ORIGINAL ARTICLE

Paternity analysis reveals wide pollen dispersal and high multiple paternity in a small isolated population of the bird-pollinated *Eucalyptus caesia* (Myrtaceae)

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Optimal foraging behaviour by nectarivores is expected to result in a leptokurtic pollen dispersal distribution and predominantly near-neighbour mating. However, complex social interactions among nectarivorous birds may result in different mating patterns to those typically observed in insect-pollinated plants. Mating system, realised pollen dispersal and spatial genetic structure were examined in the bird-pollinated *Eucalyptus caesia*, a species characterised by small, geographically disjunct populations. Nine microsatellite markers were used to genotype an entire adult stand and 181 seeds from 28 capsules collected from 6 trees. Mating system analysis using MLTR revealed moderate to high outcrossing ($t_m = 0.479\text{--}0.806$) and low estimates of correlated paternity ($r_p = 0.136 \pm \text{s.e. } 0.048$). Paternity analysis revealed high outcrossing rates (mean = 0.72) and high multiple paternity, with 64 different sires identified for 181 seeds. There was a significant negative relationship between the frequency of outcross mating and distance between mating pairs. Realised mating events were more frequent than expected with random mating for plants <40 m apart. The overall distribution of pollen dispersal distances was platykurtic. Despite extensive pollen dispersal within the stand, three genetic clusters were detected by STRUCTURE analysis. These genetic clusters were strongly differentiated yet geographically interspersed, hypothesised to be a consequence of rare recruitment events coupled with extreme longevity. We suggest that extensive polyandry and pollen dispersal is a consequence of pollination by highly mobile honeyeaters and may buffer *E. caesia* against the loss of genetic diversity predicted for small and genetically isolated populations. *Heredity* (2016) **117**, 460–471; doi:10.1038/hdy.2016.61; published online 17 August 2016

INTRODUCTION

Although the steepness and length of pollen dispersal curves varies among species, they are typically leptokurtic, with the frequency of mating events decreasing with distance between mating pairs (Levin and Kerster, 1974; Morris *et al.*, 1995). Leptokurtic pollen dispersal by animals may be attributed to optimal foraging behaviour, where animals forage to maximise energy reward relative to energy loss (Pyke, 1984). Foraging animals typically move between flowers on a single plant, and then on to a near neighbour (Harder and Barrett, 1996) that may be a genetic relative if seed dispersal is limited. Optimal foraging behaviour by nectar- or pollen-feeding animals is therefore expected to result in short-distance pollen dispersal and frequent self-pollination and biparental inbreeding.

Social interactions among nectar-feeding birds that otherwise display optimal foraging patterns on flowers, such as territoriality and the defence of nectar resources, may lead to deviations from optimal foraging behaviour (Mac Nally *et al.*, 2005; Phillips *et al.*, 2014), with potentially novel genetic consequences for plant mating patterns. The defence of nectar resources has been widely documented in honeyeaters, a key pollinator group in temperate Australia (Ford *et al.*, 1979; Hopper and Moran, 1981; Ford and Paton, 1982). For example,

large species such as Red Wattlebirds (*Anthochaera carunculata*), Western Wattlebirds (*A. lunulata*) and Spiny-cheeked Honeyeaters (*Acanthagenys rufogularis*) frequently chase away the smaller Brown Honeyeaters (*Lichmera indistincta*) and Western Spinebills (*Acanthorhynchus superciliosus*) while foraging on *Eucalyptus stoatei* (Hopper and Moran, 1981) and a similar pattern of behavioural dominance has been observed between groups of New Holland Honeyeaters and individual Western Spinebills during foraging bouts on *Anigozanthos flavidus* (Phillips *et al.*, 2014). Therefore, pollination systems involving nectar-feeding honeyeaters that frequently move between widely spaced plants as a consequence of aggressive interactions may facilitate wide outcrossing and high multiple paternity, potentially enhancing genetic diversity and evolutionary potential (Hopper, 1981). In the absence of aggressive interactions, greater pollen carryover by birds compared with insects could still influence multiple paternity and sire diversity in bird-pollinated plants (Mitchell *et al.*, 2013). High pollen carryover by birds could explain long-distance pollen dispersal between populations of tree species with generalist pollination systems (Byrne *et al.*, 2008; Mimura *et al.*, 2009).

Although field observations of honeyeater behaviour in numerous plant species support predictions of more widespread pollen dispersal

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than would be expected under insect pollination (Hopper and Moran, 1981; Ramsey, 1988; Phillips *et al.*, 2010), few studies have addressed the realised genetic consequences of this behaviour for the plants that they pollinate. Paternity studies in southwest Australian banksias suggest that departures from optimal foraging by honeyeaters can lead to wide outcrossing and very high multiple paternity (Krauss *et al.*, 2009; Ritchie and Krauss, 2012), and that population size, shape and isolation affect honeyeater visitation patterns and plant paternity (Llorens *et al.*, 2012). In *A. flavidus*, studies of seed germinability suggest that the foraging behaviour of Western Spinebills may facilitate high offspring fitness, potentially aiding the persistence of small populations (Phillips *et al.*, 2014). However, the drivers and consequences of contrasting pollen dispersal patterns in different species pollinated by nectar-feeding birds remain poorly understood.

Population genetic theory predicts that small, isolated plant populations can experience a loss of genetic diversity through genetic drift and/or inbreeding from self-pollination or mating among close relatives (Ellstrand and Elam, 1993). In extreme cases, this can lead to inbreeding depression or even population sterility (DeMauro, 1993; Husband and Schemske, 1996). For example, self-pollination can result in depressed seed set and plant growth compared with controlled outcrossed pollinations in some eucalypts (Hardner and Potts, 1995). However, multiple mating and pre- or post-dispersal selection against inbred individuals has fitness benefits that may buffer the loss of genetic diversity in small populations (Harder and Potts, 1997; Pannell and Labouche, 2013; Roberts *et al.*, 2014). Rare, localised endemics with limited dispersability may have evolved pollination strategies in response to prolonged isolation to maintain or enhance polyandry and genetic diversity (the James Effect hypothesis; sensu Hopper, 2009). For example, field observations of bird-pollinated plants in southwest Australia suggest pollination by highly mobile nectar-feeding birds as one potential mechanism facilitating wide outcrossing and thus conserving heterozygosity (Hopper, 1981, 2009). More research is needed to evaluate whether bird pollination has novel genetic consequences for the numerous honeyeater-pollinated plants in Australia, and to explore its potential relevance to other bird-pollinated plants.

The South West Australian Floristic Region (SWAFR, sensu Hopper and Gioia, 2004) has the highest known global incidence of vertebrate pollination (Phillips *et al.*, 2010). Approximately 15% of ca. 7400 plant species are pollinated by birds or small mammals, including a remarkable 40% of rare species (Keighery, 1980; Hopper *et al.*, 1990). Despite the pervasiveness of vertebrate pollination in the SWAFR, the ecological and evolutionary significance of vertebrate pollination remains unclear. *Eucalyptus caesia* Benth. (Myrtaceae) is a rare species that is endemic to 25 granite outcrops in the Central Wheatbelt of Western Australia (Figure 1). As a bird-pollinated species with accentuated persistence in small, insular populations (Moran and Hopper, 1983; Byrne and Hopper, 2008), *E. caesia* is a model system to assess the generality of near-random mating patterns identified in some bird-pollinated banksias, and to test for beneficial genetic consequences of bird pollination, as predicted by the James Effect hypothesis (Krauss *et al.*, 2009; Ritchie and Krauss, 2012). Furthermore, *E. caesia* presents an opportunity to study mating system and pollination in an intact, natural setting, whereas previous studies of pollen movement in bird-pollinated species in Australia have largely focussed on populations affected by recent habitat fragmentation (Byrne *et al.*, 2007; Breed *et al.*, 2012; Llorens *et al.*, 2012; Ritchie and Krauss, 2012).

In this study, we explore the genetic consequences of bird pollination using molecular analysis of progeny arrays in *E. caesia*.

We predict that pollination by honeyeaters will result in wide outcrossing, high multiple paternity and non-leptokurtic pollen dispersal. We then assess the extent to which spatial genetic structure of the adult population is predictable from the observed mating system. Although limited seed dispersal and extensive asexual reproduction via lignotubers suggests a strong likelihood of high spatial genetic structuring within populations, we predict that wide pollen dispersal within the study population will result in weaker spatial genetic structuring than expected if pollination and seed dispersal were both limited.

MATERIALS AND METHODS

Study species and study site

E. caesia is a granite outcrop-endemic, monoecious, winter-flowering mallee or small tree with large pink flowers (Figure 2). Whether *E. caesia* is, like most eucalypts, self-compatible but with a predominantly outcrossing mating system (House, 1997) is unknown. Two subspecies are recognised (Brooker and Hopper, 1982). *E. caesia* subsp. *caesia* extends across the species' full geographic range, whereas *E. caesia* subsp. *magna* is confined to six stands in the northeast sector of the Central Wheatbelt (Figure 1). At least eight species of honeyeaters (Meliphagidae) act as the primary pollinators of *E. caesia*, though the flowers are also visited by native and introduced insects such as the introduced honeybee (*Apis mellifera*) and occasionally small marsupials (Hopper, 1981). It is unknown whether the latter groups effect pollination, although generally high visitation rates by *A. mellifera* suggest a strong possibility for pollination by this species. Seed dispersal in *E. caesia* has not been studied but is likely by gravity, although secondary dispersal may occur through movement by wind or water, or by parrots feeding on seed capsules (House, 1997). *E. caesia* reproduces sexually, and asexually via resprouting from lignotubers (Hopper *et al.*, 1982). Surveys in multiple populations conducted over 30 years by SD Hopper indicate that flowering is asynchronous.

The present study focussed on the *E. caesia* subsp. *caesia* stand within Boyagin Nature Reserve (32°28'21.0"S 116°52'23.5"E; Figure 1), one of the largest intact reserves in the Central Wheatbelt. The number of distinct genets at the study site is unknown. Plants are distributed in distinct stands on the slopes of a granite bornhardt, called Boogin Nop by local Ballardong Noongar people. There, *E. caesia* occurs in dense thickets with other woody species (including *Hakea petiolaris*, *Allocasuarina huegeliana*, *Calothamnus ruprestris* and *Kunzea pulchella*), and as isolated plants in cracks in the granite (Figure 2).

Mapping and sample collection

To accurately assess population size, pollen dispersal distances and spatial genetic structure, a Trimble GeoExplorer differential GPS (Digital Mapping Solutions, Perth, WA, Australia) was used to obtain global positioning system (GPS) coordinates of each plant to an accuracy of <1 m. *E. caesia* has extensive asexual reproduction and stems were recorded as comprising a single plant if they arose from a common lignotuber. Leaf samples were collected from a single stem for most plants and from multiple stems of larger plants where lignotuber degradation prevented visual identification of individual genets. Thus, the number of samples collected for genetic analysis was larger than the estimated population size, with a total of 412 samples. Leaf samples were stored fresh in individual zip-lock bags before DNA extraction. A total of 72 fruits (12 per maternal plant) representing the products of the 2012/2013 flowering period were collected from each of 6 trees and dried until the seeds were released. A randomly selected sample of 16 seeds from each fruit were germinated on agar under a 20 °C day/night cycle. Some seeds failed to germinate and therefore final sample sizes were uneven across the 6 families, with final seed numbers ranging from 12 to 61 seeds per family. Maternal plants were selected to allow investigation of pollen flow into the main summit stand from smaller peripheral stands, and investigation of pollen flow among plants in the summit stand. Within the summit stand, widely spaced plants were selected as maternals to ensure that maternal plants did not comprise a single clonal patch. Generally low capsule numbers per plant across the population constrained the number of maternal plants that were suitable for inclusion in the study.

Figure 1 Distribution of *Eucalyptus caesia* subsp. *caesia* (closed circles) and *E. caesia* subsp. *magna* (open circles) in the Central Wheatbelt of Western Australia, and location of sampled plants at the study site in Boyagin Nature Reserve (32°28'21.0"S, 116°52'23.5"E), generated using Google Earth imagery (2014). Arrows indicate trees from which seeds were collected for paternity analysis. Locations of *E. caesia* populations are based on on-ground survey by SD Hopper and Western Australian Herbarium records.

Microsatellite genotyping

Genomic DNA was extracted from 2 cm² of adult leaf tissue and from entire seedlings, harvested 1–2 weeks after germination, using a CTAB (cetyl trimethyl ammonium bromide) extraction method modified by Doyle and Doyle (1987). DNA was extracted from all adult leaf samples ($n=412$) and 196 seedlings. Because of germination failure of some seeds, final progeny sample numbers were not even across the six families.

Microsatellite loci were amplified using nine primer pairs developed in other eucalypts (EGM25, EGM30, EGM47, EGM14, EGM12, EGM34, EGM35, EMBRA10 and EMBRA7) (Steane *et al.*, 2001; Bradbury *et al.*, 2013a) using the QIAGEN Multiplex PCR Kit (QIAGEN, Venio, Limburg, The Netherlands) as per the manufacturer's instructions. PCR conditions were as follows: 95 °C for 5 min, then 30 cycles of 95 °C for 30 s, 58 °C for 1 min 30 s and 72 °C for 30 s, followed by 60 °C for 30 min. A total of 30 microsatellite primer pairs were tested for amplification in *E. caesia*. The final panel of markers were

selected based on consistent amplification and scorability in combination with polymorphism, and consisted of three multiplex mixes: group A (EGM35, EMBRA10 and EMBRA7), group B (EGM12 and EGM34) and group C (EGM25, EGM30, EGM47 and EGM14). To optimise amplification peaks, the concentrations of EMBRA10 and EMBRA7 were increased from 2 to 3 and 4 μ M, respectively, and the concentration of EGM35 was reduced to 0.5 μ M.

Amplified product was visualised with an automated 3500 Genetic Analyzer with 10 μ l Hi-Di Formamide and 0.3 μ l 500 LIZ size standard (Applied Biosystems, Foster City, CA, USA). Alleles (sized base pairs) were scored manually using Geneious 7.1.4 (Drummond *et al.*, 2011). The database was checked for typing errors by using GenAlEx V6.5 (Peakall and Smouse, 2006, 2012) to generate a list of alleles and by double-checking electropherogram profiles of rare alleles. Positive and negative controls were included in each batch of samples, and randomly chosen individuals (2 samples for each 96-well plate) were genotyped in duplicate to confirm

Figure 2 *Eucalyptus caesia* subsp. *caesia* growing on slopes of a granite bornhardt in Boyagin Nature Reserve, Western Australia. Photos shown include a single multi-stemmed plant (top left), a New Holland honeyeater foraging for nectar (bottom left; photo by SD Hopper), *E. caesia* flowers (bottom right) and multiple stems comprising a single multilocus genotype (top left). Photos are by N Bezemer unless otherwise indicated.

repeatability of allele scoring. The final genetic database was double checked manually. We performed a manual check to confirm that all progeny had at least one maternal allele at each locus.

Population size and genetic diversity

Some of the 412 sampled stems were expected to be ramets of the same genet because of repeat sampling of larger plants. Replicate multilocus genotypes (MLGs) in the data set were thus identified using the identity analysis function in CERVUS V3.0 (Kalinowski *et al.*, 2007) and all subsequent analyses conducted using the condensed subset ($n = 203$) of the original adult genotypic database ($n = 412$) from which duplicated MLGs were removed.

To assess genetic diversity in the adult cohort, N (number of alleles), N_e (effective number of alleles), H_E (expected heterozygosity), H_O (observed heterozygosity) and F (fixation index) were calculated. The frequency of alleles at each locus was calculated for adults and progeny and significant differences tested for using proportions tests in R (R Core Team, 2014). All diversity statistics were calculated using GenAlEx V6.5 (Peakall and Smouse, 2006, 2012).

Mating system

Multilocus outcrossing rate (t_m), biparental inbreeding ($t_m - t_s$) and multilocus correlation of paternity (r_p) were estimated using MLTR (Ritland, 2002). Variance estimates were calculated based on 1000 bootstraps. Resampling was conducted at the family level, rather than for individuals within families, because actual mating system parameters varied between families. Multilocus outcrossing rate is an estimate of the proportion of outcrossed progeny produced by a single maternal parent, or by the population as a whole, in which outcrossing events include mating between relatives and unrelated individuals. Measures of single-locus outcrossing rate differ in that only mating between unrelated individuals is considered an outcrossing event. The difference between the two estimates ($t_m - t_s$) thus provides a measure of the frequency of mating events between close relatives (Ritland, 2002). Correlated paternity is a measure of the proportion of pairs of outcrossed siblings that are full siblings, as opposed to half siblings.

Paternity assignment

Paternity assignment was conducted by categorical allocation in CERVUS v3.0.3 (Kalinowski *et al.*, 2007). Logarithm of odds (LOD) scores were

calculated for all sampled plants, as every reproductive individual in the population is a potential sire. Confidence in assignments was measured as the difference in LOD scores (Δ) between the candidate sire with the highest score and the candidate sire with the second highest score. Strict confidence levels were set at 95% and relaxed confidence levels at 80%. Where multiple candidate sires remained nonexcluded, likelihood was used to statistically distinguish candidate sires. Where progeny–sire matches did not meet confidence thresholds, but had LOD scores of ≥ 3.0 , paternity was assigned to the most likely sire (Slate *et al.*, 2000).

Paternity assignment was used to calculate the realised outcrossing rate, multiple paternity per capsule and per tree and realised pollen dispersal distances. Distributions of realised mating and potential mating under random pollination were tested using the Kolmogorov–Smirnov test and proportions tests for each 20 m increment, and overall. Distances between maternals and potential sires represent a theoretical distribution of random mating and were calculated by taking the mean distance between each maternal and all other plants, and then averaging those values for each incremental 20 m distance class. Thus, the proportion of potential mating events reflects the number of maternal/potential sire pairs that occur within each distance class. To standardise for population size and density, realised pollen dispersal was also plotted against ranked distance between maternals and all potential sires. Ranked distance was calculated by assigning plant pairs a value from 1 to 202, representing the nearest and most distant neighbour, respectively. The resulting distribution was tested for kurtosis (K) using the kurtosis function in R. Negative kurtosis indicates a flat data distribution (platykurtosis), positive kurtosis indicates a peaked distribution (leptokurtosis) and zero kurtosis indicates normal distribution. A simple linear regression analysis was applied in R to test whether the proportion of outcross mating declined linearly with ranked distance, following the approach of Krauss *et al.* (2009).

Genetic structure and stand differentiation

Matrices of genetic and geographic distances between all plant pairs were generated in GenAlEx V6.5 and their relationship tested using a Mantel test for Matrix Correspondence. Genetic distance was calculated using the pairwise, individual-by-individual genetic distance matrix for codominant data option in GenAlEx. In addition, the genetic correlation between individuals as a function of spatial distance was assessed using the spatial autocorrelation analysis. The analysis was performed using distance classes ranging from 5 to 50 m, and evenly spaced distance classes of 20 m selected for the final analysis as this distance was deemed suitable given the maximum distance between plants (380 m). A correlation coefficient (r , where 0 = random distribution of genotypes) was calculated for all pairs of individuals within each distance class, and means and errors plotted on a correlogram. To define significance limits,

95% confidence intervals of r were generated by randomising the data and by bootstrapping. Spatial autocorrelation analysis was performed using GenAlEx V6.5.

Genetic clustering of individual MLGs was assessed using the program STRUCTURE (Pritchard *et al.*, 2000). Although STRUCTURE has been commonly applied to describe genetic relationships among populations (Kalinowski, 2011), it is an individual-based analysis and is therefore suitable to test for the presence of genetic clustering within populations. A model assuming no prior groupings was selected, and different numbers of genetic clusters (K) tested, ranging from $K = 1$ to $K = 5$. The option of correlated allele frequencies was selected as this setting is recommended where either population structure, or the ability to detect it, is subtle (Falush *et al.*, 2003). A burn-in period of 1 00 000 was used, followed by 10^6 Markov chain Monte Carlo replicates, with 50 iterations for each K value. The number of K that best fit the data was selected by comparing ΔK values (Evanno *et al.*, 2005) generated using the Python program STRUCTURE Harvester (Earl, 2012). Population genetic structure indices including F_{ST} , G_{ST} , Nei's standardised and Hederick's standardised G'_{ST} , G''_{ST} and D_{EST} were calculated in GenAlEx for the optimal K clusters from STRUCTURE. To assess whether plants in different genetic clusters were randomly or nonrandomly distributed, maps showing the location of individuals with $\geq 60\%$ inferred membership to any one of the genetic clusters identified by STRUCTURE analysis were generated in R. A principle coordinates analysis based on a matrix of genetic distance of MLGs was conducted in GenAlEx as an independent assessment of genetic clustering.

RESULTS

Genetic diversity

Allelic diversity was low, with three loci having only two alleles (EGM25, EGM47 and EGM14), whereas seven out of nine loci had four or less alleles and two loci (EGM35 and EMBRA7) had seven and nine alleles, respectively (Table 1). The total number of alleles detected across all samples was 37. Observed and expected heterozygosity across loci ranged from 0.28 to 0.79 and from 0.32 to 0.78, respectively. The fixation index was not significantly different from zero (overall mean $F = 0.016$ ($\pm$ s.e. 0.032)). There was evidence for deviation from Hardy–Weinberg equilibrium in four loci, but there were no progeny–maternal mismatches, indicating that null alleles did not compromise the results of the paternity analysis. Allele frequencies did not differ markedly between the adult stand and sampled progeny, despite the sampling effect of only six maternals (Supplementary Figure S1). However, five alleles were present in greater frequencies in

Table 1 Genetic variation at each of nine microsatellite loci for unique multilocus genotypes of *Eucalyptus caesia* subsp. *caesia* in Boyagin Nature Reserve

Locus	n	N_a	N_e	H_o	H_E	F	P
EGM25	196	2	1.47	0.281	0.322	0.128	0.076
EGM30	203	4	2.5	0.675	0.599	–0.127	0.078
EGM47	202	2	1.97	0.520	0.494	–0.053	0.479
EGM14	203	2	1.65	0.335	0.395	0.152	0.033
EGM12	203	3	1.47	0.300	0.320	0.076	0.040
EGM34	202	4	1.87	0.510	0.464	–0.099	0.002
EGM35	200	7	4.09	0.715	0.756	0.054	< 0.001
EMBRA10	199	4	2.45	0.578	0.592	0.024	0.414
EMBRA7	202	9	4.49	0.787	0.777	–0.012	0.514
Mean	201 (± 0.8)	4.1 (± 0.8)	2.44 (± 0.4)	0.522 (± 0.062)	0.524 (± 0.057)	0.016 (± 0.032)	Chi ² : ∞ d.f.: 18 Prob: High. Sign.
Total	—	37	—	—	—	—	—

Values shown are sample size (n), number of alleles (N_a), effective number of alleles (N_e), observed heterozygosity (H_o), expected heterozygosity (H_E), fixation index (F) and P -value for Fischer's exact test for deviation from Hardy–Weinberg Equilibrium (P). The s.e. values are in parentheses.

the progeny, whereas three alleles were more common in the adults. In addition, some rare alleles present in the adults were absent in the progeny.

Mating system

At the population level, multilocus outcrossing rate estimate from MLTR was 0.832, the estimate of biparental inbreeding was 0.06 and the estimate of correlated paternity was 0.136 (Table 2). Multilocus outcrossing rate estimates for each family ranged from 0.479 to 0.806, and levels of biparental inbreeding were close to zero.

Paternity assignment

Out of 196 progeny that were genotyped, 19 were assigned paternity at a 95% confidence threshold, 32 at 80% confidence and a most likely sire identified for a further 130 progeny (Supplementary Figure S2). The remaining 15 progeny had multiple potential sires with equal LOD scores, or LOD scores of <3.0, and were excluded from all further analyses.

Realised outcrossing rates varied among the 6 families (0.64–0.87; Table 2). The mean number of different sires per fruit ranged from 3.5 ($\pm$ s.e. 0.3) to 6 ($\pm$ s.e. 1.0), whereas the number of different sires assigned paternity per tree ranged from 9 to 25. The total number of different sires for all 181 seed across six families was 64.

Pollen dispersal

Mating between trees occurred over relatively large distances, given the spatial scale of the study population (Figures 3 and 4). Of 130 outcross pollination events detected by paternity assignment, 74% occurred between trees >20 m apart, 28% between trees >60 m apart and 14% between trees >100 m apart (Figure 4a). A large proportion of outcross dispersal events (59 out of 130) occurred between trees 20 to 60 m apart. There were significant differences between observed and random mating events at 5 out of 11 distance classes (all P -values <0.01). Where significant differences occurred, the proportions of observed/random mating events were 0.26/0.03 at <20 m, 0.29/0.09 at 20–40 m, 0.03/0.14 at 80–100 m, 0.02/0.12 at 100–120 m and 0.03/0.19 at >200 m. Observed mating events were higher than random

mating expectations for plants <40 m apart, indicating leptokurtic dispersal at this scale. The mean realised pollen dispersal distance was 39.4 m ($\pm$ s.e. 3.9) considering all mating events, and 54.8 m ($\pm$ s.e. 4.9) considering outcrosses only. The mean distance between maternal plants and all other plants was 120.8 m ($\pm$ s.e. 2.5). The maximum realised pollen dispersal distance for paternity assignments made at the 95% confidence threshold, 80% confidence threshold and at the most likely level were 240, 295 and 236 m, respectively, whereas the distance between maternal plants and their most distant neighbours ranged from 339 to 355 m.

Considering all progeny–sire matches, 91% of outcross mating events occurred between nonnearest neighbours, and half of all realised outcross pollination events were to plants further away than the 27th nearest neighbour (Figure 4b). There was a significant negative correlation ($R = -0.349$, $P = 0.004$) between the proportion of outcross events and ranked distance. The proportion of mating events between nearest neighbours relative to nonnearest neighbours remained low when only progeny–sire matches made at 80 and 95% confidence thresholds were considered, and no significant relationship between these mating events and ranked distance was detected. Negative kurtosis values for both data sets ($K = -0.881$ and -3.11 , respectively) indicate platykurtic frequency distributions.

Genetic structure and stand differentiation

Genetic structuring of the 203 unique MLGs was evident within patches and across the entire stand. The Mantel test revealed an overall weak but positive relationship between genetic and geographic distance between pairs of plants ($R_{xy} = 0.222$; $P(R_{xy}\text{-rand} \geq R_{xy}\text{-data}) = 0.010$). Spatial autocorrelation analysis revealed a positive association between genetic correlation and geographic distance up to 70 m, after which no significant positive correlations were observed (Supplementary Figure S3). From STRUCTURE analysis, the number of genetic clusters that best fit the data were 3 ($\Delta K_3 = 150$ and <30 for all other K) (Figure 5 and Supplementary Figure S4). The estimated proportion of membership to each group was 35.3, 29, and 35.6%. There was strong evidence for admixture among groups and admixed individuals were geographically interspersed within the

Table 2 Mating system, multiple paternity and outcrossing rate for *Eucalyptus caesia* subsp. *caesia* at Boyagin Nature Reserve, Western Australia

Maternal tree ID	Number of capsules	MLTR estimates					Calculations based on CERVUS results				
		n	t_m	$t_m - t_s$	r_p	$1/r_p$	n	Number of different sires		Proportion outcrossed	
								Seeds	Mean seeds/cap.		
1	9	67	0.681 (± 0.064)	0.111	—	—	61	6.7 (± 1.0)	4.5 (± 0.8)	25	0.64
5	2	13	0.479 (± 0.315)	–0.008	—	—	12	6.0 (± 0.0)	5 (± 1.0)	9	0.67
10	6	35	0.806 (± 0.102)	–0.038	—	—	33	5.5 (± 1.9)	3.8 (± 1.2)	18	0.77
17	4	18	0.592 (± 0.156)	–0.032	—	—	16	4.0 (± 0.7)	3.5 (± 0.3)	10	0.87
101	5	51	0.729 (± 0.069)	0.076	—	—	46	9.2 (± 0.4)	6.0 (± 0.5)	24	0.67
114	2	14	0.709 (± 0.265)	–0.118	—	—	13	6.5 (± 0.5)	6.0 (± 1.0)	10	0.87
Pop./combined	28	196	0.832 (± 0.05)	0.060 (± 0.025)	0.136 (± 0.048)	7.3	181	6.5 (± 0.6)	4.6 (± 0.3)	64	0.72

Abbreviations: cap., capsule; pop., population; r_p , multilocus correlation of outcrossed paternity; $1/r_p$, effective number of mates; t_m , multilocus outcrossing rate; $t_m - t_s$, biparental inbreeding estimate.

MLTR variance estimates were calculated from 1000 bootstraps and are shown in parentheses. Multiple paternity values were calculated on the basis of paternity assignments made in CERVUS; s.e. values are shown in parentheses.

Figure 3 Distribution of outcross pollen donors for six *Eucalyptus caesia* subsp. *caesia* located on the summit of a granite bornhardt in Boyagin Nature Reserve, Western Australia (GDA 94, Zone 50). Axis values are UTM easting (x axis) and northing (y axis). Data are based on the results of a paternity assignment ($n=39$ (Tree 1), 8 (Tree 5), 26 (Tree 10), 14 (Tree 17), 31 (Tree 101) and 12 (Tree 114)) conducted in CERVUS.

stand. Estimates of genetic distance between these clusters were exceptionally high given the spatial scale of the study site of ~12 hectares (Table 3). The percentage of variation explained by the first three axes in the principal coordinates analysis were 12.19, 10.40 and 9.88%. Genetic clustering was evident in plots of principle coordinates 1 vs 2 and 1 vs 3 (Figure 5). The STRUCTURE clusters 1 and 3 were separated by principle

coordinate 3 and STRUCTURE cluster 2 separated from clusters 1 and 3 by principle coordinate 1.

DISCUSSION

Adaptations favouring frequent cross-pollination among plants may counteract or reduce the predicted genetic effects associated with inbreeding and genetic drift in small isolated populations (Ellstrand

Figure 4 Proportion of realised outcross mating events with distance, showing (a) observed and random outcross pollination events with geographic distance (m) between plant pairs and (b) proportion of mating events with ranked distance between plant pairs. Realised mating events are based on the results of a paternity assignment of seeds ($n=130$) collected from 6 *Eucalyptus caesia* subsp. *caesia* in Boyagin Nature Reserve, Western Australia. Potential mating events are the distances between maternal trees and all potential sires. *Significant differences ($P < 0.01$) between observed and potential mating events.

and Elam, 1993). *Eucalyptus caesia* has persisted for an extremely long period, possibly since the Pliocene, as naturally small, fragmented and genetically isolated populations (Moran and Hopper, 1983; Byrne and Hopper, 2008). As documented previously (Moran and Hopper, 1983) and in the present study, genetic diversity in *E. caesia* is extremely low compared with other eucalypts, suggesting genetic drift as a significant evolutionary force (Byrne and Hopper, 2008). Wide outcrossing and/or purging of deleterious mutations causing inbreeding depression, however, may buffer the negative impacts of genetic drift. Our application of molecular markers for the assignment of paternity in *E. caesia* has confirmed predictions of wide within-stand outcrossing, high multiple paternity and a departure from a leptokurtic pollen dispersal distribution that are, at least in part, a consequence of pollination by highly mobile nectar-feeding birds. Optimal foraging theory, although relevant to many plant species, may not always apply when birds make a substantial contribution to pollination. Aggressive interactions among highly mobile nectarivorous birds may frequently lead to a departure from optimal foraging and result in high pollen carryover (Mitchell *et al.*, 2013).

We suggest that pollination by highly mobile nectar-feeding birds increases the resilience of naturally fragmented and genetically insular plant populations by buffering genetic erosion. In this sense, our results extend the argument that predictions of genetic decline in recently fragmented populations are generally not supported by empirical evidence (Kramer *et al.*, 2008) to include historically fragmented populations such as the Boyagin stand of *E. caesia*. Resilience to recent fragmentation is expected to be greater for species with highly mobile pollinators as a result of increased genetic connectivity among isolated stands (Byrne *et al.*, 2007; Ismail *et al.*, 2012; Breed *et al.*, 2013). For example, in the bird-pollinated *Eucalyptus incrassata* regular long-distance pollen flow between fragments may mitigate the effects of genetic drift (Breed *et al.*, 2012). Adaptations favouring pollen dispersal by highly mobile animals may

be equally important in mitigating the loss of genetic diversity within stands.

Our results did not, however, support the predictions of weak spatial genetic structure in the adult stand expected from wide dispersal of pollen. Rather, we found evidence for moderate spatial genetic structure, with the stand comprising three distinct genetic clusters that displayed a marked degree of geographic overlap. We suggest that this spatial genetic structure reflects a life-history strategy of extreme individual plant longevity through clonality, combined with extremely rare large-scale recruitment events that, in mallee eucalypts, typically occur when environmental variables such as climate and soil moisture are favourable and coincide with disturbance such as fire (Wellington and Noble, 1985). With the exception of recent recruitment of *E. caesia* at the study site and at Chidaarcooping Nature Reserve following wildfires, on-ground surveys of all known stands show an absence of recruitment for at least 50 years, possibly much longer (SD Hopper, personal observation). Thus, the rate of generational change is expected to be extremely slow for natural populations of *E. caesia*. Ultimately, extreme longevity combined with the genetic consequences of wide outcrossing as a result of bird pollination are likely to be important for the persistence of genetic variation in small, geographically isolated stands of *E. caesia*. This conclusion is in accordance with the James Effect Hypothesis (Hopper, 2009; Hopper *et al.*, 2016) that predicts that mechanisms conserving heterozygosity will feature prominently in rare, localised endemics, as a result of prolonged genetic isolation.

Genetic consequences of multiple mating and wide pollen dispersal

Correlated paternity in *E. caesia* ($r_p = 0.136$) was markedly lower than reported in generalist-pollinated plants, such as *Eucalyptus marginata* (0.75; Millar *et al.*, 2000) or insect-pollinated *Acer macrophyllum* (0.39; Iddrisu and Ritland, 2004) and *Acacia melanoxylon* (0.40; Muona *et al.*, 1991), and fell within the generally low range of correlated paternity values reported for other bird-pollinated plants such as

Eucalyptus rameliana (0.26; Sampson, 1998), *E. incrassata* (0.16; Breed *et al.*, 2012) and *Banksia hookeriana* (0.037; Krauss *et al.*, 2009). In contrast, multiple paternity, although high, was lower than other

plants with bird pollination systems (Llaurens *et al.*, 2008; Krauss *et al.*, 2009). Although multiple paternity is a common feature of plants with significant genetic/evolutionary benefits (Jennions and

Figure 5 Genetic differentiation in a stand of *Eucalyptus caesia* subsp. *caesia* located in Boyagin Nature Reserve, Western Australia (GDA 94, Zone 50H), showing (a) assignment of individuals to genetic clusters based on maximum likelihood analysis in STRUCTURE, (b) geographic location of individuals with ≥60% inferred membership to one of three clusters and (c) principle coordinates analysis (PCoA) plots of axes 1 vs 2 and 1 vs 3 and the total variation explained.

Table 3 Genetic differentiation between inferred clusters in a stand of *Eucalyptus caesia* subsp. *caesia* located in Boyagin Nature Reserve, Western Australia

Cluster	F_{ST}	G_{ST}	$G'_{ST} N$	$G'_{ST} H$	G''_{ST}	D_{EST}
1 vs 2	0.054	0.050	0.095	0.163	0.203	0.119
1 vs 3	0.053	0.050	0.095	0.139	0.180	0.093
2 vs 3	0.059	0.056	0.105	0.161	0.205	0.111

Petrie, 2000; Pannell and Labouche, 2013), the mechanisms through which it arises can vary drastically across species. For example, in bird-pollinated eucalypts, low flowering intensity of individuals and low population density of flowering plants may restrict bird movement and lead to the use of the same pollen sources in independent pollination events (Sampson, 1998). Conversely, low flowering intensity of individual plants or highly asynchronous flowering of the population as a whole could also lead to reduced kinship by forcing pollinators to move more frequently between plants in order to satisfy nectar or pollen requirements (Phillips *et al.*, 2014).

Low correlated paternity and high multiple paternity in our study could reflect extensive pollen carryover by highly mobile bird pollinators (Mitchell *et al.*, 2013). Indeed, high sire diversity in the hummingbird-pollinated *Ipomopsis aggregata* is attributed to extensive pollen carryover, in contrast to the insect-pollinated *Mimulus ringens* with reduced sire diversity and restricted pollen carryover (Mitchell *et al.*, 2013). Here, it is important to note that multiple pollinator visits to *M. ringens* flowers increases the number of mates (Karron *et al.*, 2006). Future studies could thus expand the findings of Mitchell *et al.* (2013) by contrasting the influence of single and multiple visits by different pollinator groups on multiple paternity and sire diversity. Whether high sire diversity in *E. caesia* results from simultaneous or sequential polyandry, and the influence of different pollinator groups, requires experimental testing. High multiple paternity may also arise through density-dependent effects. For example, *Arabidopsis halleri* has exceptionally high multiple paternity (average 22.4 sires per 26.7 offspring per maternal) despite clonality and insect pollination, as a result of extremely high population density (Llaurens *et al.*, 2008). In contrast to the largely density-dependent polyandry observed in plants such as *A. halleri*, we suggest that multiple mating in *E. caesia* is coupled with a high diversity of sires because of frequent movement of honeyeaters between widely spaced plants. From an evolutionary perspective, multiple mating with a diverse array of plants may reduce pollen limitation (Knight *et al.*, 2005), promote pollen competition (Delph and Havens, 1998) and lead to the production of a more genetically variable progeny (Karron and Marshall, 1990) or progeny cohorts with increased survival rates (Breed *et al.*, 2014), potentially increasing the genetic resilience of small, isolated populations such as *E. caesia* at Boyagin Reserve.

The platykurtic distribution of pollen dispersal distances found in *E. caesia* is a departure from the typically leptokurtic dispersal distribution predicted for animal-pollinated plants (Harder and Barrett, 1996). In *E. caesia*, there was no significant difference between observed and random mating events between plants 40–80 and 120–200 m apart, indicating that the likelihood of mating was influenced by, but not strongly dependent on, distance for plants > 40 m apart. Conversely, there was a significantly higher proportion of observed to potential mating events between plants < 40 m apart, indicating a frequency of short-distance pollen dispersal in excess of random mating expectations. Realised outcrossing rates of 0.64–0.87 indicated that self-pollination is not uncommon in *E. caesia*. However, studies in other

eucalypts with mixed mating systems suggest post-germination selection against inbred progeny should remove most if not all selfed plants from the standing population (James and Kennington, 1993; Harder and Potts, 1997).

With respect to the frequent short-distance pollen dispersal we detected, our results differed with those of the honeyeater-pollinated *B. hookeriana*, where mating between near neighbours occurred less frequently and the overall pollen dispersal distribution was panmictic (Krauss *et al.*, 2009). This may reflect differences in the behaviour or composition of honeyeater species between sites because of variation in resource availability (Mac Nally *et al.*, 2005), or different spatial distributions of plants, or a greater contribution to pollination from insects. In eucalypt species such as *E. globulus*, pollination by insects may result in predominantly short-distance pollen dispersal, whereas flower visitation by birds or bats is more likely to explain longer dispersal events (Southerton *et al.*, 2004). We therefore predict that bird pollination, rather than insect pollination, is responsible for the long dispersal curve and high frequency of mating events between widely spaced plants in the Boyagin stand of *E. caesia*. Whether insects or birds or both groups are implicated frequent mating between plants < 40 m is uncertain and requires investigation.

The relative contribution of birds and insects such as the introduced honeybee to mating and pollen dispersal in *E. caesia* is unknown. Following their introduction to Australia ca. 150 years ago, introduced honeybees are now ubiquitous across the SWAFR, and are efficient pollinators of some plants but disruptive to others (Paton, 1993; Yates and Ladd, 2004). Our field observations of *E. caesia* indicate that although honeybees are frequent visitors to plants and consume substantial amounts of nectar, movement between plants is rare. Similarly, nearly 10 h of honeybee observations saw no movement between *Callistemon rugulosus* plants, whereas territorial New Holland Honeyeaters (*Phylidonyris novaehollandiae*) moved between plants on average 7.3 times per hour (Paton, 1993). These results may be biased because of the difficulty in following the movements of small insects, particularly considering that honeybees do forage over extensive areas (Visscher and Seeley, 1982). Honeybees often fail to contact the reproductive structures of bird-adapted plants, such as *E. stoatei* (Hopper and Moran, 1981) and *Banksia menziesii* (Ramsey, 1988), making their effectiveness as pollinators of *E. caesia* questionable, particularly as the flowers are large enough to permit access to nectaries without contacting the stigma. A pollinator exclusion experiment that allows insect access to flowers is required to resolve whether insects pollinate *E. caesia*, and whether short-distance pollen dispersal among *E. caesia* results predominantly from the activity of birds or insects. Studies that compare patterns of plant visitation and empirically test the extent of pollen carryover by insects and birds within a single plant species would be both novel and informative (Mitchell *et al.*, 2013).

Spatial genetic structure and geographic interspersal of genetic clusters

Given the wide realised pollen dispersal and small spatial extent of the study population, genetic differentiation of the three genetic clusters recognised by STRUCTURE was surprisingly high. Indeed, the genetic differentiation we observed falls within the range of values reported for geographically isolated populations, or across species' ranges, in the southwest region and that are attributed to isolation by distance effects (Bradbury *et al.*, 2013b; Nistelberger *et al.*, 2015). Alternatively, high population genetic differentiation may reflect colonisation or recruitment history (Jones *et al.*, 2006; Pardini and Hamrick, 2008). For example, in *Albizia julibrissin* (Fabaceae), different genetic groups were

spatially and temporally clustered and corresponded to episodes of recruitment (Pardini and Hamrick, 2008). In contrast, the three genetic clusters of *E. caesia* were interspersed rather than aggregated—a pattern that we suggest reflects gradual erosion of spatial genetic structure through extensive pollen dispersal and subsequent recruitment of widely outcrossed individuals, and a high survivorship of adult plants through lignotuber resprouting. On the other hand, weak spatial structuring of genetic groups could also reflect long-distance seed dispersal or the historical effect of a seed shadow (Jones *et al.*, 2006). Spatial autocorrelation analysis indicated genetic patch sizes of 70 m in the study population that may partially reflect greater seed dispersal distances than expected for eucalypts as seeds may be transported downslope by wind or water (Byrne and Hopper, 2008). Empirical tests of seed dispersal distances on granite rocks and canopy and soil-seedbank longevity of *E. caesia* are required.

Extreme longevity of individual plants, facilitated by asexual reproduction, is likely to influence spatial genetic structure in *E. caesia*. We found a mean of 7 stems per unique MLG, and the largest putative clonal patch comprised 55 stems over an area 8.8 m wide and 11.5 m long. Such patches of *E. caesia* may be thousands of years old and among the oldest known eucalypts (Smith *et al.*, 2003). The relative contribution of recruitment history, seed/pollen dispersal and extensive clonal reproduction on the fine-scale spatial genetic structure of *E. caesia* requires further study. Following a recent fire that has triggered seedling recruitment at the study site, an opportunity exists to test the hypothesis that geographic interspersal of genetic clusters results from recruitment of highly outcrossed individuals resulting from extensive pollen dispersal. This episode also presents an opportunity to assess the degree to which extensive asexual reproduction in between rare episodes of sexual reproduction contributes to accentuated persistence of *E. caesia*.

Conservation implications

Our study represents one of the most detailed analyses of mating and pollen dispersal in a bird-pollinated plant in a natural landscape. We extended the findings of recent studies in banksias (Krauss *et al.*, 2009; Ritchie and Krauss, 2012) by indicating that non-leptokurtic pollen dispersal and high multiple paternity, most likely as a result of extensive pollen dispersal by honeyeaters, may characterise bird-pollinated plants. However, disturbances such as habitat fragmentation may disrupt pollinator visitation, leading to reduced cross-pollination, predominantly near neighbour mating and high correlated paternity in bird-pollinated plants (Coates and Hamley, 1999; Llorens *et al.*, 2012; Coates *et al.*, 2013). The frequency of honeyeater movement among plants may vary with plant population size (Yates *et al.*, 2007; Phillips *et al.*, 2014), and pollinator behavioural characteristics may vary with a range of factors such as population shape and level of isolation (Llorens *et al.*, 2012). Overall, the results of our study suggest that genetic erosion leading to population decline is not necessarily inevitable in small, genetically isolated populations of bird-pollinated plants. Rather, wide pollen dispersal within stands by honeyeaters could maintain or even enhance genetic diversity within stands even when population diversity remains intrinsically low. Small populations of naturally or recently fragmented bird-pollinated plants—characteristics of many of the 40% of threatened species in the SWAFL that are pollinated by vertebrates—should be considered viable and remain a high conservation priority. Although wide outcrossing occurred in the study population, the very strong genetic differentiation evident among geographically isolated populations (Byrne and Hopper, 2008) indicates that the protection of each *E. caesia* population is necessary to retain species-level genetic diversity.

DATA ACCESSIBILITY

Sampling locations and microsatellite genotypes are available from the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.43rv3>.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

N Bezemer designed and performed the research, analysed the data and wrote the paper. SL Krauss, RD Phillips, DG Roberts and SD Hopper supervised the research, contributed to experimental design, provided advice on statistical and laboratory analyses and reviewed drafts of the paper.

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