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Pollen diversity matters: revealing the neglected effect of pollen diversity on fitness in fragmented landscapes

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Abstract

Few studies have documented the impacts of habitat fragmentation on plant mating patterns together with fitness. Yet, these processes require urgent attention to better understand the impact of contemporary landscape change on biodiversity and for guiding native plant genetic resource management. We examined these relationships using the predominantly insect-pollinated *Eucalyptus socialis*. Progeny were collected from trees located in three increasingly disturbed landscapes in southern Australia and were planted out in common garden experiments. We show that individual mating patterns were increasingly impacted by lower conspecific density caused by habitat fragmentation. We determined that reduced pollen diversity probably has effects over and above those of inbreeding on progeny fitness. This provides an alternative mechanistic explanation for the indirect density dependence often inferred between conspecific density and offspring fitness.

Keywords: density dependence, global change, plant genetic resources, plant mating systems, revegetation

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Introduction

Many tree species promote long-distance gene flow, particularly through the movement of pollen (Petit *et al.* 2005; Kremer *et al.* 2012). For this reason, tree populations tend to be buffered against the negative genetic effects of habitat disturbance (e.g. logging, clearing) commonly exhibited by other organisms (Vranckx *et al.* 2011). In this 'paradox of forest fragmentation genetics' (Kramer *et al.* 2008), high intrapopulation genetic diversity is usually maintained by trees over several generations, which can equate to 100s of years, even in seemingly challenging situations (Lowe *et al.* 2005; Kramer *et al.* 2008; Bacles & Jump 2011; Vranckx *et al.* 2011). However, within tree populations, reduced conspecific density following habitat disturbance is often observed to change individual mating patterns (e.g. inbreeding, pollen diversity) and, for animal-pollinated species, pollinator behaviour (Lowe *et al.* 2005; Dick *et al.* 2008; Eckert *et al.* 2010). These changes in mating patterns drive immediate gains or losses of genetic diversity and are expected to directly impact the fitness of future generations (Yasui 1998; Keller & Waller 2002; Lowe *et al.* 2005; Bacles & Jump 2011; Breed *et al.* 2012a). Impacts to mating patterns can be highly context-dependent (e.g. local vs. landscape-scale variation in spatial arrangement of the plants) and greatly affected by attributes of pollination vectors (Dick *et al.* 2008; Kramer *et al.* 2008; Bacles & Jump 2011; Breed

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et al. 2012b). Nevertheless, a pattern of inverse density dependence between offspring fitness and conspecific density is routinely inferred in studies of forest fragmentation, from the observation that trees in higher-density contexts tend to receive higher pollen diversity and exhibit lower levels of inbreeding, which is associated with higher offspring fitness (Courchamp *et al.* 1999; Breed *et al.* 2012a).

Elevated inbreeding generally imposes a fitness cost, known as inbreeding depression, due to the increased probability that phenotypes of deleterious recessive alleles are expressed (Crnokrak & Barrett 2002; Szulkin *et al.* 2010). As trees predominantly outcross (Petit & Hampe 2006), they can accumulate many deleterious recessive alleles, accruing a high genetic load (Crnokrak & Barrett 2002) and thus have great potential for expressing inbreeding depression. Inbreeding depression is more commonly expressed in more stressful environments (Fox & Reed 2010) and is likely to become more severe with an increase in environment-dependent stress caused by global change (Beaumont *et al.* 2011). With changes to inbreeding rates driven by habitat disturbance, statistical associations between heterozygosity at neutral genetic markers and fitness are expected. These heterozygosity-fitness correlations (HFCs) describe how variation in inbreeding associates with variation in fitness (Szulkin *et al.* 2010).

A factor that has received less attention than inbreeding is how lower effective tree density and altered pollinator behaviour may reduce pollen diversity received by plants as the number or diversity of pollen sources declines. The diversity of pollen detected in progeny arrays can be described indirectly by the commonly estimated mating system parameter, correlated paternity (r_p) (Ritland 2002) or through direct measures such as estimation of the number of half-sibships within progeny arrays (Berger-Wolf *et al.* 2007) or average relatedness within sibships (Lynch & Ritland 1999). Levels of correlated paternity and the numbers of half-sibships within progeny arrays are measured independently of selfing (Ritland 2002; Ashley *et al.* 2009) and should be mostly independent from other forms of inbreeding. Indeed, decreases in heterozygosity have been shown to have weak effect on correlated paternity (Breed *et al.* 2012a). Additionally, numbers of half-sibships within progeny arrays should only decrease when fewer unique paternal genotypes contribute to progeny arrays, regardless of their relatedness (Ashley *et al.* 2009).

Measures of pollen diversity are expected to correlate with fitness, as reduced pollen diversity results in a higher probability of unfit combinations of pollen and ovules (Skogsmyr & Lankinen 2002 and references therein). The mechanisms of this fitness effect are not mutually exclusive and include female choice for more compatible pollen (i.e. the acquisition of 'good genes') or a heterosis effect (Yasui 1998; Skogsmyr & Lankinen 2002). The fitness benefits of high pollen diversity are difficult to investigate independent of inbreeding avoidance because both processes can result in a positive heterosis effect (Skogsmyr & Lankinen 2002). However, higher levels of pollen diversity may dampen inbreeding depression by reducing the number of recessive deleterious alleles involved in reproduction (Armbruster & Gobeille 2004); thus, pollen diversity can have fitness effects independent of inbreeding.

While a great deal of theoretical and empirical work has been conducted on the effects of isolation on mating systems and pollination/gene flow dynamics (Ghazoul 2005; Lowe *et al.* 2005), few studies have investigated the downstream fitness costs to progeny. In this study, we co-analysed genetic diversity, mating patterns and progeny growth rates of open-pollinated progeny arrays of the predominantly insect-pollinated *Eucalyptus socialis* (red mallee) along a gradient of habitat disturbance, including low-density isolated trees, small remnant patches (medium density) and large intact woodland (high density). Groups of progeny arrays were sourced from mother trees located at two sites, Monarto and Yookamurra (Fig. 1). Within these sites, trees were sampled at three levels of density related to recent habitat disturbance in the Murray-Darling Basin of Australia (Bradshaw 2012). Within Monarto, we sampled trees at both low and medium density, related to their landscape context. Within Yookamurra, all sampled trees were from a large intact woodland with high *E. socialis* density. Monarto and Yookamurra were separated by 70 km of primarily agricultural land. By sampling both low- and medium-density landscape contexts at Monarto, we are controlling for population level effects that potentially influence tree responses to density (e.g. shifts in pollinator community).

In this study, we investigate how reduced density of *E. socialis* associates with selfing, biparental inbreeding and measures or pollen diversity. We predict that inbreeding would be greater and pollen diversity would be lower in lower-density contexts. Using progeny growth (over 15 months) as an indicator of progeny fitness, we then investigate how inbreeding and pollen diversity impact growth. We predict that greater inbreeding and lower pollen diversity should negatively impact on progeny fitness (Yasui 1998; Szulkin *et al.* 2010). While we found that inbreeding was correlated with reduced fitness in some cases, we found that reduced pollen diversity best explained variation in fitness. Consequently, pollen diversity may have effects over and above those of inbreeding on progeny fitness.

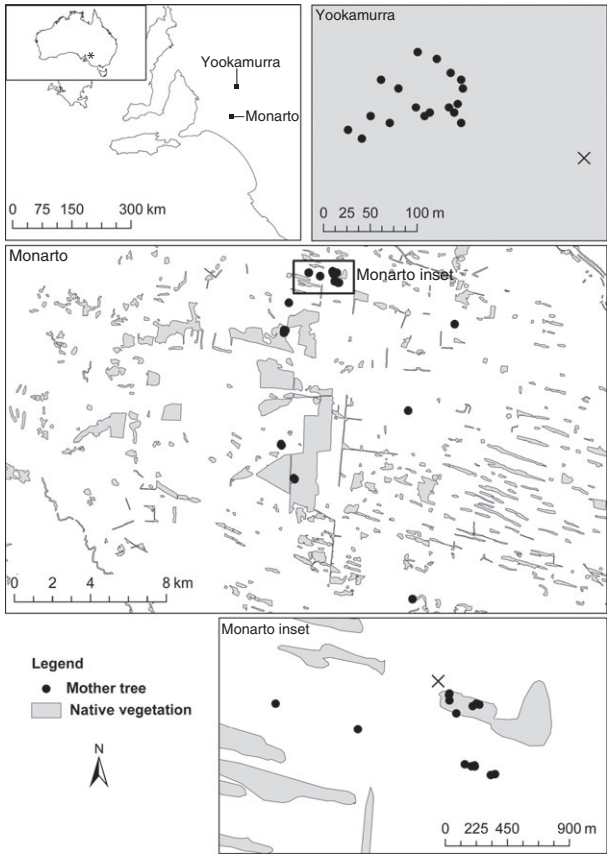

Fig. 1 Map showing location of *Eucalyptus socialis* mother trees (filled circles), with inset maps showing location of study sites (Monarto and Yookamurra) and details of the spatial arrangement of mother trees. Shaded areas represent native vegetation and all maternal plants sampled in shaded area were from the woodland contexts. The crosses (×) in the Yookamurra and Monarto inset maps indicate the location of the common garden experiments.

Furthermore, pollen diversity may provide an important additional mechanistic explanation for indirect density dependence between conspecific density and offspring growth across the disturbance gradient in this case.

Materials and Methods

Study species

Eucalyptus socialis is a sclerophyllous tree and one of many *Eucalyptus* species common throughout the deep sand and sand-over-limestone soils of the Murray-Darling Basin in southern Australia (Parsons 1969; Nicolle 1997). *Eucalyptus socialis* generally grows from 2 to 8 m high and possesses small hermaphroditic flowers pollinated primarily by small insects and, to a lesser degree, by birds and small marsupials (Slee *et al.* 2006). *Eucalyptus socialis* is likely to have a mixed mating system with preferential outcrossing based on observations of closely related eucalypts (Sampson & Byrne 2008; Mimura *et al.* 2009; Breed *et al.* 2012b). Although eucalypt flowers are protandrous (i.e. male reproductive phase precedes female phase within flowers), the development of flowers within and between inflorescences is sequential and gradual. Therefore, flowers in male or female phase may be in close proximity, allowing geitonogamous selfing to occur (i.e. pollination from another flower on the same plant; House 1997). Serotinous fruit (i.e. seeds released in response to an environmental trigger) are held over numerous years, with drying triggering seed release. Seeds are small (<2 mm diameter) and gravity-dispersed. Based on observations of the closely related and ecologically similar *E. incrassata* (Parsons 1969), it is likely that ants generally exhaust the soil seed bank, except during particularly heavy seed release, such as postfire (Wellington & Noble 1985a,b).

Seed collection and landscape contexts

Open-pollinated seeds were collected from mother trees selected from three landscape contexts from two sites in the Murray-Darling Basin (Fig. 1). Monarto mother trees (n = 29; Fig. 1) were within a single landscape and either isolated pasture trees (n = 13; low density) or from small remnant woodlands (n = 16; medium density). Isolated pasture trees were in very small clusters (often single trees) of vegetation either in agricultural land or between public roads and agricultural land. Small remnant woodlands were natural habitats surrounded by agricultural land. Yookamurra mother trees (n = 18; high density; Fig. 1) were from a large intact woodland with no history of known anthropogenic disturbance. In all cases, care was taken to avoid sampling near neighbours (see Appendix A for a pairwise distance matrix between maternal plants, Supporting information).

Population level effects may derive from divergence in environmental (e.g. pollinator community, rainfall), genetic (e.g. mating system) and phenotypic (e.g. flowering time) traits between these sites. However, divergence in *E. socialis* environmental traits is expected to be low between Monarto and Yookamurra because only small environmental differences are present between sites (including abiotic and biotic factors; summarized in Appendix B, Supporting information), which should result in only weak divergent selection (Coyne & Orr 2004). Additionally, *E. socialis* gene flow across the region is expected to be high (Petit & Hampe 2006; Breed *et al.* 2012b; results presented below), reducing the effectiveness and efficiency of selection (Lenormand 2002). However, as we cannot rule out possible divergence in reproductive traits or important unmeasured differences between sites, we primarily focus our

comparisons within Monarto (trees in low- vs. medium-density landscape contexts) as trees from this site were all from the same landscape (maximum distance between trees <12 km). We then extend our comparison to include trees from Yookamurra, acknowledging that it is possible that differences may be present.

Density of large intact and small remnant woodlands was estimated by counting conspecifics in seven replicates of 30 × 10 m transects, then extrapolating this to trees per hectare. We estimated density of isolated pasture trees by counting the number of conspecifics within a 30-m radius of each tree (i.e. nearest-neighbours), then extrapolating this to trees per hectare. The 30-m radius was a manageable distance given the reasonably evenly spaced distribution of the isolated pasture trees. At Monarto, small remnant woodlands had higher density than isolated pasture trees (small remnant woodlands = mean 11.43 ± SE 0.48 trees per ha; isolated pasture trees = mean 2.16 ± SE 0.83 trees per ha; Table 1). The large intact woodland at Yookamurra was characterized by higher *E. socialis* density than both groups of Monarto landscape context trees (large intact woodland = mean 23.33 ± SE 0.76 trees per ha).

Table 1 Genetic variability of *Eucalyptus socialis* samples from three landscape contexts in the Murray-Darling Basin, Australia. Progeny array sizes and growth data are also reported (*n*, number of samples; AR, rarefied allelic richness; H_E and H_O, unbiased expected and observed heterozygosity, respectively; *F*, fixation index; progeny array, mean number of offspring per progeny array; growth, mean height of progeny; standard deviations in parentheses)

Study group	Yookamurra large intact woodland	Monarto small remnant woodlands	Monarto isolated pasture trees
Mother trees			
n	18	16	13
Density (trees per ha)	23.33 (0.76)*	11.43 (0.48)*	2.16 (0.83)*
AR	5.19 (1.39)	4.95 (1.45)	5.01 (1.54)
H_E	0.80 (0.19)	0.78 (0.22)	0.80 (0.16)
H_O	0.81 (0.16)	0.76 (0.17)	0.80 (0.19)
F	−0.06 (0.22)	−0.04 (0.20)	−0.06 (0.19)
Progeny			
n	197	197	175
Progeny array size	10.94 (3.24)	12.31 (5.45)	13.46 (4.59)
AR	4.69 (1.40)	4.75 (1.33)	4.72 (1.46)
H_E	0.75 (0.21)	0.76 (0.20)	0.75 (0.23)
H_O	0.66 (0.22)	0.60 (0.23)	0.55 (0.23)
Growth (cm)	56.28 (18.36)	37.91 (15.92)	36.06 (14.28)

*Standard errors reported.

Common garden experiment

Twenty replicates of approximately 20 seeds from each mother tree were sown on February 1, 2010, under semi-controlled glasshouse conditions in Adelaide, South Australia (S34°55′05″, E138°36′18″). All progeny were moved to full sun at Mt. Lofty Botanic Gardens, South Australia (S34°59′03″, E138°43′08″), after 4 weeks in glasshouse conditions. Seedling crates were shifted and rotated approximately weekly to avoid confounding effects of location in glasshouse/nursery. Thinning to a single central progeny was performed by pulling out subsidiary progeny over the subsequent weeks prior to planting. Glasshouse and nursery environments may allow inferior seedlings to survive when compared to seedling survival under natural woodland conditions. However, this bias should be consistent across density groups and progeny arrays, and under glasshouse/nursery environments, we are controlling for additional biases (e.g. competition, demographic or environmental stochastic effects).

Growth of progeny was assessed by common garden experiments located at Monarto (Monarto $n_{\text{small remnant woodlands}} = 197$ progeny; $n_{\text{isolated pasture}} = 175$ progeny; common garden location shown in Fig. 1 inset A) and Yookamurra (Yookamurra $n_{\text{large intact woodland}} = 197$ progeny). All progeny were planted 4 months postgermination. We implemented a randomized complete block design with families replicated within each block and rows representing a single block. Common garden experiment plots were located in close proximity to mother trees to avoid potential confounding population effects (e.g. local adaptation; Fig. 1). The fitness proxy observed during trials was aboveground stem height (distance from ground to distal stem). Observing growth should allow an examination of differences that probably affect growth in later life (beyond maternal effects). However, using aboveground stem height as a fitness proxy has limitations, as established seedlings may have experienced strong selection at pre- or early postzygotic stages, and therefore, cryptic early-acting inbreeding depression may have acted on seedlings observed in this study. In this study, germination rates were not observed to be different across groups, but detailed data were not available for further analysis. Additionally, our methods did not assess belowground growth, which may influence the observed patterns.

Microsatellite genotyping

Leaf tissue was collected from maternal trees in the field and from each progeny prior to planting and DNA was extracted using the Machery-Nagel Nucleospin Plant II Kit at the Australian Genome Research

Facility (AGRF, Adelaide, Australia). Eight direct-labelled microsatellite markers were selected from the set of EST-derived markers by Faria *et al.* (2010; EMBRA1382; EMBRA2002; EMBRA914; EMBRA1990; EMBRA1284; EMBRA1928; EMBRA1468; EMBRA1363a, b). A BLAST search was performed for each microsatellite sequence using accession numbers in Faria *et al.* (2010), resulting in no significant hits with genes of known function. EMBRA1363 produced two unlinked and scoreable PCR products that we treated as separate loci. PCR was performed in a single 10-μL multiplex PCR containing 1 μL template DNA (*ca.* 20 ng/μL), 5 μL 2 × Qiagen Multiplex PCR Master Mix (Qiagen, Hilden, Germany), 3 μL of nuclease-free water, 1 μL of primer mix with each primer at 2 μM concentration. Standard Qiagen Multiplex PCR conditions were used with an initial activation step at 95 °C for 15 min, 40 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 90 s and extension at 60 °C for 60 s, with final extension at 60 °C for 30 min. LIZ500 size standard was added to samples, and fragments were separated on an AB3730 genetic analyser with a 36-cm capillary array (Applied Biosystems, Foster City, MA, USA) at AGRF. Alleles were sized using GENEMAPPER software (Applied Biosystems) and double-checked manually.

Data analysis

Each mother tree was presumed to reflect preclearance dynamics as all trees sampled were estimated to be >80 years old (Clarke *et al.* 2010; Vranckx *et al.* 2011; most land clearance occurred <80 years ago; Bradshaw 2012). Maternal genotypes (derived from maternal leaf tissue) were used to test for null alleles in MICRO-CHECKER (Oosterhout *et al.* 2004). GENEPOP on the web (http://genepop.curtin.edu.au) was used for tests for heterozygote deficit/excess and linkage disequilibrium, applying sequential Bonferroni correction for multiple testing where appropriate. Additionally, the per-locus probability of paternity exclusion (Q) and combined probability of paternity exclusion (QC) were estimated in GENALEX (Peakall & Smouse 2006). Pairwise population genetic differentiation parameters G_{ST_est} (Nei & Chesser 1983) and D_{est} (Jost 2008) were estimated in SMOGD (Crawford 2010).

Genetic diversity. We estimated the following genetic diversity parameters for mother tree and progeny groups using GENALEX: number of alleles (A), Nei's unbiased expected and observed heterozygosity (H_E and H_O, respectively; Nei 1973). In addition, the fixation index (F) was estimated for all mother tree groups. To account for differences in sample size, we estimated the rarefied mean number of alleles per locus (AR) using HP-RARE (Kalinowski 2005). We estimated individual observed progeny multilocus heterozygosity (H_i) and scaled the measure to between 0 and 1 by $H_i = \Sigma h_{ij}/n_i$, where h is a heterozygote for the ith individual at the jth locus for n successfully genotyped loci. We calculated family heterozygosity as $H_f = \Sigma H_{fi}/n_f$, where H_{fi} is the progeny multilocus heterozygosity for the ith individual in the fth family of sample size n. All samples that failed amplification at more than five loci were excluded ($n = 44$) (see Appendix C for more details on missing genotype data, Supporting information).

Mating system. We estimated the following mating system parameters from the progeny array genotypes in MLTR (Ritland 2002): multilocus outcrossing rate (t_m), biparental inbreeding (difference between the single-locus and multilocus estimates of outcrossing rate; $t_m - t_s$), proportion of effective selfing rate that results from true uniparental selfing (correlation of selfing among loci, r_s, where $1 - r_s$ = proportion of effective selfing rate that results from biparental inbreeding) and multilocus correlated paternity (r_p), the proportion of progeny that are full-sibs. Families were bootstrapped 1000 times to calculate variance estimates for each parameter. Family-level mating system parameters were estimated in the same way except that individuals within families were bootstrapped 1000 times to calculate variance estimates. Additionally, we estimated the probability that each progeny was the product of an outcross event using individual analysis in MLTR.

To investigate the role of the diversity of pollen donors in more detail, we estimated the relatedness of outcrossed progeny within families (r_o) using the Lynch & Ritland (1999) method in GENALEX. Additionally, the number of half-sibships within progeny arrays (k) was estimated in KINALYZER (Berger-Wolf *et al.* 2007; Ashley *et al.* 2009) using the 2-allele algorithm. These family-level parameters were included in the statistical analyses outlined below.

Mating system parameters derived for families rather than groups of families (e.g. the mothers in each landscape/density context) are expected to have increased variance around means due to reduced sample sizes. However, using this approach, it is possible to derive statistical relationships between individual mating system parameters and fitness, rather than relying on *post hoc* comparisons of mating system values and fitness means. As these family-level estimates of mating system parameters have higher levels of variance than mating system parameters estimated for groups of families, we bootstrapped the regression slopes of the family-level analyses 10 000 times in R v. 2.15.0 (R Development Core Team 2012). We only bootstrapped regression slopes for models that were either the best fitting model

or had $\Delta AIC_c < 4$ and ranked above the null model. Family-level mating system parameter variance estimates from MLTR (Ritland 2002) are presented in Appendix D in the Supporting information. No variance estimates were calculated for relatedness of outcrossed progeny within families (r_o) or the number of half-sibships within progeny arrays scaled to progeny array size (k_n). Mating system parameter estimates for groups of families are presented in Fig. 2.

Heterozygosity-fitness correlations (HFCs). We used Gaussian general linear models and Gaussian mixed-effect models in a maximum likelihood, multi-model inference framework in R v. 2.12.1 (Burnham & Andersen 2002; R Development Core Team 2012) to analyse relationships among genetic predictors and growth for *E. socialis* progeny grown in common garden experiments. GLMs were performed using the glm function with family = Gaussian in the stats package. Mixed-effects models were performed in the lme4 package (Bates & Maechler 2010; Appendix E, Supporting information). We used Akaike's Information Criterion corrected for small sample sizes (AIC_c) for model selection (Burnham & Andersen 2002).

We investigated HFCs for each mother tree group following Szulkin *et al.* (2010). We tested for relationships between mean individual observed heterozygosity (H_i) and growth. Following Zuur *et al.* (2009), we began by evaluating the need for including family or block (i.e. planting row) as random effects, but we found little support for either random effect (see Appendix E for more details on mixed-effect model methods, Supporting information). Thus, we used general linear models to test for the effect of individual heterozygosity on growth. All fitted models met the assumptions of Gaussian linear models (Crawley 2007). We estimated the slope of the growth ~ H_i relationship ($\beta_{\omega i,Hi}$) and the variance explained ($r^2_{\omega i,Hi}$). However, as a correlation between heterozygosity and fitness does not indicate how much variation is explained by inbreeding, the inbreeding load ($\beta_{\omega i,f}$) and variance in fitness explained by inbreeding ($r^2_{\omega i,f}$) were also estimated following Szulkin *et al.* (2010). HFCs rely on a correlation between observed heterozygosity at the genotyped markers and heterozygosity at functional loci (i.e. correlation due to identity disequilibrium), and as such, the interlocus heterozygosity correlation (g_2) for mother tree groups was estimated in RMES (David *et al.* 2007), where a significant correlation indicates the presence of identity disequilibrium. HFCs also rely on variation in inbreeding, and as such, the inbreeding estimate (f) was derived from the selfing rate (s) where $s = 1 - t_m$, and then $f = s/(2 - s)$ (David *et al.* 2007).

For all mother tree families, we used GLMs to test for hypothesized relationships between growth and five genetic predictors: multilocus outcrossing rate (t_m), biparental inbreeding ($t_m - t_s$), correlated paternity (r_p), outcrossed progeny relatedness (r_o) and the number of half-sibships (k) within progeny arrays scaled to progeny array size ($k_n = k/n$). Block was not included in these models because there was no support for including block in individual-level analyses of growth for any group (see Appendix E, Supporting information).

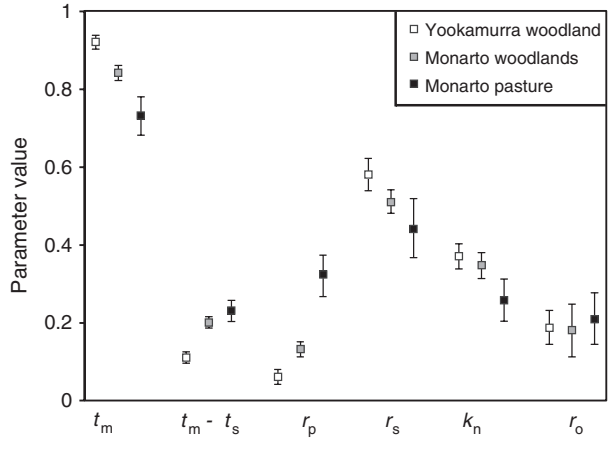

Fig. 2 Mating pattern estimates for groups of *Eucalyptus socialis* plants from three landscape contexts in the Murray-Darling Basin of Australia. Samples were from a large woodland context (open squares), small remnant woodland context (grey squares) and isolated pasture trees (black squares) (t_m, multilocus outcrossing rate; $t_m - t_s$, difference between the singlelocus and multilocus estimates of outcrossing rate; r_s, correlation of selfing among loci; r_p, multilocus correlation of outcrossed paternity; k_n, the number of half-sibships within progeny arrays scaled to progeny array size; r_o, outcrossed progeny relatedness; error bars show 95% confidence intervals).

Results

Genetic marker quality

We genotyped open-pollinated progeny from 16 mother trees from small remnant woodlands (n = 197) and 13 isolated pasture mother trees (n = 175) located at Monarto (progeny array size data reported in Table 1). Additionally, we genotyped open-pollinated progeny from 18 mother trees from a large intact woodland (n = 197) located at Yookamurra. A total of 112 different alleles were identified across all mother trees (summary of per-locus data in Appendix F in the Supporting information). The combined probability of paternity exclusion if neither parent is known indicates good resolution for the genetic markers used (QC = 1.00; Appendix E, Supporting information). No significant excesses or deficits of heterozygotes were observed in

the groups of mother trees (Table 1, Appendix E, Supporting information), and we found no significant null alleles at any loci within these groups. No significant linkage disequilibrium was observed between pairs of loci scored in mother trees after adjustment for multiple testing.

Genetic diversity and population differentiation

There were no significant differences in allelic richness and expected heterozygosity between progeny and mother trees in each group (all *t*-test *P*-values > 0.05; Table 1). However, progeny were significantly more homozygous than mother trees, particularly progeny from isolated pasture trees (*t*-test: isolated pasture trees $t = 3.27$, d.f. = 16, *P*-value < 0.01; small remnant woodlands $t = 2.33$, d.f. = 16, *P*-value < 0.05; large intact woodland $t = 2.30$, d.f. = 16, *P*-value < 0.05; Table 1). Genetic differentiation between mother tree groups was very weak and not significant (all genetic differentiation values < 0.05; all *P*-values > 0.05; see Appendix G in Supporting information for more details).

Mating patterns and growth

Eucalyptus socialis was primarily outcrossed, but a significant shift towards mixed mating occurred in lower-density landscape contexts (Fig. 2). Within Monarto, isolated pasture trees expressed significantly higher correlated paternity and selfing than small remnant woodlands. This trend continued in the large continuous woodland (i.e. Yookamurra) where trees experienced significantly less selfing and correlated paternity than Monarto remnant woodland and pasture tree groups. The large continuous woodland experienced significantly less biparental inbreeding than Monarto groups (measured by $t_m - t_s$), and maternal plants at this site experienced less effective inbreeding due to biparental inbreeding (measured by $1 - r_s$). Fewer half-sib groups were present in the isolated pasture trees families than both the large intact woodland and the remnant woodland, but there was no significant difference in relatedness amongst outcrossed progeny across mother tree groups (r_o).

Progeny from the large intact woodland grew significantly taller than progeny from both small remnant woodlands and isolated pasture trees (one-way ANOVA: $F = 54.42$, d.f. = 2, *P*-value < 0.01; Table 1).

When analysed across families from all mother tree groups, correlated paternity and the number of half-sibships within progeny arrays scaled to progeny array size had the strongest effects and explained most variation in growth (r_p had a negative effect on growth: per cent deviance explained = 16.6%; k_n had a positive effect on growth: per cent deviance explained = 15.2%; ΔAIC_c between top two models = 0.78; ΔAIC_c to next best model = 5.13; ΔAIC_c to null model = 6.05; Table 2, Fig. 3). We were unable to directly include the variance estimates of the response variables in the GLM analyses, but we are confident that the trends are significant as the 2.5% and 97.5% bootstrapped percentiles did not overlap zero (Crawley 2007). The difference between the singlelocus and multilocus estimates of outcrossing rate (i.e. biparental inbreeding) and outcrossed progeny relatedness both had negative effects on growth, but their effects were much weaker than correlated paternity and the number of half-sibships within progeny arrays scaled to progeny array size ($t_m - t_s$ and r_o: per cent deviance explained = 6.7%; ΔAIC_c to best fitting model = 5.13). Outcrossing rates and the correlation of selfing among loci did not associate with growth (t_m and r_s: both explained <0.5% per cent deviance and both were ranked lower than the null model).

Correlations between heterozygosity and growth

Heterozygosity exhibited a positive relationship with growth in both small remnant woodlands and isolated pasture trees (Tables 2 and 3), but not in the large intact woodland. An interlocus correlation of heterozygosity (i.e. identity disequilibrium as measured by g_2) was significant in both small remnant woodlands and isolated pasture trees, but not the large intact woodland group. Consequently, the relationship between inbreeding and fitness (using progeny growth as the quantified variable; $r^2_{\omega i,f}$) and inbreeding load ($\beta_{\omega i,f}$) was only estimated for small remnant woodlands and isolated pasture trees (Monarto site). The inbreeding load in small remnant woodlands translated to an average change in growth after one generation of selfing (i.e. $f = 1/2$) of -35.11 cm $\times$ $1/2 = -17.56$ cm. After one generation of selfing in isolated pasture trees, the average change in growth was estimated at -8.12 cm. However, the inbreeding load values for both small remnant woodlands and isolated pasture trees overlapped when $g_2 \pm 2$ standard deviations was used (g_2 SD = 0.014 and 0.023, respectively; Table 3).

Discussion

We demonstrate that the mating patterns of *Eucalyptus socialis* were increasingly impacted by reduced conspecific density associated with greater habitat disturbance. Most importantly, we found that reduced pollen diversity was the effect that best explained variation in seedling growth and probably has effects over and above those of inbreeding. Thus, in this case, an alternative

Table 2 General linear model comparisons of relationships between genetic predictors and growth of *Eucalyptus socialis*. We combined data from all groups to investigate family-level trends

Model	% DE	*w*AIC	ΔAIC_c	*k*	ß (2.5 and 97.5% percentiles)
Family-level					
Growth ~ r_p	16.59	0.53	0	3	−18.36 (−32.81 to −4.75)
Growth ~ k_n	15.17	0.36	0.78	3	46.40 (88.48 to 2.83)
Growth ~ $t_m - t_s$	6.75	0.04	5.13	3	
Growth ~ r_o	6.74	0.04	5.13	3	
Growth ~ 1	0	0.03	6.05	2	
Growth ~ t_m	0.25	0.01	8.23	3	
Growth ~ r_s	0.04	0.01	8.33	3	
Individual-level					
Growth $_{\text{yookamurra large woodland}}$ ~ 1	0.0	0.8	0.0	3	
Growth $_{\text{yookamurra large woodland}}$ ~ H_i	0.1	0.3	2.2	2	
Growth $_{\text{Monarto small woodlands}}$ ~ H_i	8.4	1.0	0.0	3	24.52 (37.86 to 10.83)
Growth $_{\text{Monarto small woodlands}}$ ~ 1	0.0	0.0	12.1	2	
Growth $_{\text{Monarto isolated pasture trees}}$ ~ H_i	6.0	1.0	0.0	3	16.27 (26.47 to 6.02)
Growth $_{\text{Monarto isolated pasture trees}}$ ~ 1	0.0	0.0	6.9	2	

We kept mother tree groups separate for individual-level analyses (% DE, per cent deviance explained by model; *w*AIC, weight showing relative likelihood of model *i*; ΔAIC_c, indicator of differences between model AIC_c and minimum AIC_c in the model set, respectively; *k*, number of parameters; ß, unstandardized regression slope with 2.5 and 97.5% bootstrapped percentiles in parentheses in models that were either the best fitting model or had $\Delta AIC_c < 4$ and ranked above the null model; t_m, outcrossing rate; $t_m - t_s$, biparental inbreeding; r_p, correlated paternity; k_n, the number of half-sibships within progeny arrays scaled to progeny array size; r_o, outcrossed progeny relatedness; H_i progeny individual heterozygosity; 1, null model).

mechanistic explanation for the indirect density dependence, often inferred between conspecific density and offspring fitness, appears to be acting.

Habitat disturbance and density effects on mating system and genetic diversity

The insect-pollinated *E. socialis* population studied here had a mixed mating system with outcrossing rates ranging from 73% to 92% across mother tree groups. This degree of outcrossing is comparable with other studies on eucalypts, where 60–95% outcrossing has generally been observed (Sampson & Byrne 2008; Mimura *et al.* 2009; Breed *et al.* 2012b). We show that decreasing conspecific density, caused by increasing habitat disturbance, had an increasingly negative effect on *E. socialis* mating patterns. We observed this trend for progeny arrays from maternal plants located at different densities within the same site (Monarto site, low vs. medium density; i.e. controlling for population differences in pollinator communities or mating system traits) and continued when we extended our comparison to the relatively undisturbed (Bradshaw 2012) and environmentally similar high-density woodland site at Yookamurra. Overall, these trends support the commonly found positive relationship between conspecific density and greater outcrossing (Lowe *et al.* 2005; Eckert *et al.* 2010).

The observed trend of more disrupted mating patterns with increasing habitat disturbance is probably explained by fewer opportunities for outcrossing because of vegetation loss in surrounding landscapes and alterations to pollinator behaviour in response to reduced tree density (Fig. 1). Reduced tree density is expected to result in pollinators spending more time at individual trees due to the relative cost of moving between canopies (Ottewell *et al.* 2009), leading to increased geitonogamous selfing (i.e. pollen fertilizes ovules in the same canopy but on different inflorescences). Geitonogamous selfing is possible as *E. socialis* flowers are protandrous but may differ in male–female phase within a single canopy (House 1997). Furthermore, with reduced tree density, pollinators are more likely to restrict their foraging to neighbouring trees, probably leading to reduced pollen donor diversity and increased correlated paternity observed in more isolated trees (Ottewell *et al.* 2009). It is also likely that *E. socialis* populations show fine-scale spatial genetic structure similar to other eucalypts (e.g. ecologically similar and bird-pollinated *E. incrassata* sp-statistic = 0.005; Breed *et al.* 2012b; primarily insect-pollinated *E. globulus* Mantel's $r = 0.133$; Jones *et al.* 2007; primarily insect-pollinated *E. aggregata* and *E. rubrida* significant spatial autocorrelation at short distances; Field *et al.* 2011). Under this assumption, lower conspecific density should also lead to an increase in biparental inbreeding,

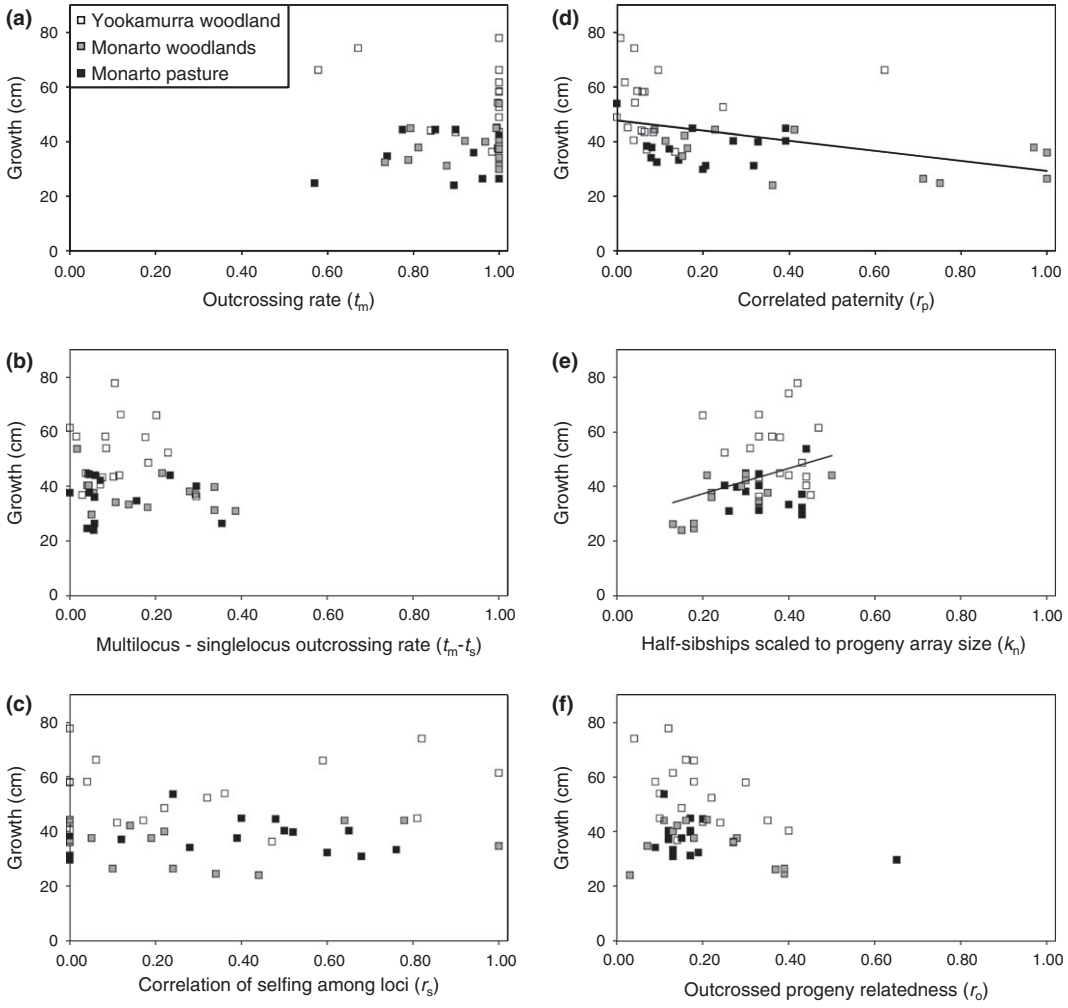

Fig. 3 Scatterplots showing relationships between growth and family-level genetic parameters, where (a) shows outcrossing rate, (b) difference between the singlelocus and multilocus estimates of outcrossing rate, (c) correlation of selfing among loci, (d) correlated paternity, (e) half-sibships scaled to progeny array size and (f) outcrossed progeny relatedness from large woodland (open squares), small remnant woodlands (grey squares) and isolated pasture trees (black squares). Growth is shown on the *y*-axis and genetic parameter values shown on the *x*-axis. Linear trend lines between genetic parameters and growth shown for relationships where $\Delta AIC_c < 4$ (ΔAIC_c values presented in Table 2).

Table 3 Heterozygosity–fitness correlation comparisons for mother tree groups of progeny following methods presented by Szulkin *et al*. (2010; *H* mean individual heterozygosity with variance in parentheses; *f*, inbreeding estimate derived from the MLTR selfing rate where $f = s/(2 - s)$; g_2, interlocus heterozygosity correlation inferred from RMES (David *et al*. 2007) with standard deviations in parentheses; $r^2_{\omega i,Hi}$, the variation in fitness explained by heterozygosity with values ± 2 g_2 standard deviations in parentheses; $\beta_{\omega i,Hi}$, regression slope of fitness–heterozygosity regression with values ± 2 g_2 standard deviations in parentheses; $r^2_{\omega i,f}$, variation in fitness explained by inbreeding; $\beta_{\omega i,f}$, regression slope of fitness–inbreeding, the inbreeding load)

Mother tree group	*H*	*f*	$r^2_{\omega i,Hi}$	$\beta_{\omega i,Hi}$	g_2	$r^2_{\omega i,f}$	$\beta_{\omega i,f}$
Yookamurra large intact woodland	0.66 (0.04)	0.04	<0.01	−2.11*	0.01†		
Monarto small remnant woodlands	0.60 (0.04)	0.09	0.08	24.52*	0.05 (0.023)‡	0.18 (0.12 to 0.42)	−35.11 (−79.24 to −22.54)*
Monarto isolated pasture trees	0.55 (0.05)	0.16	0.06	16.27*	0.11 (0.014)‡	0.09 (0.07 to 0.16)	−16.31 (−28.53 to −11.42)*

*In units of plant growth (cm).
†No significant interlocus heterozygosity correlation with *P*-value = 0.144.
‡Significant interlocus heterozygosity correlation with *P*-value < 0.001.

as pollinators would probably restrict their foraging to near neighbours, increasing the amount of fertilizing pollen from closely related individuals (Zhao *et al.* 2009; Dubreuil *et al.* 2010). However, we did not estimate fine-scale spatial genetic structure here due to insufficient sampling of adults ($n = 47$; Cavers *et al.* 2005) although this information is relevant when considering the patterns of mating between related individuals.

The mating system responses to reduced conspecific density observed here were much more pronounced than those found in closely related *E. incrassata* (Breed *et al.* 2012b; *E. incrassata* low density = 1.70 trees per ha, $t_m = 0.94$, $t_m - t_s = 0.16$, $r_p = 0.16$; *E. incrassata* high density = 12.62 trees per ha, $t_m = 0.94$, $t_m - t_s = 0.19$, $r_p = 0.18$; *E. socialis* density and mating pattern data reported in Table 1, Fig. 2). Unlike *E. socialis*, *E. incrassata* is primarily pollinated by birds, which are potentially more resilient to changes in tree density than the insects that pollinate *E. socialis* (Dick *et al.* 2008; Bacles & Jump 2011). A study of the more temperate, but similarly insect-pollinated, *E. globulus* reported mating system responses to altered density comparable to those reported here (*E. globulus* high density = 340–728 trees per ha, $t_m = 0.89$–0.86, $t_m - t_s = 0.04$–0.06, $r_p = 0.03$–0.06; low density = 3.3–3.6 trees per ha, $t_m = 0.65$–0.79, $t_m - t_s = 0.04$–0.06, $r_p = 0.12$–0.20; Mimura *et al.* 2009).

Unlike the observed heterozygosity decline in the progeny groups, which probably occurred due to inbreeding, we reported no change in allele-based genetic diversity metrics (allelic richness, expected heterozygosity) between progeny and adults in any landscape context. These findings support the 'paradox of forest fragmentation genetics' (Lowe *et al.* 2005; Kramer *et al.* 2008; Bacles & Jump 2011) where, in general, tree populations only experience reduced allelic diversity where multiple generations have passed since fragmentation (Lowe *et al.* 2005; Finger *et al.* 2011; Vranckx *et al.* 2011). Additionally, the overlapping generations of many tree populations result in large genetic inertia, which further dampens the effect of random genetic drift. However, as observed here and reported previously (Lowe *et al.* 2005; Eckert *et al.* 2010; Breed *et al.* 2012a; Finger *et al.* 2012), changes to tree inbreeding and pollen diversity can provide mechanisms that immediately reduce genetic diversity, via decreasing observed heterozygosity.

Mating pattern and heterozygosity–fitness correlations

Unlike outcrossing and other measures of inbreeding, little attention has been given to the association between pollen diversity and progeny fitness in fragmented systems. This is particularly surprising for trees as strong outcrossing is the norm (Petit & Hampe 2006), which limits the detection of associations between outcrossing and fitness. In our study, correlated paternity (r_p) and the number of half-sibships within progeny arrays (k_n), rather than inbreeding or inbreeding-related parameters (t_m, $t_m - t_s$, $1 - r_s$ and r_o), had the strongest correlation with progeny growth. There are only a few cases where pollen diversity has been studied in fragmented tree systems in conjunction with proxies of fitness of open-pollinated progeny, and these have generally reported nonsignificant relationships (Rocha & Aguilar 2001; Cascante *et al.* 2002; Fuchs *et al.* 2003; O'Connell *et al.* 2006; Mathiasen *et al.* 2007; Breed *et al.* 2012b). However, the ability to test statistical associations between fitness and correlated paternity in these studies were most probably limited by the small sample sizes.

The fitness benefits of greater pollen diversity (i.e. lower correlated paternity, more half-sibships per progeny array) derive from exposure to a higher diversity of pollen, which facilitates the acquisition of more 'good genes' driven by female choice for more compatible pollen and/or a heterosis effect (Yasui 1998; Skogsmyr & Lankinen 2002). This sorting of advantageous genes at such small spatial scales may occur at many stages of reproduction, including prefertilization (via pollen germination, penetration of the cuticle and pollen tube growth) and postfertilization (Skogsmyr & Lankinen 2002). Across generations of affected plants, reduced pollen diversity may be another mechanism for erosion of genetic diversity within populations, in addition to the well-established effects of inbreeding and genetic drift. Additionally, there is a great opportunity for pollen diversity to affect fitness as trees commonly receive pollen from numerous pollen donors (Skogsmyr & Lankinen 2002). Thus, progeny that are affected by low pollen diversity may have lower fitness, a trend that is supported by results presented here and from findings from other plant systems (reviewed in Skogsmyr & Lankinen 2002).

Higher pollen diversity can improve offspring fitness independently of inbreeding avoidance, as it can reduce the number of recessive deleterious alleles involved in reproduction (Armbruster & Gobeille 2004). Indeed, the two best predictors of growth in this study were pollen diversity measures that are relatively independent of inbreeding (correlated paternity, r_p, and the number of half-sibships within progeny arrays, k_n), which supports the action of pollen diversity on fitness independently of inbreeding. Selfed progeny are excluded when calculating these estimates (Ritland 2002; Ashley *et al.* 2009), and both estimates are robust to biparental inbreeding (Ashley *et al.* 2009; Breed *et al.* 2012a). Concordantly, we observed no significant correlations between any pollen diversity measure (r_p, k_n, r_o) and any inbreeding measure (t_m, $t_m - t_s$, r_s; Appendix D, Supporting

information). Despite it not being possible to totally disentangle the fitness effects of pollen diversity from biparental inbreeding using the metrics estimated here, the trends across the significant results found here point to a strong role of pollen diversity effects over and above those of inbreeding. Further study would be required to address this issue in detail by, for example, direct paternity analysis to quantify relatedness amongst male and female parents and assess these impacts on fitness.

After correlated paternity and the number of half-sibships within progeny arrays, biparental inbreeding had a weaker though significant effect on growth. Despite its secondary effect, inbreeding warrants discussion as it is much better studied than pollen diversity and is routinely observed in natural plant populations (Keller & Waller 2002). A fraction of progeny was selfed or sired by close relatives across all groups. Indeed, inbreeding occurred to a greater extent in small remnant woodlands and isolated pasture trees than in the large intact woodland (Monarto pasture: $t_m = 0.73$, $t_m - t_s = 0.23$; Monarto woodlands: $t_m = 0.84$, $t_m - t_s = 0.20$; Yookamurra: $t_m = 0.92$, $t_m - t_s = 0.11$). Accordingly, we observed a significant interlocus correlation of heterozygosity (as measured by g_2; David *et al.* 2007) and a negative relationship between inbreeding and fitness (Szulkin *et al.* 2010) in progeny from both small remnant woodlands and isolated pasture trees. In contrast, progeny from the large intact woodland (i.e. Yookamurra) were almost completely outcrossed and experienced low biparental inbreeding; thus, no significant interlocus correlation of heterozygosity was observed, preventing estimation of a relationship between inbreeding and fitness. Inbreeding effects have been previously documented for outcrossed eucalypts. For example, a temporal purging of self-pollinated plants over a 10-year period was observed in the closely related and allopatric *E. globulus* (Costa e Silva *et al.* 2010). The sympatric *E. incrassata* expressed inbreeding depression related to stress caused by fungal infection and much higher-observed heterozygosities in adult compared to progeny cohorts (Breed *et al.* 2012b). This heterozygosity difference was probably explained by weak selection acting on progeny but lifespan accumulated mortality of inbred adults, similar to the *E. globulus* case. Indeed, it should prove interesting to monitor inbreeding coefficients and inbreeding depression across additional life stages of *E. socialis* (Barrett & Harder 1996).

Management implications

Rapid growth in carbon and biodiversity markets has created great demand for revegetation and restoration plantings (Galatowitsch 2009). Consequently, the economic climate is favourable to replace large areas of cleared native vegetation, providing an opportunity to greatly benefit biodiversity conservation and reinstate disrupted ecosystem services globally (Vesk & Mac Nally 2006). However, revegetation projects have had mixed success (Godefroid *et al.* 2011), with failures often attributed to the use of poor genetic resources (Broadhurst *et al.* 2008).

Here, we show how the genetic resource quality of a commonly used revegetation species in southern Australia, *Eucalyptus socialis*, declines with conspecific density, contributed mainly by changes in pollen diversity. Currently, issues raised in the Small Population Paradigm (*sensu* Caughley 1994) guide the majority of seed-collecting policies (e.g. avoiding populations that have undergone strong genetic drift; Falk *et al.* 2001; Guerrant Jr *et al.* 2004; Kramer & Havens 2009; Maschinski & Haskins 2012). However, reduced pollen diversity, as demonstrated in this study, and increased inbreeding, as described elsewhere (Eckert *et al.* 2010; Breed *et al.* 2012a), as a result of landscape changes can have more direct impacts on seed quality and genetic diversity losses than genetic drift. Consequently, collecting seeds from maternal plants in higher-density stands or from less isolated contexts is a strategy that has been proposed to avoid collecting significant amounts of selfed seed (Broadhurst *et al.* 2008), and under most circumstances, this collection method should also minimize the collection of seeds from maternal plants that suffer from low pollen diversity.

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M.F.B. and A.J.L. designed the study, M.F.B., M.H.K.M. and J. B.C.H. collected field data, M.F.B. and M.H.K.M. generated genetic data, M.F.B. and J.B.C.H. performed analyses. M.F.B. wrote the first draft of the manuscript, and all authors contributed substantially to revisions.

Data accessibility

Growth, block, genetic diversity and mating system data are available through Dryad at doi: 10.5061/dryad.bs42p.

Supporting information

Additional supporting information may be found in the online version of this article.

Appendix A Pairwise distance matrix of maternal plants.

Appendix B Similarity of experimental sites, Monarto and Yookamurra.

Appendix C Missing genotype data.

Appendix D Family-level mating system parameter variance estimates from MLTR.

Appendix E Detailed methods of statistical mixed-effect model analyses.

Appendix F Per locus data summary.

Appendix G Genetic differentiation among mother tree groups.

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