillea caleyi* seedlings at RA and (b) three patches of post-fire seedlings at JJM

Despite the large genetic differences among clumps and patches of seedlings at RA and JJM, there was very little fine-scale variation in genetic diversity. At both sites, A_O, P_L and H_E were either identical or very similar among clumps or patches (Fig. 4).

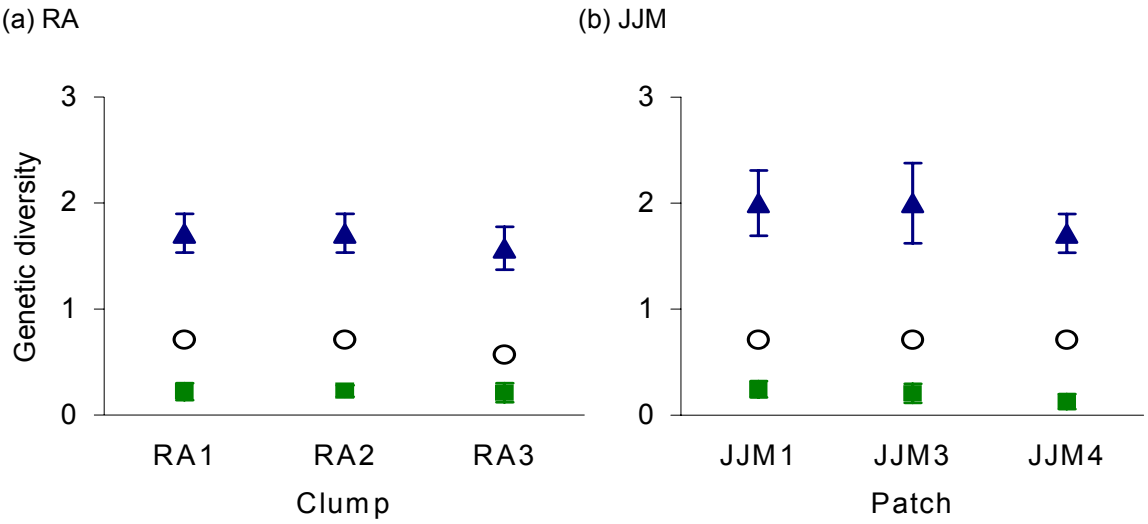

Figure 4. Three measures of genetic diversity in post-fire *Grevillea caleyi* seedlings within (a) three clumps of seedlings at RA and (b) three patches at JJM

▲ = mean number of alleles per locus (A_O), ○ = proportion of loci polymorphic (P_L), ■ = mean expected heterozygosity (H_E). A_O and H_E are presented with standard error bars.

Spatial and temporal variation in the mating system

All estimates of the mating system indicated that *G. caleyi* was highly inbred. Heterozygote deficits were widespread among loci and populations for both adults and seedlings (Table 3). Values of *f* were nearly always positive for both adults and seedlings, and were significantly greater than zero in many cases. Among seedlings, *f* was as high as 0.596 (at JJM), while *f* among adults reached a maximum of 0.500 (JJM3). The fixation index could not be calculated at DF due to the lack of variation.

Fresh seed collected from four *G. caleyi* plants at JJM had low to moderate estimated rates of multi-locus outcrossing for the two patches combined ($t = 0.35 \pm 0.19$), and for the patches considered separately (JJM1: $t = 0.67 \pm 0.23$; JJM4: $t = 0.40 \pm 0.19$). There were five detectable outcrosses among the 20 seed analysed from JJM1 (all five were from one maternal plant), and six among the 20 seed from JJM4 (five from one maternal plant and one from the other). For several reasons, however, the estimates of *t* are problematic. The estimates were only calculated from two or four families of seed, and thus from a very low sample size. However, this should be viewed in terms of the small number of adults present at this site: in fact, seed were sampled from 40% of the plants at JJM1 and 67% of the plants at JJM4. The estimate for patches combined probably violated the assumption for the mixed mating model of random movement of pollen among plants (Brown *et al.* 1975), demonstrated by the results of parentage analyses (see below) and by the large degree of genetic differentiation among patches. Therefore, the results must be interpreted in terms of the localised movement of pollen rather than mate choice from a wide range of available pollen. The mating system estimates also violated the assumption that there is no inter-plant variation in outcrossing rates (Brown *et al.* 1975), because only two plants produced most of the outcrossed seeds. The violation of these assumptions should downward-bias the estimates of *t* (Brown *et al.* 1975), which may have been the case for the patches combined. However, these assumptions are also violated in many other plants with a mixed mating system (*e.g.* Young and Brown 1998; England *et al.* 2001).

Variation among life stages

Seedlings consistently had larger values of *f* compared with adults at RA and JJM (Fig. 5). This was particularly noticeable at RA, where *f* was negative for adults (*f* = -0.167) but was significantly greater than zero for seedlings (*f* = 0.221; *P* = 0.017). *f* was significantly greater than zero for both seedlings and adults at JJM, but was larger in seedlings than adults (0.596 *vs.* 0.442). Within individual patches at JJM, *f* was significantly greater than zero only for seedlings, even though it was slightly larger in adults than seedlings at both JJM3 and JJM4. However, the large *f* for adults at JJM3 was calculated from a single variable locus (*Gi9*).

For seedlings, single-locus estimates of *f* were significantly greater than zero for at least one locus in every population or patch (Table 3). In contrast, only one population or patch (JJM) produced a single-locus *f* that was significantly greater than zero for adults. Despite this, however, the variation in single-locus *f* among adults and seedlings was not significant for any population or patch, as measured by paired *t*-tests (*P*> 0.09 in every case).

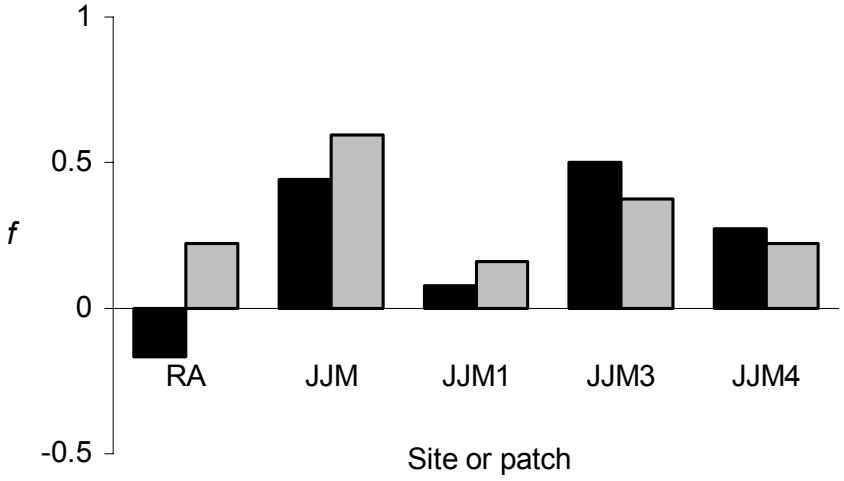

Figure 5. Wright's fixations index, *f*, for *Grevillea caleyi* adults (■) and seedlings (■)

Plants were sampled from two *Grevillea caleyi* populations (RA and JJM) and separately for three patches within JJM.

When mean fixation indices were calculated for the *G. caleyi* seed from two patches at JJM, *f* was large at JJM1 (*f* = 0.196) and JJM4 (*f* = 0.426) and for patches combined (*f* = 0.485). At JJM1, *f* calculated from fresh seed was more than twice as large as that obtained for adults (0.077) and slightly larger than *f* for post-fire seedlings (0.159). At JJM4, *f* for fresh seed was double that obtained for seedlings (0.222) and adults (0.273). For the two patches combined, *f* in fresh seed was midway between that for adults (*f* = 0.308) and seedlings (*f* = 0.571).

Spatial variation in the mating system

There was some variation in *f* among clumps of seedlings at RA and among patches of adults and seedlings at JJM (Table 3). At RA, *f* varied noticeably among clumps of seedlings, with mean *f* indicating a significant excess of heterozygotes at RA3 (*f* = -0.429, *P* < 0.001) but a slight deficit of heterozygotes at RA2 (*f* = 0.154, *P* = 0.33). However, the variation in *f* among clumps was not significant (*F* = 1.96, *P* = 0.19).

Within patches at JJM, *f* for seedlings ranged from 0.159 to 0.375, and was significantly greater than zero at JJM3 (*P* < 0.001) and JJM4 (*P* = 0.013) but not JJM1 (*P* = 0.098). The

range in *f* among patches was greater for adults (*f* = 0.077 to 0.500) than for seedlings; however, the large value for *f* at JJM3 (0.5) was calculated only from a single locus. The variation in *f* among patches was not significant for either seedlings ($F = 0.81$, $P = 0.47$) or adults ($F = 0.50$, $P = 0.63$).

Parentage of seedlings and paternity of seed

No parentage analysis was performed at DF, as all sampled seedlings were genetically identical to all sampled adults. At the remaining sites, the parentage of some seedlings could not be explained by the adults that were sampled pre-fire (Table 5). Some of these seedlings were easily identified when they contained alleles not present in any adult plants; others required more complex parentage analyses. Some seedlings had no possible parents among the pre-fire adults sampled, with additional seedlings having only one possible parent among the adults (Table 5). This was most pronounced at RA, where six of the 20 seedlings had both parents missing from among the adults, and another six seedlings had only one possible parent among the adults. These data show that at least 45% and 13% of the parents of RA and JJM seedlings, respectively, were not found amongst the sampled pre-fire adults. It should be stressed, however, that these are conservative figures, because 'possible' parents are not necessarily the true parents. The true numbers of 'missing' parents are likely to be much higher, due to the lack of power in the microsatellite markers.

The parentage analyses conducted for seedlings provided strong evidence for variation among adults in their relative contributions to the seed bank. For example, two of the five pre-fire adults at JJM1 could fully account for the parentage of 18 of the 20 seedlings in the patch, either through selfing or by mating with each other. At JJM4, selfing by only one adult could have produced 17 of the 20 seedlings in the patch. This trend was less obvious at JJM3, where one or both parents of 12 seedlings either had died before pre-fire sampling, or were from another patch. However, two genetically indistinguishable pre-fire adults were still able to account for the parentage of seven of the remaining eight seedlings.

Paternity analyses of fresh *G. caleyi* seed at JJM revealed an apparent lack of pollen movement among patches. Of the 11 seed that were detectably outcrossed, all had a possible father within the same patch as the maternal parent. For each maternal plant, all of its outcrossed seed appeared to have been sired by a single plant from the same patch, revealing non-random distribution and/or success of pollen within the population. Interestingly, the outcrossed seed in each patch were probably all fathered by the other maternal plant whose seeds were genotyped. Each of these fathers produced few or no outcrossed seed of their own, indicating that the production of outcrossed seed varies greatly among plants.

Table 5. Summary of parentage analyses performed for post-fire *Grevillea caleyi* seedlings

At RA and JJM, all adults present several years pre-fire were sampled and some seedlings were sampled post-fire. Parentage analyses were used to determine whether one or both parents of each seedling might have been present among the pre-fire adults.

Site	No. of adults sampled	No. of seedlings sampled	No. with 2 possible parents	No. with 1 possible parent	No. with no possible parents	Minimum no. parents missing
RA	5	20	8	6	6	18 (45%)
JJM	14	60	47	8 + 2*	3	16 (13%)

* 2 seedlings had two possible parents among the adults, but both were from another patch (JJM); assuming no seed dispersal, this indicates that the maternal parent was missing from among the adults.

Discussion

The results of this study mostly support the results of the limited published literature on the genetic characteristics of persistent seed banks, with some exceptions. Post-fire seedlings from the three populations of *G. caleyi* showed (1) equal or greater genetic diversity than the pre-fire adults, (2) similar allele frequencies to the pre-fire adults, with minor exceptions, and (3) greater homozygosity than pre-fire adults.

The maintenance of allele frequencies among life stages and the presence of greater genetic diversity and additional alleles in the seed bank indicate that the seed bank of this species has the capacity to buffer, to some extent, the loss of genetic diversity that may accompany reductions in population sizes. In addition, the results of parentage analyses were consistent with multi-generational contributions to the seed bank, which should further slow post-fragmentation genetic changes. In one *G. caleyi* population, however, the greater among-patch genetic structure in seedlings compared with adults was contrary to expectations from other studies, and may indicate some genetic effect of the recent fragmentation.

Estimates of the mating system indicated that all life stages were highly inbred, and that inbreeding increased from adult plants to seedlings to fresh seed, but it is difficult to draw conclusions regarding apparent increases in inbreeding with successive temporal estimates. However, the large individual variation in contribution to the seed bank, outcrossing and pollen success, with the lack of pollen flow among patches at JJM, indicate a likely mechanism for any recent increases in fine-scale genetic structure and inbreeding.

Genetic diversity, allele frequencies and parentage in the seed bank

All of the *G. caleyi* populations surveyed contained low levels of genetic diversity in their post-fire seedlings, reflecting the low levels of genetic diversity generally found in adult populations of this species (Llorens in prep.). The major pattern that emerged from this study was the close genetic similarity between post-fire seedlings and pre-fire adults; this was demonstrated by low values for F_{ST} among life stages, non-significant tests for allelic and genotypic heterogeneity, and the fact that for each population, every allele sampled from the pre-fire adults was also represented in post-fire seedlings. The only exceptions to these findings was a single patch at the *G. caleyi* site JJM, which showed significant F_{ST} and allelic heterogeneity among adults and seedlings, and had a single low-frequency allele in the adults that was not represented in seedlings. The close genetic similarities between seedlings and adults indicate that the seed bank of *G. caleyi* was highly effective at preserving both the composition and frequencies of alleles present in pre-fire adults.

While other studies have also found that the seed bank preserves the allelic composition of the adult generation, most have found significant variation in allelic frequencies among the seed bank and adult plants (*e.g.* Cabin 1996; Cabin *et al.* 1998; Aparicio *et al.* 2002), which contrasts with this study. This may indicate a relative lack of selection occurring on seedlings or young adults in *G. caleyi*, which is consistent with the high rates of survivorship observed among *G. caleyi* seedlings at some sites several years post-fire (NSW NPWS 2001). That several populations or patches had very low abundances of adult plants demonstrates that populations of *G. caleyi* are able to maintain their genetic composition and allele frequencies for at least a generation following a severe bottleneck among the adults. A major reason for this is likely to be the capacity of the seed bank to increase the effective population size to levels well above adult population size (Auld and Scott submitted).

Despite the close genetic similarity among pre-fire adults and post-fire seedlings, however, there was a consistent trend for seedlings to show slightly greater allelic diversity than pre-fire adults. The only exception to this, DF, did not show any microsatellite diversity in either the adults or seedlings. At RA and JJM, *G. caleyi* seedlings contained several alleles not represented in the adults, and possessed a greater proportion of polymorphic loci than the adults. Seedlings contained a 20% greater number of alleles than did adults at RA, and 12.5% at JJM. Both sites showed slightly greater expected heterozygosity (H_E), a measure of allelic diversity, in seedlings than adults. However, more populations would have to be sampled to assess the generality of these results; unfortunately, the limited occurrence of recent fires meant that I was only able to sample three populations of *G. caleyi*.

As well as the presence of additional alleles and greater diversity in the seedlings, the results of parentage analyses provided additional evidence that the seed bank of *G. caleyi* may act as a reservoir of genetic diversity, accumulated through previous seed cohorts. A significant proportion of the seedlings at RA and JJM had parents that were not present at the time of pre-fire sampling. This was particularly the case at RA, where the parents of at least 45% of the seedlings were not present among the pre-fire adults, and at JJM3, where one or both parents of 60% of seedlings were not among that patch's pre-fire adults. In addition, the large amounts of spatial structuring within populations, and the apparently very limited movement of pollen, imply that my parentage analyses were probably too liberal because they included all adult plants as potential parents of seedlings, rather than just those in the near vicinity of each seedling. Therefore, it is likely that where the analyses identified potential parents that were more than a few metres from a seedling, these were unlikely to have been the real parents.

The lack of significant genetic variation among adults and seedlings at these sites indicates that the missing parents had gene frequencies similar to those of the sampled adults. However, this was clearly not the case for *GmI* at JJM3 or *Gm37* and *Gi9* at RA, where the missing parents had relatively high frequencies of alleles that were absent from the sampled pre-fire adults. For these loci, tests for allelic heterogeneity had *P* values of between 0.068 and 0.093, indicating some difference in allelic frequencies. It is likely that the generally low levels of allelic diversity at these sites limited my power to detect genetic differences among seedlings and adults.

Many authors have hypothesised that the seed bank may store diversity from previous generations, and hence slow the rate of evolution (*e.g.* Templeton and Levin 1979; Hairston and De Stasio 1988; Ellstrand and Elam 1993). However, although several studies have found genetic differences between the seed bank and adults of other species, I could only find four that have identified greater genetic diversity in the seed bank (Alvarez-Buylla and Garay 1994; McCue and Holtsford 1998; Aparicio *et al.* 2002; Morris *et al.* 2002), and five that have identified alleles in the seed bank of a population that were not present in adults (Tonsor *et al.* 1993; Alvarez-Buylla and Garay 1994; McCue and Holtsford 1998; Mahy *et al.* 1999; Aparicio *et al.* 2002). However, in three of the latter studies, the seed bank's additional alleles were counteracted by the presence of alleles in the adults that were absent from the seed bank. These studies also suffered from the fact that they sampled only a fraction of the adults from each population or plot; therefore, the presence of additional alleles in the seed bank is likely to have been due to a sampling effect.

Although Alvarez-Buylla and Garay (1994) did not sample an entire population of *C. obtusifolia*, they sampled all adults within a very large (5 ha) study plot. Therefore, *G. caleyi* appears to be only the second species for which a store of additional alleles has been found in the seed bank after sampling all extant adult plants. It may be the first species, however, for

which such evidence has been found within small or fragmented populations, or in a rare plant. This study may therefore provide the first direct evidence that when a rare plant is faced with reduced population size, its seed bank may act as a reservoir that preserves genes previously lost from adult plants.

There are several possibilities that could account for the greater genetic diversity and presence of additional alleles in the seedlings at RA and JJM, and the missing parents of seedlings at both sites. First, some adults may have died prior to the pre-fire sampling, so that estimates of genetic diversity at these sites may have been sampled from a subset rather than the whole adult population. This was especially likely at RA, where the site had remained unburned for so many years that most of the pre-fire adults had probably died prior to sampling, particularly given the much wider spatial distribution of post-fire seedlings compared with sampled pre-fire adults. However, significant pre-sampling adult deaths were much less likely at JJM, which was much younger than RA at the time of pre-fire sampling. A census of adult plants taken three years prior to pre-fire sampling, when the plants at JJM were about 7 years old (below the age at which *G. caleyi* plants generally senesce), revealed only three additional adult plants (Scott *et al.* 1995), although it is not known which patch they were from. Even though I may not have sampled all adult plants at RA and JJM, however, this need not diminish the discovery of greater diversity in seedlings, because the seedlings were also sub-sampled from a larger population; indeed, it is highly likely that I sampled a lower proportion of the seed bank than of the pre-fire adults. This was particularly the case for JJM, at which many more seedlings emerged than could survive as adult plants, due to the extremely small size of the habitat patches. In addition, many seed may have failed to recruit as seedlings in all populations due to lack of fitness, or for other reasons such as the patchy distribution of germination cues, vertical location of seeds in the soil profile, or polymorphism in response to gemination cues. Following the fire, such seeds would have either died or formed part of the residual seed bank.

Second, the greater genetic diversity, additional alleles and missing parents of seedlings may reflect multi-generation contributions to the seed bank and the relatively large size of the seed bank compared with adult plants. *Grevillea caleyi* is known to possess a residual seed bank that remains viable following a fire (Auld 1994), so that at any one time the seed bank may potentially contain seeds from at least two generations of adults. Up to 45% of the seed bank of *G. caleyi* may remain dormant after a fire but germinate following a subsequent fire (Auld 1994). Therefore, the missing parents and additional alleles may have been present among adults that lived two or more generations ago. Another possible reason for the absence of some alleles in the pre-fire adults, and the greater diversity in the seedlings, is the effect of random sampling when only a small number of adults are produced from a large seed bank. This was particularly likely at sites with small habitat patch sizes (*e.g.* JJM) or very high seedling density, where the population would thin out as plants became larger and crowded.

Third, the additional alleles in the seed bank and the missing parents of seedlings may have originated in another population. It is highly unlikely that this occurred via seed dispersal from another population, given the absence of a known mechanism for seed dispersal (Auld and Denham 1999), and the large number of seed that would had to have been dispersed to account for the missing parents. A more likely possibility is that additional alleles and paternal parents were introduced to populations in the form of pollen. While such inter-population outcrossing events may be occasionally facilitated by bird pollinators, however, there is no evidence that they occur more than very rarely (Llorens in prep.). Indeed, low inferred rates of outcrossing in *G. caleyi* (Llorens in prep.) make inter-population outcrossing less likely. In addition, inter-population gene flow via pollen could not explain the presence of seedlings for which both parents were unaccounted.

Fourth, the additional alleles in seedlings may have been created through mutations occurring in soil-stored seed (Levin 1990). However, such an event would occur extremely rarely at best, and was unlikely in these cases given that all additional alleles were also found in nearby populations.

Of these four possibilities, the first two are most likely to account for the presence of additional alleles in the seed bank, and seedlings of unexplained parentage. As there is evidence to support both, it seems likely that some seedlings were the progeny of pre-fire adults that died prior to sampling, while others were produced two or more generations ago but remained dormant in the seed bank until the most recent fire. It should also be noted that while the majority of post-fire seedlings at each site had possible parents among the pre-fire adults, those adults were not necessarily the seedlings' true parents. Therefore, the actual number of seedlings that were produced by unsampled pre-fire adults or adults from previous generations cannot be known, but may be considerably higher than the minimum estimates given here. Whether or not this was the case, it is nevertheless clear that the parents of the *G. caleyi* seed stored in a population's seed bank may never have all been alive at a single point in time.

There was one minor exception to the overwhelming trend that seedlings contained equal (DF) or greater (RA, JJM) amounts of genetic diversity compared with adults. In one of the three patches at JJM (JJM4), the allele GmD^{164} was present in a single pre-fire adult plant, but was absent from the seedlings. This may indicate the loss of the allele from JJM4, but the allele is also likely to be present in one of the patch's 25 unsampled seedlings or in the residual soil seed bank. However, as the adult in question was heterozygous at *GmD*, half of its progeny would be expected to carry the GmD^{164} allele. Therefore, it appears that this adult did not make a significant contribution to the following generation of *G. caleyi* at JJM4. This was confirmed by parentage analysis, which showed that 85% of the post-fire seedlings at JJM4 may have been produced by only one of the patch's three pre-fire adults, through selfing alone. It is significant that this adult was larger in size than the other two adults, and was observed to produce many more fruit than the other two adults over a several year period (T. Llorens pers. obs.). This demonstrates the combined effect that an extreme bottleneck (three adult plants) and individual variation in reproduction may have on the loss of alleles from a population. It should be noted, however, that the seedlings at JJM4 contained two rare alleles not found in the adults. Therefore, despite the possible loss of one allele from the patch, the seed bank had preserved two alleles not expressed in the pre-fire adult population.

It is highly likely that the greater levels of genetic diversity contained in the seedlings, and hence the seed bank, of *G. caleyi* may enable populations to maintain genetic diversity and slow the rate of other genetic changes following reductions in population size. This is important for *G. caleyi*, which experiences dramatic natural fluctuations in population size due to variation in fire frequency. With recent human-induced habitat fragmentation, the diversity-maintaining properties of this species' seed bank have gained even greater significance, and may explain why even very small populations do not appear to show any loss of genetic diversity (Llorens in prep.). However, this power of the seed bank is likely to be limited, and may not prevent the loss of diversity from populations that remain very small over many generations, particularly given the large amounts of inbreeding and individual variation in reproduction (Llorens in prep.); this may have happened in the past at DF, which had no detectable microsatellite variation. In addition, the significance of additional alleles in the seed bank should probably not be emphasised too strongly, because the alleles were so rare that they may disappear from the populations in the next generation or two due to random

sampling. This seems likely at JJM, given the small number of plants that appeared to be contributing to the seed bank, and the high rates of inbreeding.

Unfortunately, the conclusions able to be drawn from this study regarding the ability of the seed bank to buffer genetic changes are limited not only by the small number of populations sampled, but by the fact that I could only sample one generation of adults and seedlings. Several generations would need to be sampled to determine how long after fragmentation that changes in genetic diversity and allele frequencies occur, but for *G. caleyi* such sampling would have to span several decades. In addition, it should be noted that, due to adult mortality increasing with age, there may be a relationship between time since fire (*i.e.* plant age) and the genetic diversity found in adult plants. In future studies of a similar nature with *G. caleyi*, it would be preferable to sample adult plants about four or five years after a fire, when most plants should be reproductive and could be considered adults. This would mean that all of a population's adults, and therefore potential parents of its seed, could be included in sampling, thus allowing for a more valid comparison of adult and seed bank diversity. However, it is possible that the sampling of relatively old populations of *G. caleyi* in this study did not greatly underestimate seed bank diversity, because seedlings showed very similar allele frequencies and only slightly or no greater diversity than adults, despite the probable death of some adult plants and the very large difference in abundance of pre-fire adults compared with post-fire seedlings. This may suggest that there is little selection operating on seedlings or adults.

Genetic structure in the seed bank

There was a striking amount of genetic differentiation among clumps of *G. caleyi* seedlings at RA and among patches of seedlings at JJM. The PCoA analyses revealed an apparently high degree of relatedness among seedlings within patches or clumps, with many seedlings genetically indistinguishable. The degree of genetic structuring among clumps and patches of seedlings (F_{ST} = 0.332 and 0.570, respectively) was similar to that observed among *G. caleyi* populations separated by several kilometres (F_{ST} = 0.43; Llorens in prep.). This was not unexpected, because adults sampled from two plots within the BQE population were highly differentiated (F_{ST} = 0.450), despite being separated by only 26 m of continuous bushland that contained *G. caleyi* plants (Llorens in prep.). However, at RA and JJM, the distances separating some clumps or patches were even smaller than at BQE; there was less than 10 m between each clump at RA, and 9, 48 and 58 m between patches at JJM. This demonstrates that the processes that produce large-scale genetic structuring among *G. caleyi* populations operate on a very small scale. It also shows that despite the seed bank's capacity to prevent the loss of genetic diversity from small populations, it does not have the power to prevent genetic structuring from occurring within continuous populations, much less among fragmented subpopulations.

With a highly inbreeding mating system and little pollen or seed movement over medium or long distances (Auld and Denham 1999), it is probably not surprising that fine-scale genetic structuring has developed within *G. caleyi* populations, even over less than 10 metres, as occurred at RA. It is also not surprising that genetic structuring appeared to be more severe at JJM - where habitat fragmentation has accentuated the physical distance between groups of plants and each patch contained very few plants - given the greatly increased action of genetic drift in small populations (Wright 1969). However, the large amount of structuring at RA is particularly interesting, because the seedlings may represent only the second generation of plants at this site following the inadvertent translocation, in soil, of *G. caleyi* seeds. As the seeds were probably considerably mixed during translocation, this appears to demonstrate

how quickly fine-scale genetic structure can develop in this species, probably from lack of seed movement and selfing.

Templeton and Levin (1979) and Hairston and De Stasio (1988) proposed that a persistent seed bank, particularly a multi-generation one, should greatly slow the rate of evolution. In support of this prediction, several studies have found that the seed bank contained less spatial genetic structuring than adult plants (*e.g.* Tonsor *et al.* 1993; Cabin *et al.* 1998; McCue and Holtsford 1998), indicating that the seed bank slowed the differentiation of populations that may otherwise occur through random or deterministic processes. Surprisingly, however, the opposite trend occurred for *G. caleyi* at JJM, where the degree of genetic structuring among patches, while always high, was greater in seedlings (F_{ST} = 0.570) than in pre-fire adults (F_{ST} = 0.394). Therefore, it appears that while the seed bank may indeed have slowed the differentiation of patches in the first generations following fragmentation, the degree of genetic structuring among patches may now be increasing over time. This may be explained, and even expected to occur, because of the generally lower levels of outcrossing and fewer pollinators in small populations (Llorens in prep.). In addition, all outcrosses detected in this study among seed from JJM involved plants from the same patch, no doubt a result of the highly localised, within-patch foraging of pollinators (Llorens in prep.).

Unfortunately, it is difficult to generalise from the among-patch genetic structuring of seedlings and adults at JJM, both because I was only able to sample one population in this manner, and because the population would need to be sampled over several generations to form a more accurate picture of changes in among-patch structuring. However, if the result is real, it would indicate that the seed bank has limited capacity to prevent genetic differentiation when faced with several generations of small effective population sizes (N_E). Although this study demonstrated that the seed bank may increase N_E, the very small areas of available habitat mean that N_E can never be large at JJM, and each adult generation will be very small. In addition, most of the alleles re-introduced to each patch from the seed bank were at such low frequencies in the seedlings that they were unlikely to prevent further differentiation of patches. This will particularly be the case if there is high mortality of seedlings or young adults, which seems inevitable given the high densities of seedlings in areas of habitat able to support few adult plants. Eventually, the seed bank will merely preserve the genetic differences among patches, and perhaps prevent any future genetic homogenisation of patches by swamping the effects of occasional among-patch outcrossing events.

Inferred mating systems

The high fixation indices in seedlings and adults of *G. caleyi*, and the low estimates of outcrossing in seed, indicated a highly inbred mating system both at present and in the past. These results were consistent with the high estimates of inbreeding inferred from adult plants within many populations and from direct investigation of the mating system of *G. caleyi* (Llorens in prep.). Such high rates of inbreeding may be readily explained by the large amounts of selfing, short-distance movements of pollinators, and tight family spatial structuring that occurs in *G. caleyi* (Llorens in prep.).

The parentage and paternity analyses conducted at JJM for seedlings and seed, respectively, provided additional insights into the development of such large amounts of inbreeding within populations of *G. caleyi*. Parentage analyses of seedlings revealed that within each patch, most seedlings were likely to have been the selfed progeny of only one pre-fire adult from the same patch. Paternity analyses indicated that the most likely father of each outcrossed seed at JJM was a single plant from the same patch as the maternal parent. In addition, there was

considerable variation among maternal plants in the proportion of their seeds that were outcrossed. These results suggest that (1) there is extreme individual variation in male mating success, the mating system expressed by a plant, and relative contribution to the seed bank, and (2) most mating occurs on a very local scale, either as selfing or as outcrossing with near neighbours that are probably close relatives. The highly local nature of matings was also supported by the larger *f* values obtained for adults and seedlings within the whole JJM site compared with individual patches; this was consistent with the effect of combining several independent mating groups, producing a Wahlund effect.

In combination with the short-distance pollen dispersal and apparent lack of seed dispersal in *G. caleyi*, the highly inbreeding mating systems indicate that near neighbours are also likely to be close relatives; therefore, even most outcrossing events may promote inbreeding. This is especially likely to be the case in very small populations or patches, such as at JJM, where there are few near neighbours with which to mate. When there is also large variation among individuals in outcrossing, paternity and seed production, as was certainly the case at JJM, it seems inevitable that genetic changes will occur. Populations should become more inbred and lose diversity, particularly for populations that remain small for several generations, as JJM is likely to. This should occur despite the buffering effect provided the seed bank, which is likely to be reduced with each generation of individually-skewed contributions of seed and increased inbreeding. Indeed, the results of this study indicated that inbreeding may be increasing over time. Seedlings of *G. caleyi* generally displayed a larger or more significant multi-locus *f* than adults, and estimates of *f* for fresh seeds at JJM were greater than for seedlings or adults. However, several studies have reported similar results for other species (*e.g.* Cabin *et al.* 1998; McCue and Holtsford 1998), indicating that *G. caleyi* may be part of a more general phenomenon among plants in which homozygosity decreases from the fresh seed to seed bank to adult stage. If this is the case, then it is possible that the increases in inbreeding inferred for *G. caleyi* may not indicate changes in the mating system over subsequent generations. To confirm that the mating system really is becoming more inbred in small populations, at least another generation of adults, seed and seedlings would need to be surveyed, so that different generations of the same life stage are compared.

Tonsor *et al.* (1993) proposed three possible explanations that could account for the relatively common observation of greater homozygosity in earlier life stages. First, the mating systems that produced the seed and seedlings may have had higher levels of inbreeding than the mating systems that produced the pre-fire adults. Mating systems are likely to include more inbreeding as population sizes decrease (Barrett and Kohn 1991). This may occur through selfing, biparental inbreeding or mating with relatives, either due to the available mates being more closely related than in a larger population, or in response to changes in pollinator availability or behaviour (Llorens in prep.). There is evidence to suggest that adult populations had decreased in size either since becoming fragmented during a previous generation (JJM), or during the inter-fire interval (RA). For *G. caleyi*, a very long inter-fire interval would mean that, if a handful of plants survived and reproduced for many years after the majority of plants had died, the surviving plants would make disproportionately large contributions to the seed bank. Their seeds are also more likely to be inbred due to a lack or low density of mates, particularly if pollinators are affected by the changes in vegetation structure that occur with time since fire. Variation in contribution to the seed bank is also likely to play a role: the mating system analysis indicated that of the pre-fire adults at JJM1 and JJM4, those that appeared to have contributed most to the seedling population were also producing seed that were significantly more inbred. Adults that were parents of very few, or no, seedlings had much higher rates of outcrossing. The situation at RA regarding inter-generational differences in inbreeding was more difficult to interpret, because so many of the post-fire seedlings were clearly not produced by the sampled pre-fire adults, indicating

deficiencies in pre-fire sampling, and nothing was known about the characteristics of the pre-fire population prior to the senescence of most of its plants.

Second, the seed bank may be displaying a temporal Wahlund effect. This may occur if the seed bank was produced by several generations of adults that varied in gene frequency, or if the mating system varied over time within a single generation. The former may occur to some extent in all populations of *G. caleyi*, because the seed bank contains seeds produced by at least two generations of adults. However, this is not well-supported by the general lack of variation in allele frequencies among adults and seedlings observed in this study. The latter possibility - that the mating system varies over time - is particularly plausible for these populations, particularly regarding reductions in population size and changes to pollinators that occur following habitat fragmentation or during long inter-fire intervals, as discussed above.

The third possible reason for the greater homozygosity in seedlings than adults is the operation of selection against inbred individuals. If the more homozygous seedlings are also more inbred, they may suffer inbreeding depression that prevents them from reaching either adulthood or old age (Charlesworth and Charlesworth 1987). There is much evidence to indicate that this occurs in other species, particularly in relation to inbred seed having a lower seed mass, which in turn affects seed germination and seedling survival (*e.g.* Kalisz 1989; Cabin *et al.* 1997). However, at present there is no evidence for *G. caleyi* that selection pressures are greater on inbred than outbred individuals; an experimental approach is required to address this issue.

Conclusion: Conservation genetic implications of the seed bank

The methods of sampling used for this study have raised difficulties in interpretation of the results. As mentioned previously, it is difficult to draw conclusions on changes to the mating system over time, or the effect of the seed bank in preventing the loss of genetic diversity from populations, by only sampling one generation of each life stage. Unfortunately, almost all published studies of the genetics of the seed bank have also only examined the seed bank and adult plants over a single generation. Although studies of rare plants or those affected by habitat changes are the most likely to provide insights into the role of the seed bank in buffering genetic changes to fragmented populations, I could only find three genetic studies of the seed bank of a rare plant, each of which found greater diversity in the seed bank than in adults. Of these, two had sampled populations at a single point in time and concluded that the seed bank had prevented the loss of genetic diversity and other genetic changes, despite small population sizes (McCue and Holtsford 1998; Aparicio *et al.* 2002). However, Morris *et al.* (2002) sampled temporal variation from three vertical strata of the age-stratified seed bank of *Astragalus bibullatus* and found evidence for increased inbreeding and decreased gene flow among populations in the youngest soil layer. This indicated that habitat fragmentation, caused by land use changes in the recent past, has had a detrimental effect on the genetic characteristics of this species' seed bank. Such a result may not have been detected by a simple comparison of adults and seeds, indicating that many more multi-generation seed bank studies are required. Unfortunately, however, this is likely to be very difficult to achieve when working with long-lived species that may have decades between generations, such as for *G. caleyi*.

The soil seed bank has great significance for the conservation genetics of *G. caleyi*, both from an evolutionary perspective and for the short- to medium-term conservation of genetic diversity. This significance arises from three important properties of the species' seed bank:

(1) the seed bank's ability to increase the effective population size to levels well above adult population size; (2) the longevity of the seed bank, which enables the storage of considerable amounts of genetic diversity long after adult plants have died; and (3) the residual seed bank that remains dormant after a fire, enabling mixing of generations and slowing of genetic changes.

This study has demonstrated the ability of the seed bank to maintain genetic diversity within *G. caleyi* populations despite severe bottlenecks of the adult cohort, and to store alleles that would otherwise have been lost from the population. Such features are particularly important for this species, which probably experiences dramatic natural fluctuations in population size due to variation in the fire regime. With the recent increase in human-induced habitat fragmentation, the diversity-maintaining properties of the seed bank have gained even greater significance. However, these powers of the seed bank are limited, and are unlikely to prevent the loss of diversity from populations that remain small over several generations. If N_E remains small for several generations, the loss of alleles and changes in allelic frequency should be expected, due to the combined effects of large amounts of inbreeding, lack of gene flow among populations or subpopulations, individual variation in reproduction, and genetic drift.

There is evidence for past losses of genetic diversity from *G. caleyi* populations, perhaps due to prolonged periods of low population size. The lack of microsatellite variation in either adults or seedlings at DF may have resulted from a past bottleneck that was so prolonged that it exhausted the seed bank's capacity to store genetic variation. In addition, data from JJM indicate that the seed bank appears to have very limited ability to prevent the further genetic differentiation of small populations or fragmented subpopulations, in the face of strong inbreeding and lack of pollen or seed dispersal. Therefore, the seed bank may enable the retention of diversity and retardation of other genetic changes only in the short- to medium-term, unless a concerted management effort is made to prevent adult and seed bank populations from falling or remaining below some (unknown) critical size. However, it is not known to what extent reduced genetic diversity or increased population structuring will affect the long-term viability of a population. A lack of genetic diversity may theoretically impair a population's ability to adapt to short-term selection pressures or respond evolutionarily in the long term to environmental changes (Franklin 1980). In addition, the likely increase in inbreeding within a small population after the buffering power of the seed bank has been exhausted may have more immediate impacts on the population's viability, if more inbred individuals have lower fitness.

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