

## ORIGINAL ARTICLE

Contrasting impacts of pollen and seed dispersal on spatial genetic structure in the bird-pollinated *Banksia hookeriana*SL Krauss<sup>1,2</sup>, T He<sup>1,3</sup>, LG Barrett<sup>3,4</sup>, BB Lamont<sup>3</sup>, NJ Enright<sup>3,5</sup>, BP Miller<sup>1,2,3</sup> and ME Hanley<sup>3,6</sup><sup>1</sup>Botanic Gardens and Parks Authority, Kings Park and Botanic Garden, Perth, Western Australia, Australia; <sup>2</sup>School of Plant Biology, The University of Western Australia, Nedlands, Western Australia, Australia; <sup>3</sup>Department of Environmental Biology, Centre for Ecosystem Diversity and Dynamics, Curtin University of Technology, Perth, Western Australia, Australia; <sup>4</sup>Department of Ecology and Evolution, University of Chicago, Chicago, IL, USA; <sup>5</sup>School of Environmental Science, Murdoch University, Murdoch, Western Australia, Australia and <sup>6</sup>School of Biological Sciences, University of Plymouth, Drake Circus, Plymouth, UK

In plants, pollen- and seed-dispersal distributions are characteristically leptokurtic, with significant consequences for spatial genetic structure and nearest-neighbour mating. However, most studies to date have been on wind- or insect-pollinated species. Here, we assigned paternity to quantify effective pollen dispersal over 9 years of mating, contrasted this to seed dispersal and examined their effects on fine-scale spatial genetic structure, within the bird-pollinated shrub *Banksia hookeriana* (Proteaceae). We used 163 polymorphic amplified fragment length polymorphism markers to assess genetic structure and pollen dispersal in a spatially discrete population of 112 plants covering 0.56 ha. Spatial autocorrelation analysis detected spatial genetic structure in the smallest distance class of 0–5 m ( $r=0.025$ ), with no significant structure beyond 8 m. Experimentally quantified seed-dispersal distances for 337 seedlings showed a leptokurtic distribution around a median of

5 m, reaching a distance of 36 m. In marked contrast, patterns of pollen dispersal for 274 seeds departed strikingly from typical near-neighbour pollination, with a distribution largely corresponding to the spatial distribution of plants. We found very high multiple paternity, very low correlated paternity and an equal probability of siring for the 50 closest potential mates. Extensive pollen carryover was demonstrated by multiple siring in 83 of 86 (96.5%) two-seeded fruits. Highly mobile nectar-feeding birds facilitate this promiscuity through observed movements that were effectively random. As the incidence of bird-pollination is markedly greater in the Southwest Australian Floristic Region than elsewhere, our results have broad and novel significance for the evolution and conservation for many species in Gondwanan lineages.

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## Introduction

The processes that affect, and are affected by, the spatial arrangement of genetic variation within natural populations, and their consequences for the evolution of biodiversity, are fundamental issues in ecology and evolutionary biology. Spatial genetic structure is determined largely by the interplay between dispersal, selection, genetic drift and the spatial arrangement of individuals (Wright, 1969; Loveless and Hamrick, 1984). In seed plants, the relationship between genetic structure and dispersal is complicated by the possibility of gene movement via both seeds and pollen (Levin and Kerster, 1974). Disentangling the relative contributions of these two agents to total dispersal and to spatial genetic structure

remains a major challenge (Fenster, 1991; Ennos, 1994; Ouborg *et al.*, 1999; Fenster *et al.*, 2003; Petit *et al.*, 2005).

According to models of isolation by distance (Wright, 1946; Rousset, 1997), spatially limited dispersal in the absence of selection results in a negative relationship between genetic similarity of individuals and geographic distance (Malecot, 1969; Epperson, 1990, 2007). For plants with typically leptokurtic dispersal (Levin and Kerster, 1974; Loveless and Hamrick, 1984; Vekemans and Hardy, 2004), local genetic structure arises such that populations consist of a continuum of smaller genetic neighbourhoods correlated with the dispersal curve (Wright, 1946; Turner *et al.*, 1982; Crawford, 1984; Epperson, 2007). A convenient and common measure of this spatial genetic structure is Wright's genetic neighbourhood ( $N_b$ ). This is defined as the area from which the parents of central individuals may be treated as if drawn at random, and can be determined by the variances of pollen and seed dispersal around a mean of 0 (Wright, 1946; Crawford, 1984).

However, the immobility of plants restricts their mating opportunities severely (Crawley, 1997) and

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random mating is rarely achieved in natural populations. Pollen dispersal is restricted by extrinsic forces such as plant density and pollinator movements, so that most pollen grains are typically deposited on nearest neighbours. Consequently, the probability of siring is generally assumed to decrease rapidly with distance between mates (Levin and Kerster, 1974; Webb, 1998). Pollen dispersal between nearest neighbours that are more related than on average across a population promotes biparental inbreeding, and a reinforcement of local spatial genetic structure and genetic neighbourhoods (Turner *et al.*, 1982). These extrinsic forces may be modified by intrinsic plant reproductive characteristics, such as self-incompatibility, mate choice, or inbreeding depression (Waser, 1993), possibly extending the genetic neighbourhood beyond that determined by pollen dispersal alone. Variability in individual reproductive success will also reduce effective genetic neighbourhood size below census size.

Consequently, the interactions between population genetic structure, seed dispersal, pollen dispersal and their effects on realized mating, genetic variation and the evolution of populations are complex, and an understanding relies mostly on the accuracy of estimates of spatial genetic structure and dispersal (Levin, 1981; Ouborg *et al.*, 1999). Highly polymorphic neutral molecular markers facilitate powerful direct, or 'real-time', approaches for elucidating these interactions. For example, there is now a battery of assignment methods (Jones and Ardren, 2003; Manel *et al.*, 2005) that utilize molecular markers to generate information on realized patterns of pollen dispersal through paternity assignment to individual pollen donors. When the pool of candidate parents becomes large, simple exclusion analysis can become impractical, and categorical allocation methods that use likelihood approaches to select the most likely parent from a pool of non-excluded parents are a solution (Meagher, 1986). For example, Gerber *et al.* (2000) developed and implemented for dominant markers such as amplified fragment length polymorphism (AFLP) a variation of the original likelihood-based approach for determining parentage.

Remarkably, few studies have assessed pollen dispersal and its consequences on genetic structure for species pollinated by birds. For example, in a major review of studies of ecological determinants of genetic structure in plant populations, only 2 of 163 (1.2%) species considered were bird/bat pollinated (Loveless and Hamrick, 1984). This is largely a consequence of the rarity of nectar-feeding birds in temperate latitudes of the northern hemisphere and temperate rainforests of the world, where wind pollination (anemophily) of trees and insect pollination (entomophily) of shrubs dominates, with pollination by vertebrates (zoophily) being generally uncommon (Ford *et al.*, 1979; Proctor *et al.*, 1996).

The incidence of vertebrate pollination syndromes in Australia, however, is markedly greater than elsewhere, perhaps a result of a large allocation of expendable energy on the profuse production of nectar (Orrians and Milewski, 2007). In the highly diverse Southwest Australian Floristic Region (SWAFR), an international biodiversity hotspot (Myers *et al.*, 2000), 15% of 7380 plant species are considered to be bird and/or mammal pollinated, a striking 40% of which are threatened endemics (Hopper and Gioia, 2004). In contrast, the

other major bird-pollinated regions of South Africa and Central America have only around 4% of their floras bird pollinated (Bawa, 1990; Orrians and Milewski, 2007). Observations of highly mobile behaviour by these Australian birds (Collins and Rebelo, 1987; Ramsey, 1989; McGoldrick and MacNally, 1998), and estimates of components of the mating system for bird-pollinated plants (Sampson, 1998; Coates *et al.*, 2007), suggest potentially novel genetic consequences from wide pollen dispersal and promiscuity in these plants.

Here, our objectives were to examine the relationship between spatial genetic structure, effective seed dispersal, realized patterns of mating and effective pollen dispersal in a spatially discrete population of the Western Australian bird-pollinated shrub *Banksia hookeriana* Meissner (Proteaceae). We used AFLP markers and multivariate spatial autocorrelation analysis (SAA) to characterize genetic structure, and compared this pattern to direct measurements of effective pollen and seed dispersal. Observations of avian foraging behaviour allowed us to interpret these data with respect to inter-plant movements by the principal pollen vector. As a result, we generate new insights into the role of dispersal in shaping genetic structure, and to the extrinsic forces influencing effective mating patterns, in a bird-pollinated plant species.

## Materials and methods

### Study species

*Banksia hookeriana* is a woody, non-sprouting (fire-killed), serotinous (canopy-stored seed) shrub up to 3 m tall × 3 m wide, that is restricted to the fire-prone northern sandplain scrub-heaths of southwestern Australia, 250–330 km north of Perth (Miller *et al.*, 2007). As a consequence of the undulating landscape within which it occurs, *B. hookeriana* exists at least in part of its range as discrete populations physically separated by uninhabitable interdunal swales of distances ranging from 0.2 to > 1 km (He *et al.*, 2004; Krauss *et al.*, 2006). Individuals of *B. hookeriana* commence flowering at 4–5 years of age. Flowers are protandrous and grouped into large cream-to orange-coloured inflorescence of about 1000 florets (Lamont *et al.*, 2003), with typically many inflorescences per plant that promote geitonogamy. Flowering is largely synchronous, with all flowering plants within a population even-aged and overlapping to some extent in flowering. Plants are capable of pollination by nectar-feeding birds and mammals and insects, particularly bees. Although seemingly self-compatible (Sedgley *et al.*, 1996), complete outcrossing occurs in at least one population of *B. hookeriana* (Barrett *et al.*, 2005). Ovaries contain two ovules, of which one but usually two develop into seeds. Viable seeds are held dormant within serotinous woody follicles on large infructescences (cones) in the crown of plants for more than 5 years, and up to 12 years of seed production may be stored in this fashion until fire triggers their release (Enright *et al.*, 1996). Fire intervals in *B. hookeriana* populations average ~ 13 years (Miller *et al.*, 2007), so most seeds are released following fire events. Seeds of *B. hookeriana* are winged and dispersed primarily by wind, with a capacity for long-distance dispersal. Population genetic assignment tests in *B. hookeriana* have indicated a surprisingly high

frequency (immigration rates of 7%) and distance (up to 2.4 km) of long-distance dispersal of seeds within a metapopulation area of  $3 \times 2$  km (He *et al.*, 2004). Populations establish in a single cohort following fire and consequently are easily aged either directly by counting stem node scars or with the help of available fire history maps.

### Seed dispersal

Seed dispersal was quantified experimentally *in situ* using seedling distributions around manipulated source plants. The study site was selected on the basis of an immediate post-burn environment and absence of *B. hookeriana*. The day following the burning of the study site in a wildfire, a total of 11 12-year-old plants from a nearby unburnt population were harvested at their bases and transported to the study site. Individual plants were tied to wooden stakes to mimic their natural habit and were placed 200–300 m apart along a 2.5 km transect. Seed-bearing cones were torched with a portable gas burner, triggering release of seeds over the next few days. Plants were inspected for seed release 2 weeks later, by which time 90% of seeds had been released. Seedling establishment was systematically surveyed 9 months later. The locations of all seedlings, dead or alive, were recorded within a 50 m radius of the source plant using a compass and measuring tape. The dispersal distance of each seedling was assigned to 5 m distance classes and averaged over all 11 plants.

### Genetics—study population and sampling

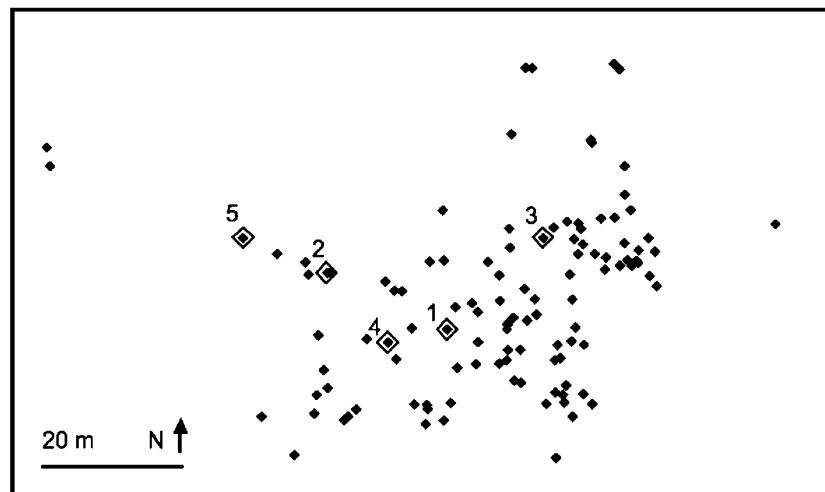
For the characterization of spatial genetic structure and pollen dispersal, we selected a discrete population containing 120, 15-year-old *B. hookeriana* individuals within an area of 0.56 ha ( $70 \times 80$  m), with density,  $d = 210$  plants  $\text{ha}^{-1}$  (Figure 1). Owing to the poor health of eight plants, only 112 could be genotyped. There were three *B. hookeriana* plants 35–45 m from the edge of the study population, and then no others within 250 m. The location of each *B. hookeriana* individual within the study area was mapped, and the average distance between all pairs of plants was 31.6 m (s.e. = 0.5), with a range from 0.3 to 128 m. For each plant, leaf material was collected

and stored in silica gel until DNA was extracted. The serotinous (canopy stored) seed bank was sampled by selecting five plants that displayed the most complete record of on-plant seed storage from nine successive years of mating. Cones representing each of nine years of seed production were collected for each of the five plants. Seeds were germinated on moistened filter paper at 18 °C and seedlings planted into pots containing a 5:1 mixture of sand and fine mulch before DNA was extracted from fresh leaves according to He *et al.* (2004).

AFLP was performed as described by Zawko *et al.* (2001), except that six AFLP primer pairs were used: M-CAG/E-ACT, M-CAG/E-AGG, M-CAG/E-ACC, M-CAG/E-AAC, M-CAG/E-ACA and M-CAT/E-ACC. Electropherograms were scored for presence (1) and absence (0) of fragments between 75 and 500 base pairs, and assembled into a binary data matrix. We utilized only AFLP markers with presence frequencies ranging 0.05–0.95 to minimize the risk of using rare false polymorphisms, with little impact on analysis power.

### Spatial genetic structure

Spatial genetic structure within the study population was assessed by spatial SAA using GenAlEx V6 (Peakall and Smouse, 2006), following the procedures of Smouse and Peakall (1999) that allow the multivariate analysis of individual spatial genetic structure for multilocus data sets. Evenly spaced distance classes of 5 m were chosen. For all pairs of individuals within a distance class, a correlation coefficient  $r$  was calculated using formula 15 in Smouse and Peakall (1999). Upper and lower confidence limits, as generated by 999 random permutations of the data, bound the 95% confidence interval (CI) about the null hypothesis of no spatial structure. In addition, 95% CIs about  $r$  were estimated by bootstrapping. Although caution is required with the interpretation of patch size from correlograms because it is dependent on the properties of sampling and analysis, a complete sampling of all plants within the population, a large number of polymorphic markers, and a stabilizing profile justified the identification of a critical distance in this study (Krauss and Koch, 2004; Vekemans and Hardy, 2004).



**Figure 1** Distribution of 112 *Banksia hookeriana* plants within the study population at Eneabba, Western Australia. The five plants from which seeds were collected for paternity assignment are indicated.

We also calculated cumulative estimates of  $r$  (along with associated errors about  $r$  and the null hypothesis) for increasing distance class sizes using GenALEX V6 (Peakall and Smouse, 2006). That is,  $r$  is calculated for all pairs of individuals in the smallest distance class (0–5 m), the two smallest distance classes combined (0–10 m), and so on up to the largest distance class. When significant positive structure is present, the estimated value of  $r$  will decrease with increasing size of distance class. The distance class size at which the estimate of  $r$  is no longer significant is an estimate of the true extent of detectable positive genetic structure.

In addition, we used SPAGeDi (Hardy and Vekemans, 2002) to characterize spatial genetic structure through their  $S_p$  statistic, which is primarily dependent on the rate of decrease of pairwise kinship coefficients between individuals with the logarithm of the distance in two dimensions. Under certain conditions, this statistic estimates the reciprocal of the neighbourhood size (Vekemans and Hardy, 2004). The  $S_p$  statistic allows a comparison to other species that is not possible with SAA, and we contrasted our value of  $S_p$  to those of 47 other plant species summarized by Vekemans and Hardy (2004).

### Paternity analyses

All paternity analyses were calculated using the software FaMoz (Gerber *et al.*, 2003) according to the categorical allocation likelihood methods described in Gerber *et al.* (2000). As the maternal plant was known for each offspring, a logarithm of the likelihood ratio (LOD score) was determined as the likelihood of an individual being the sire of a given offspring divided by the likelihood of these two individuals being unrelated. In our calculations, a mistyping error of 3% was accepted based on our previous results in generating replicate AFLP fingerprints, and reported mistyping rates of AFLP in other studies (Duhovnikoff and Dodd, 2003). We then conducted simulation-based assessments of confidence in paternity assignment by determining an LOD score threshold, as outlined in Gerber *et al.* (2000, 2003). Therefore, a criterion was determined for deciding whether a given individual would be chosen as the true paternal parent, and the individual with the highest LOD score that was also greater than the threshold was chosen as the true sire for a particular offspring. When all parent–offspring relationships fell below the likelihood threshold, offspring paternity was unassigned.

### Pollen dispersal

The proportion of realized pollen-dispersal distances compared with the proportion of plant pairs was plotted for 5 and 10 m distance classes for each of the five maternal plants, and for all maternal plants combined. The agreement of the distributions of realized pollen dispersal and plant pair number was assessed by the Kolmogorov–Smirnov two-sample test (Siegel, 1956). For the combined data set (all five maternal plants), we tested whether the mean difference between the fraction of realized pollen-dispersal events and the fraction of plant pairs within each distance class was significantly different from 0 by  $t$ -test. To standardize for distance, we also plotted siring success as a function of the ranked distance between mates for each maternal plant as well

as the combined data set, and assessed the relationship by linear regression analysis.

A two-parameter Weibull function, defined as

$$f(x) = \frac{c}{b} \left(\frac{x}{b}\right)^{c-1} e^{-(x/b)^c}$$

was used to model the realized dispersal distance distribution for both seed and pollen. The scale parameter,  $b$ , approximates to the mean dispersal distance, whereas a shape parameter of  $c=2$  corresponds to a normal distribution, and  $c=1$  to an exponential model. Decreasing  $c$  corresponds to fatter-tail functions, and consequently an increasing degree of leptokurtosis. A maximum likelihood method was employed to determine the  $c$  and  $b$  parameters of the Weibull distribution, using STATISTICA for Windows (Statsoft Inc., 1998).

A measure of the diversity of sires is the correlation of outcrossed paternity ( $r_p$ ), or the proportion of pairs of outcrossed progeny that are full sibs, as opposed to half-sibs (Ritland, 1989). This correlation arises from repeated matings to the same near-neighbours, or from the tendency of pollinator pollen loads to be derived from single plants. We used paternity assignments to directly estimate rates of correlated paternity. We used  $r_p$  to calculate a paternity pool as  $1/r_p$ , defined as random mating to an effective pool of neighbours, or the ‘effective number of sires’—that is, the number of sires necessary to cause the observed level of correlated mating paternity assuming equal mating chances and independent mating events (Ritland, 1989).

### Effective population size

The relative contributions of seed and pollen dispersal to the genetic neighbourhood ( $N_b$ ) inferred by Wright (1946) was estimated by calculating the axial variance ( $\sigma_{\text{axial}}^2$ ) for each from the sums of dispersal distances ( $d^2$ ), where  $\sigma_{\text{axial}}^2 = (\sum d^2/n)/2$ , and  $n$  is the sum of dispersal events measured (Crawford, 1984). The contributions of seed and pollen dispersal to gene dispersal ( $\sigma_g^2$ ) are additive, and the haploid pollen (p) component halved, so that  $\sigma_g^2 = (\sigma_s^2) + (\sigma_p^2)/2$  (Vekemans and Hardy, 2004). When dispersal is normally distributed around a mean of 0, and outcrossing rates are unity, the neighbourhood area can be estimated as  $N_a = \pi(2\sigma_{\text{axial}(p)}^2) + (4\sigma_{\text{axial}(s)}^2)$  (Crawford, 1984). Estimates of effective neighbourhood size ( $N_b$ ) were derived by multiplying  $N_a$  by the density of breeding plants  $\text{m}^{-2}$ .

### Bird pollinator movements

Movements of bird pollinators between *B. hookeriana* plants were examined in an adjoining population of 103 plants within a  $35 \times 35$  m area. As this was towards the end of the flowering season, 28 plants were currently flowering (nectar yielding), and only 50 plants had flowered during the current (relatively dry) season. All flowering plants were marked to enable identification, and the number of inflorescences on each plant actively yielding nectar noted. All plants were mapped, and interplant distances calculated. Pollinator movement observations were conducted for a total of 9 h over 2 days in September 2007, between 0700–1100 and 1500–1800 hours as pollinator activity during the middle of the day was much reduced. Once a bird had begun feeding within the observational area, we noted the number of inflorescences visited on each individual

plant, the time spent feeding on each inflorescence and whether the bird left the area or moved to another marked plant, in which case the marked plant was noted and observations of inflorescences visited and time spent feeding continued. As the next nearest flowering plant for all individuals was within the observational area, we were thus able to determine whether inter-plant distance (that is, nearest-neighbour proximity) or forage availability (that is, the number of inflorescences available on each flowering plant) influenced the movements of nectar-foraging birds. We observed a total of 74 pollinator visits, all by white-cheeked honeyeaters (*Phylidonyris nigra*).

## Results

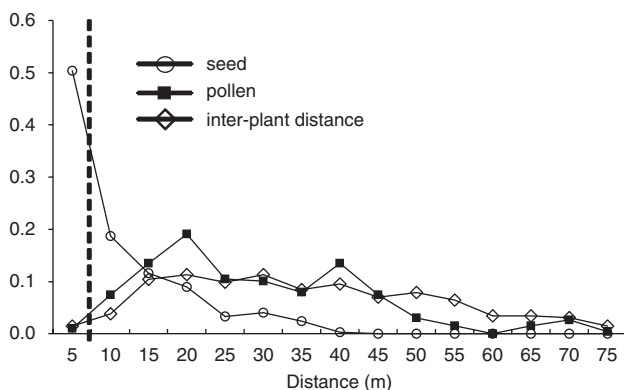
### Seed dispersal

In total, 337 seedlings were located around the 11 parent plants. The seed-dispersal distribution kernel was positively skewed, with mean dispersal distance of 8.12 m (s.d. = 8.34 m; s.e. = 0.46 m; kurtosis = 1.05), median of 4.9 m and range from 0.3 m to a maximum detected seed-dispersal distance of 36 m (Figure 2). A two-parameter Weibull distribution best fitted the frequency distribution of seed dispersal, with scale parameter  $b = 10.5$ , and shape parameter  $c = 0.96$ . The axial variance of realized seed dispersal ( $\sigma_{\text{axial}(s)}^2$ ) was 68 m.

### Population genetic structure

Six AFLP primer pairs generated 290 markers from 112 adults and 326 seedlings, of which 163 (56%) were polymorphic (frequencies between 0.05 and 0.95). Genetic dissimilarity, measured by the number of polymorphic markers between all pairs of adults, varied from 11 to 49, with a mean of 27. A detailed comparison of genetic diversity between adults and offspring over 9 years of mating is given elsewhere (Barrett *et al.*, 2005).

There was a positive genetic correlation between individuals, when compared with permuted  $r$ , in the smallest distance class of 5 m ( $P$  ( $r < \text{permuted } r$ ) = 0.01) (Table 1). Bootstrap estimates of the 95% error about



**Figure 2** Effective seed- and pollen-dispersal in *Banksia hookeriana*, showing the proportion of dispersal events for each 5 m distance class. Vertical dashed line indicates the largest distance at which positive genetic structure was detected by spatial autocorrelation analysis of 163 polymorphic amplified fragment length polymorphism (AFLP) markers in a population of 112 plants of *B. hookeriana*. Frequency distribution of inter-plant distance within this study population is also shown.

$r$  showed that  $r$  was greater than 0 ( $P < 0.05$ ). There was no significant correlation at any other distance class, indicating a stabilizing profile. Genetic patch size, defined as where  $r$  first passes from significant to non-significant within the correlogram, was 8 m. On average, target plants had five neighbours within this distance, although this ranged from 1 (plant 5) to 10 (plant 3) across the five maternal plants. The graph of  $r$  calculated for increasing distance classes confirms weak but positive significant genetic correlation between pairs of individuals at the smallest distance class (5 m), and no significant genetic correlation (or only weakly so) at larger distances (Figure 3). This result supports the conclusion of patch size between 5 and 10 m. However, in absolute terms, all  $r$  values, even that at 5 m, were low, reflecting overall weak genetic structure. This conclusion of weak (but detectable) spatial genetic structure was supported by the estimate of  $S_p = 0.004$  (s.e. = 0.0012) from SPAGeDi (Hardy and Vekemans, 2002). Neighbourhood size, as the inverse of  $S_p$ , was 250, greatly extending beyond the limits of the study population.

### Pollen dispersal

The paternity exclusion probability (EP) for 163 polymorphic markers was high (EP = 0.998). Simulation tests using FaMoz gave a threshold likelihood ratio value of 2.5. Using this threshold, maximum likelihood assignment for 326 offspring gave 52 (16%) with no compatible sire among the sampled population, and 274 (84%) with a most-likely sire within the study population. For these 274 offspring assigned paternity, 114 (42%) had only one potential sire above the threshold. For the remainder, more than one potential sire surpassed the threshold, and we assigned paternity to the most likely sire. For the offspring of these five maternal plants, the percentage of sires within the study population (84%) equates approximately to the definition of a genetic neighbourhood size as a circle whose radius is equivalent to twice the standard deviation of the gene dispersal distance, or 86.5% of the parents of an individual at its centre (Wright, 1946; Levin and Kerster, 1974).

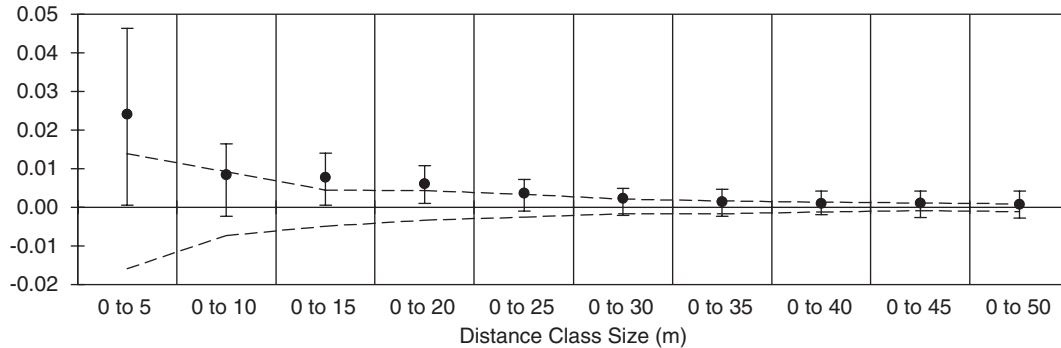
Of the 326 seedlings, 178 were from 89 follicles that each contained two seeds. For three of these, both seeds were assigned the same sire, whereas different sires were assigned for each seed in 58 follicles, paternity was assigned for just one seed in 25 of the follicles, and paternity was not assigned for either seed in the remaining three follicles. Consequently, 83 of 86 (96.5%) of two-seeded fruits were shown to be multiply sired. In no case was paternity assigned to the maternal plant (that is, self), indicating complete outcrossing in this population. All seeds had a minimum of 7, and up to 34, non-maternal markers, with a mean of 21.

Within the two-seeded *B. hookeriana* fruits, correlated paternity ( $r_p$ ) could only be either 1 or 0, but averaged a very low 0.035 across 86 two-seeded fruits: only 3.5% of two-seeded fruits contained full-sibs. Over the whole plant, correlated paternity ( $r_p$ ) varied from 0.018 to 0.059 across five maternal plants, and averaged 0.037 (s.e. = 0.001). That is, on average, only 3.7% of all seed pairs on a *B. hookeriana* plant were full sibs, and this percentage varied little across five maternal plants, despite marked differences in the spatial arrangement of plants around each maternal plant (Figure 1). Our

**Table 1** Spatial autocorrelation analysis results for 163 polymorphic AFLP markers scored for a population of 112 plants of *Banksia hookeriana*

Distance class	0–5	5–10	10–15	15–20	20–25	25–30	30–35	35–40	40–45	45–50	50–55	55–60
<i>n</i>	204	430	602	670	753	726	598	484	424	345	270	212
<i>r</i>	0.025	0.001	0.007	0.002	−0.003	−0.001	−0.005	−0.001	0.001	−0.002	0.001	0.000
<i>U</i>	0.013	0.010	0.008	0.009	0.007	0.009	0.007	0.010	0.011	0.010	0.013	0.014
<i>L</i>	−0.012	−0.009	−0.009	−0.008	−0.008	−0.008	−0.010	−0.010	−0.011	−0.012	−0.014	−0.012
<i>P</i>	0.010	0.440	0.050	0.310	0.800	0.590	0.930	0.550	0.420	0.630	0.440	0.420

AFLP, amplified fragment length polymorphism. Shown, for each distance class (in metres), are number of pairwise comparisons (*n*), genetic correlation (*r*), upper (*U*) and lower (*L*) bounds for the 95% confidence interval about the null hypothesis of no spatial structure (*r* = 0), and the probability (*P*) of a one-tailed test for positive autocorrelation.



**Figure 3** Genetic correlation coefficient (*r*) for increasing distance class sizes, 95% CI about the null hypothesis of a random distribution of genotypes, and 95% confidence error bars about *r* as determined by bootstrapping (with 999 simulations), for 163 polymorphic amplified fragment length polymorphism (AFLP) markers scored in a population of 112 plants of *Banksia hookeriana*.

**Table 2** Multiple paternity, as determined by maximum likelihood paternity assignment across five families of *Banksia hookeriana*

Maternal plant	No. of offspring assigned paternity	No. of sires	Maximum effective pollen grains provided by a single sire (% of total offspring)	Mean # offspring sired ± s.d.
1	45	32	4 (8.8%)	1.4 ± 0.8
2	48	23	9 (18.8%)	2.1 ± 1.9
3	68	28	10 (14.7%)	2.4 ± 2.3
4	61	32	9 (14.8%)	1.9 ± 1.6
5	52	34	5 (9.6%)	1.5 ± 0.9
Mean	55	30	7 (12.7%)	1.9 ± 1.6

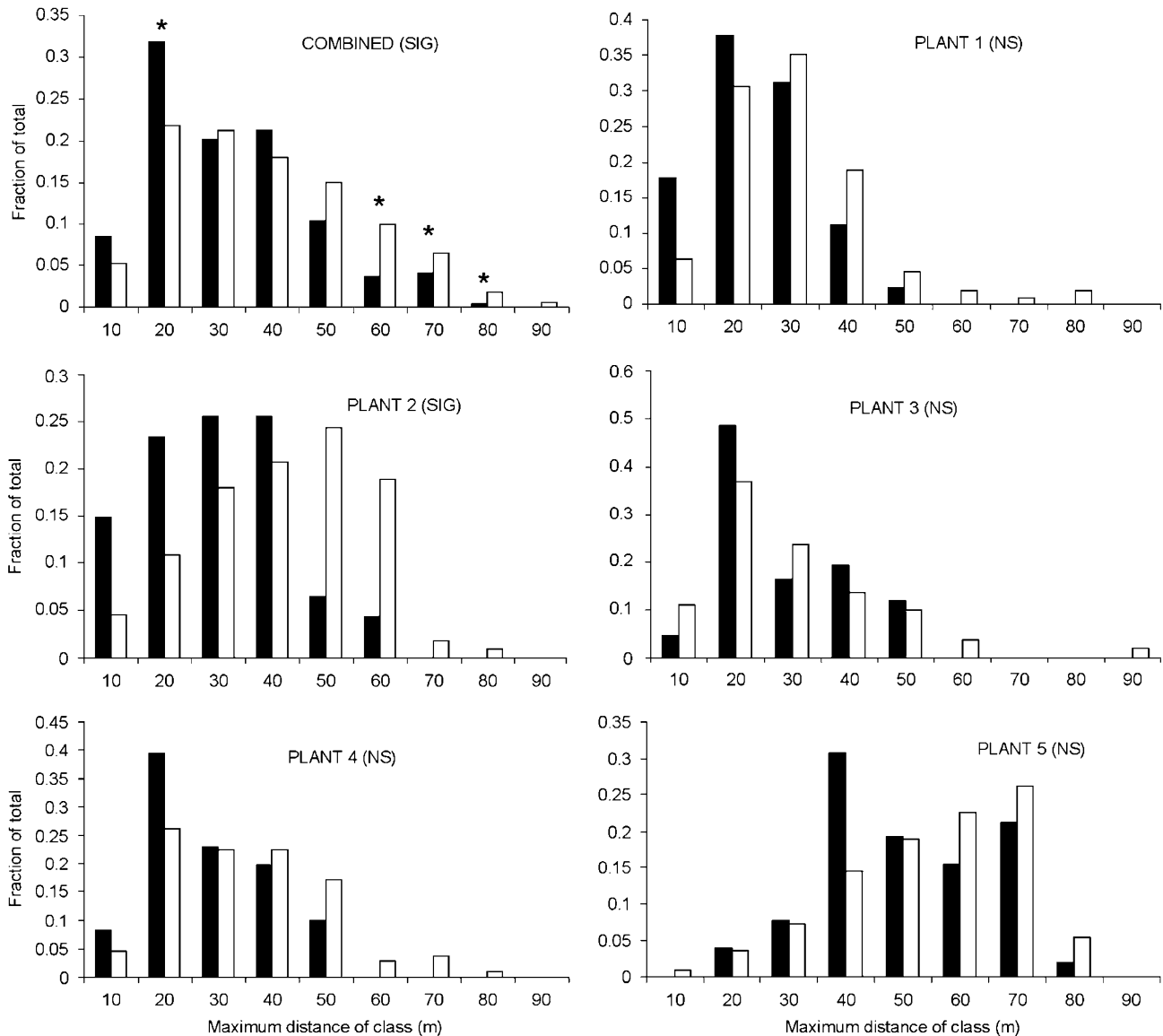
calculation of  $r_p = 0.037$  equates to an estimate of the effective pool of random mating ( $1/r_p$ ) in *B. hookeriana* of 27 plants.

In total, 75 of 111 (67%) plants sired at least one of the 274 offspring assessed. Multiple paternity within each of the five families was high, with an average of 30 sires for 55 offspring (Table 2). The most successful sire was different across the five families, and ranged from 4 of 45 offspring (8.8%) to 9 of 48 offspring (18.8%) (Table 2).

Pollen-dispersal distance was estimated for each offspring as the linear distance between the maternal plant and known sires as determined by paternity assignment, and categorized into 5 m distance classes (Figure 3). Combining the data for all maternal plants, the distribution of the frequency of mating for each distance class was skewed (skewness coefficient =  $0.764 \pm 0.147$ ) with most mating events occurring at an intermediate distance (15–20 m), rather than in the smallest distance classes (Figure 2). Overall, 10% of the mating pairs were separated by less than 10 m. The average distance between all pairs of mates was 29.9 m (average realized pollen-dispersal distance), and the average geographic

distance between all pairs of plants was 31.3 m. A two-parameter Weibull distribution function best fitted the frequency distribution of realized pollen dispersal, with scale parameter (*b*) of 29.5 (equivalent to the average pollen-dispersal distance), and shape parameter (*c*) of 1.9. The axial variance of realized pollen dispersal ( $\sigma_{axial(p)}^2$ ) was 493 m. Combined with the axial variance for seed dispersal, and an assumption that dispersal is normally distributed around a mean of 0, the estimate of effective neighbourhood size ( $N_b$ ) was 83 plants.

Realized pollen-dispersal distributions varied among maternal plants, and appear strongly influenced by the position of the maternal plant within the population (Figure 4). However, in only one maternal plant (two) was the pollen-dispersal distribution significantly different from the physical distribution of plant pairs (Kolmogorov–Smirnov two-sample test,  $P < 0.001$ ; Figure 4), indicating no detectable departure from random mating for the offspring from four of five families. Combining all maternal plants, there was a significant departure from random mating (Kolmogorov–Smirnov two-sample test,  $P < 0.001$ ), with a higher than expected



**Figure 4** Frequency distribution showing the fraction of 274 effective pollen dispersal events (solid bars) and plant pairs (unfilled bars) for each distance class (upper boundary given) for the combined data set of five maternal plants combined and separate (1–5) in a population of 112 plants of *Banksia hookeriana*. 'SIG' indicates a significant departure ( $P < 0.001$ ) between the two distributions, 'NS' indicates no significant departure ( $P > 0.05$ ) between the two distributions, as determined by Kolmogorov–Smirnov two-sample tests. Asterisk indicates significant difference ( $t$ -test,  $P < 0.05$ ) between fraction of effective pollen dispersal and plant pairs within a distance class for the combined data set.

number, under random mating, of mates in the 10–20 m distance class, and fewer than expected under random mating between 50 and 80 m (Figure 4). These departures may reflect non-random pollinator movements at these distances. Overall though, the probability of mating is only weakly associated with distance between mates, at least up to an average inter-mate distance of 50 m. Within this study population, that equates to an average for a maternal plant of 50 potential mates of equal age with more or less equal probability of paternity over the 9 years of mating measured.

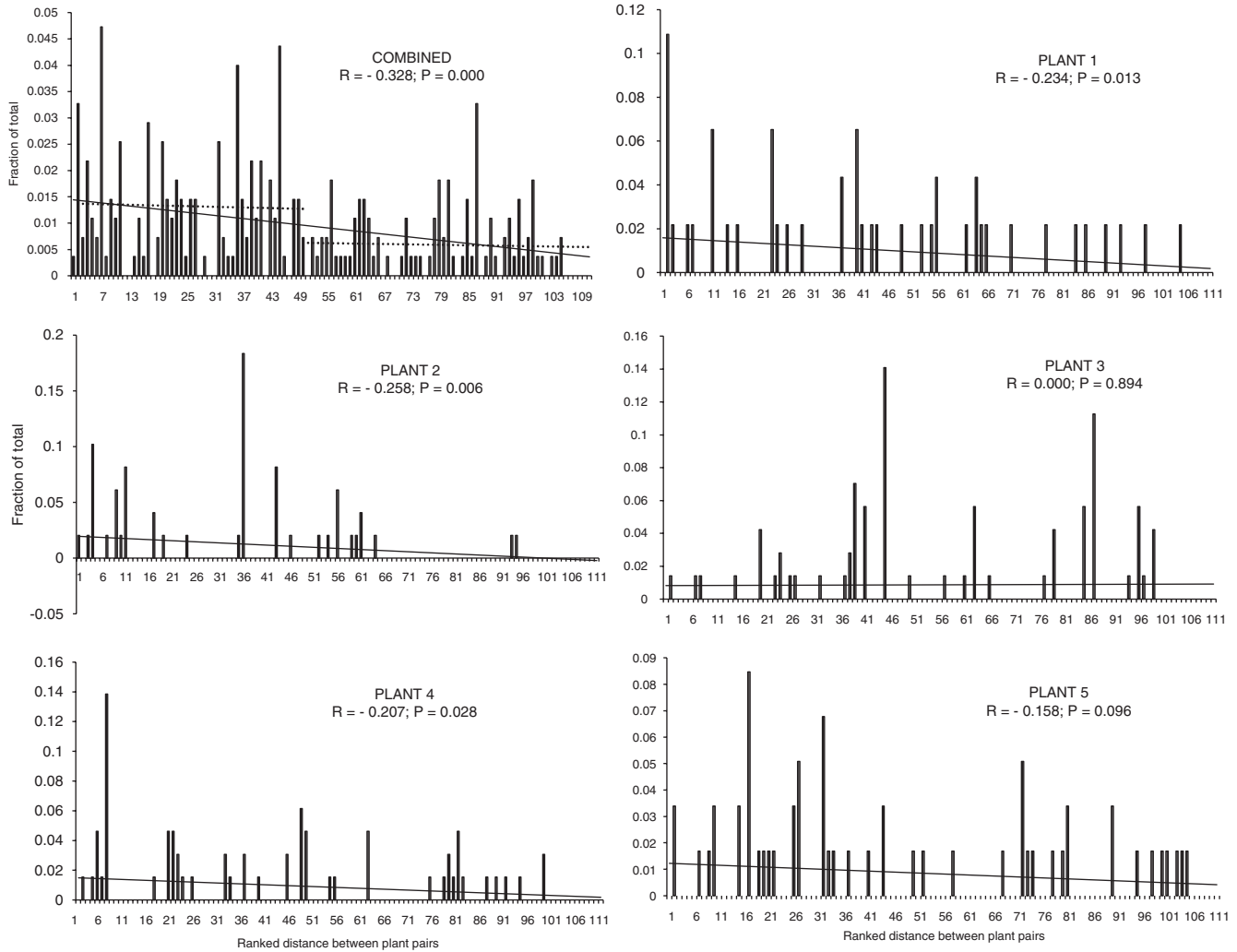
Mating success as a function of the ranked distance between mates showed a weak but significant negative linear relationship ( $r = 0.328$ ;  $P = 0.0004$ ) (Figure 5). However, this negative relationship is largely realized only beyond the 50th mate pair, as there was no significant relationship for ranked distance and mating success

between the first and 50th mate pairs ( $r = -0.035$ ;  $P = 0.907$ ). Up to the 50th mate pair ranked according to distance, the probability of mating for each pair averaged 0.014. For the 51st–111th mate pair, no relationship was seen between ranked distance and siring success, although the probability of mating for each pair averaged only 0.006. A weak, but significant, negative relationship between mating success and ranked distance between mates was found for maternal plants 1, 2 and 4 (Figure 5). However, there was no such relationship for maternal plants 3 and 5 (Figure 5).

#### Bird pollinator movements

Of 74 individual bird visits observed, 50 (68%) left the population after visiting only a single plant, whereas 24 (32%) moved to another plant within the population. The





**Figure 5** Frequency distribution showing the fraction of 274 effective pollen dispersal events against ranked distance between all plant pairs, for the combined data set of five maternal plants combined and separate (plants 1–5) in a population of 112 plants of *Banksia hookeriana*. Regression coefficient ( $r$ ) is shown, and when  $P < 0.05$ ,  $r$  is significantly different from 0. For the combined data set, dotted regression lines indicate no relationship between ranked distance and siring success within ranked distance 1–50, and within ranked distance 51–112.

total number of movements observed within the population was 35. Of these, significantly fewer ( $\chi^2 = 10.83$ ;  $P < 0.001$ ) were to the nearest flowering neighbour (8, 23%), whereas 27 (77%) were to a non-nearest neighbour. The average distance between all pairs of flowering plants, between nearest flowering neighbours, and of pollinator movements within the population were 16.6 ( $\pm 8.0$  s.d.), 3.3 ( $\pm 1.4$  s.d.) and 7.4 ( $\pm 3.6$  s.d.) m, respectively. There was a significant correlation ( $r = 0.439$ ;  $P < 0.05$ ) between the number of open inflorescences on a plant and the number of bird visits to a plant. No relationship was found between movement and direction. Taken together, these observations suggest that bird movements within this population were influenced more by flowering intensity than plant proximity.

## Discussion

Effective pollen dispersal in *B. hookeriana* showed a striking departure from the predominantly nearest-neighbour mating that is typical of plants pollinated by

wind and insect vectors. Furthermore, we found exceptionally high multiplicity of paternity, very low correlated paternity, and a probability of mating locally that showed a weak linear, rather than leptokurtic, relationship with ranked distance between mates detected across the entire population of 112 plants. An average probability of siring was found to be equivalent for the 50 most proximate potential sires around a maternal plant, and is to our knowledge an observation of random mating unmatched in the pollen-dispersal literature.

## Genetic neighbourhood

Consistent with this panmixis, the contribution of pollen dispersal to the estimate of Wright's neighbourhood size ( $N_{b(p)} = 4\pi(\sigma_p^2/2)$ ) was 65 plants. As a consequence of this panmixis, and despite highly restricted and typically leptokurtic primary seed dispersal, the ratio of effective to census population size ( $N_b/N = 83/112 = 0.74$ ) was strikingly greater than the average ( $N_e/N = 0.1$ ) for more than 100 species of animals and plants reported by



Frankham (1995). However, comparison with the indirect estimate of neighbourhood size (inverse of  $S_p$ ) suggests that the direct estimate,  $N_b$ , may significantly underestimate effective population size, in contrast to studies on other species that have found equivalent direct and indirect estimates (Fenster, 1991; Fenster *et al.*, 2003). A marked departure from the assumption of normally distributed dispersal in the calculation of  $N_b$ , as well as a population that is not in drift migration equilibrium, may largely explain the discrepancy between these direct and indirect estimates. In addition, these direct and indirect estimates are 'real-time' and historical measures respectively, and accumulated dispersal over many generations is apparently even greater than that observed directly. Therefore, the indirect estimate accounts for all possible causes of variation in effective and census population size, whereas the direct estimate is restricted to the spatial limitation of local seed and pollen dispersal with distance (Vekemans and Hardy, 2004).

Neighbourhood size, estimated indirectly as  $1/S_p = 250$ , greatly exceeded the limits of the study population, and highlights the importance of metapopulation dynamics in this species. This is strongly supported by our observations of high between population pollinator movement rates, high levels of inter-population pollen dispersal (16% of offspring with no compatible sire from the sampled population) and surprisingly high inter-population seed-dispersal rates, where 7% of individuals were inferred to be seed immigrants (He *et al.*, 2004). The direct estimate of neighbourhood size does not incorporate this gene flow, whereas the indirect estimate does.

### Spatial genetic structure

Spatial genetic structure in this population of *B. hookeriana*, as measured by the  $S_p$  statistic, was among the weakest yet recorded (Vekemans and Hardy, 2004). For 47 plant species reviewed by Vekemans and Hardy (2004),  $S_p$  ranged from 0.0003 to 0.263, with an average ( $0.030 \pm 0.049$  (s.d.)) an order of magnitude greater than that detected for *B. hookeriana* here ( $S_p = 0.004$ ). Again, this result is striking given the restricted primary seed dispersal and considering the average  $S_p$  for six gravity-dispersed seed species of 0.028 (Vekemans and Hardy, 2004). Although  $S_p$  indicated overall weak genetic structure in our *B. hookeriana* population, the impact of narrow seed dispersal was apparent in the SAA, which resolved spatial genetic structure to a distance of 8 m (an average patch size of five plants), and a stabilizing profile with no structure beyond. An explanation for this spatial genetic structure therefore lies in the contrasting impacts of pollen and seed dispersal, where  $\sigma_p^2/\sigma_s^2 = 7.25$ , and the random mating that is facilitated primarily by the behaviour of highly mobile nectar-feeding birds as pollinators.

### Pollen dispersal

The pattern of pollen dispersal we detected in *B. hookeriana* departs from theoretical models of pollen dispersal, as well as from empirically determined pollen-dispersal distributions estimated for insect- and wind-pollinated species (Levin and Kerster, 1974; Levin, 1981; Proctor *et al.*, 1996; Smouse *et al.*, 1999; Sork *et al.*, 2002; Austerlitz *et al.*, 2004; Smouse and Sork, 2004; Vekemans

and Hardy, 2004). The shrub *Personia mollis* (Proteaceae), for example, is pollinated by a suite of bees such as *Leioproctus* spp., and paternity analysis has identified the typically leptokurtic pollen-dispersal curve in this species, where first-order nearest neighbours, and first- to third-order nearest neighbours, sired 50 and 70%, respectively, of all seeds (Krauss, 2000). Similarly, in the wind-pollinated *Pinus sylvestris*, first-order nearest neighbours, and first- to third-order nearest neighbours, sired 25 and 42% of all seeds, respectively (Robledo-Arnuncio and Gil, 2005). In contrast, first-order nearest neighbours in *B. hookeriana* sired on average two orders of magnitude fewer (0.5%) of the total seeds assessed, whereas first- to third-order nearest neighbours sired only 4.4% of all seeds. Whereas pollen-dispersal distances can be very high for widely dispersed plants (for example, 23 km for species pollinated by euglossine bees in tropical rainforests; Janzen, 1971), the dispersal kernel remains typically leptokurtic (Janzen, 1971; Bawa, 1990; Proctor *et al.*, 1996). Although some recent paternity studies have shown surprisingly long pollen-dispersal distances (Kramer *et al.*, 2008), nearest-neighbour pollination, whereas not universal (Broyles and Wyatt, 1991; Dick *et al.*, 2003), is clearly a generally observed phenomenon in pollination biology (Webb, 1998), from which *B. hookeriana* is a notable exception.

A genetic consequence of nearest-neighbour pollination is higher correlated paternity, where full-sib pairs are usually sired by nearest neighbours. This relationship serves to amplify the genetic structuring effects of nearest-neighbour pollination. In contrast, near random mating in *B. hookeriana* resulted in very low correlated paternity ( $r_p = 0.037$ ), that to our knowledge is the lowest recorded, and a value that is an order of magnitude less than typical measures (Sampson, 1998). A departure from nearest-neighbour pollination has been previously observed (Broyles and Wyatt, 1991) when pollen has been packaged into discrete bundles (pollinia). In this case, however, multiple paternity within fruits is almost non-existent, and correlated paternity is near to or equal to unity (that is, all seeds within a fruit are full sibs), with significant consequences for spatial genetic structure.

### Pollen vectors

The panmixis we detected requires extensive pollen carryover and/or near random pollinator foraging behaviour, in terms of both distance and direction. Such features are generally considered unlikely in zoophilous plant species (Levin and Kerster, 1974). Our observation that 96% of two-seeded fruits were multiply sired indicates extensive pollen carryover in this population. In *B. hookeriana*, pollination is achieved primarily through nectar-feeding birds in the family Meliphagidae. These include the White-Cheeked Honeyeater (*P. nigra*), Tawny-crowned Honeyeater (*P. melanops*), Red Wattlebird (*Anthochaera carunculata*) and the Brown Honeyeater (*Lichmera indistincta*) (Brown *et al.*, 1997). Indeed, nectar-feeding birds routinely pollinate many *Banksia* species (Ford *et al.*, 1979; Hopper, 1980; Collins and Rebelo, 1987; Taylor and Hopper, 1988; Ramsey, 1989; Day *et al.*, 1997; Wooller and Wooller, 2004). Although the honey possum, *Tarsipes rostratus* is also an important pollinator for many southwestern Australian banksias (Wiens *et al.*, 1979; Bradshaw and Bradshaw, 2002), studies of foraging

movements of *T. rostratus* suggest that it does not generally achieve the long-realized pollen-dispersal distances inferred here (Bradshaw and Bradshaw, 2002). Insects (predominantly native bees such as the banksia bee *Hylaeus alcyoneus* (Houston, 2000) and the introduced honeybee *Apis mellifera*) visit flowers, but winter flowering of *B. hookeriana* restricts the abundance of insects as potential pollinators.

### Consequences of pollination by nectar-feeding honeyeaters

A major feature of bird-pollinated plants in temperate southern Australia is a patchy, asynchronous flowering of different species, which is a principal driver of mass nomadism of nectarivorous birds among regions and habitats (McGoldrick and MacNally, 1998). Thus, most honeyeaters are highly mobile generalist foragers well adapted to the heterogeneous flowering landscape over which nectar is produced (Ford *et al.*, 1979; McGoldrick and MacNally, 1998). In our study, this was demonstrated by 77% of all within-population bird movements being beyond nearest neighbours, and 68% of observed movements leaving the local population. Energy requirements for individual nectar-feeding birds demonstrate a need for movement between a large number of plants, and often species (Collins and Rebelo, 1987). High mobility and aggressive spatial displacement behaviour of honeyeaters competing for the same resource largely explains the patterns of pollen dispersal and pollen carryover we detected. In contrast, most (but not all) hummingbirds display conspicuous aggressive behaviour in the defence of small-feeding territories, and coupled with nearest-neighbour optimal foraging, this restricts the dispersal of pollen for plants pollinated by these birds (Pyke, 1980; Proctor *et al.*, 1996).

Part of the pattern of pollen dispersal can also be attributed to limited co-flowering within the population, obliging birds to move beyond the next plant on those grounds alone. Our observation of 27% co-flowering in this study can be compared with 32, 61 and 69% co-flowering plants in other studied populations of *B. hookeriana* at the peak of flowering (with 98% flowering over the entire season; Lamont *et al.*, 2003). Aggregation of flowers into a few dense inflorescences is typical of many bird-pollinated species, decreasing the chances of co-flowering and increasing the distance between potential mates. Birds often flew over flowering plants in search of those with greater nectar rewards. However, we have also demonstrated that the accumulation of 9 years of mating smoothes out the year-to-year pollen pool variation that may result from sporadic flowering (this study; Barrett *et al.*, 2005). Indeed, in *B. hookeriana*, genetic variation in the aerial seed bank approached a maximum after only a few years of mating, suggesting that random mating is achieved quickly (Barrett *et al.*, 2005).

In Australia, nectarivores are a conspicuous component of avian communities as a result of the profuse production of nectar and relatively few fleshy-fruited plants (Orians and Milewski, 2007). Consequently, bird pollination is a prominent feature of the flora of temperate Australia, especially in the ancient Gondwanan families Proteaceae and Myrtaceae (Ford *et al.*, 1979; Keighery, 1980). Over 110 species of birds have been

recorded visiting the flowers of 1000 species of plants in more than 64 genera and 16 families (Ford *et al.*, 1979; Keighery, 1980; Brown *et al.*, 1997). Many of these genera (for example, *Banksia*, *Grevillea*, *Melaleuca*, *Eucalyptus*) are highly diverse and abundant. Significantly, the large number of species pollinated by nectarivores suggests that the wide pollen-dispersal patterns we have detected, and its consequences for mating, genetic variation and genetic structure, may be a common feature of pollination for many plant species in temperate Australia. We suggest, then, that nectar-feeding bird pollination in southern temperate zones is a significant pollination syndrome with poorly understood, yet potentially novel, genetic consequences. These included wide outcrossing, multiplicity of paternity, low correlated paternity, weak genetic structure and the maintenance of genetic variation that may not be accurately predicted if applying generally accepted models of pollen dispersal based largely on wind and insect pollination.

Our observations raise intriguing suggestions that the honeyeater pollination syndrome can be characterized by ecological attributes that distinguish it from other pollination syndromes in promoting wide outcrossing. These include flowers typically in dense inflorescences, plants in a population tending not to all co-flower, highly mobile pollinators displaying movements beyond nearest plant neighbours, pollinators carrying high pollen loads from multiple plant visits and aggressive spatial displacement among birds promoting highly dynamic activity. Our direct observations of the majority of all bird movements to beyond the study population suggests that long-distance pollen flow among populations may also be a characteristic feature of this syndrome. In support, our genetic data showed that 52 (16%) offspring did not have a compatible sire within the known pollen pool, and Byrne *et al.* (2007) found an average of 33% of seedlings sired by pollen sources outside of the study population, at distances of up to 5 km, in the Western Australian bird-pollinated shrub *Calothamnus quadrifidus*. In addition, our study population was relatively small (He *et al.*, 2004), and wide outcrossing and multiplicity of paternity is likely to be even more pronounced in typically larger populations.

### Conservation implications

An immediate challenge of some urgency is to understand how human-induced alterations to the natural distribution and abundance of plant species and their bird pollinators affect dispersal and plant-pollinator interactions (Saunders and Ingram, 1995; Kearns *et al.*, 1998; Paton, 2000). Recent genetic studies on other bird-pollinated plants in the SWAFR have demonstrated an impact on the mating system, with increased selfing and increased correlated paternity associated with reduced population size following habitat fragmentation (Coates *et al.*, 2007). Introduced, non-native pollinators such as European honeybees (*A. mellifera*), and increased competition for declining vertebrate pollinators, strongest in biodiversity hotspots (Vamosi *et al.*, 2006), affect pollen limitation and seed set in many species with already low conversions of flowers to fruits (Ayre and Whelan, 1989; England *et al.*, 2001), increasing extinction risk. Our results highlight the additional conservation genetic concerns from bird-pollinator disruption that may result

in a significant increase in near-neighbour pollen dispersal, correlated paternity and inbreeding through bi-parental mating. In these widely outcrossing species, genetic load is high, and inbreeding depression strong (Heliyanto *et al.*, 2006). These potentially detrimental genetic impacts will be of greatest concern in species characterized by promiscuity, likely to be a feature of the highly diverse bird-pollinated flora of the SWAFR.

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