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Wide outcrossing provides functional connectivity for new and old Banksia populations within a fragmented landscape --Manuscript Draft--

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Abstract:	<p>Habitat fragmentation affects landscape connectivity, the extent of which is influenced by the movement capacity of the vectors of seed and pollen dispersal for plants. Negative impacts of reduced connectivity can include reduced fecundity, increased inbreeding, genetic erosion and decreased long-term viability. These are issues for not only old (remnant) populations, but also new (restored) populations. We assessed reproductive and connective functionality within and among remnant and restored populations of a common tree, <i>Banksia menziesii</i> R.Br. (Proteaceae), in a fragmented urban landscape, utilizing a genetic and graph theoretical approach. Adult trees and seed cohorts from five remnants and two restored populations were genotyped using microsatellite markers. Genetic variation and pollen dispersal were assessed using direct (paternity assignment) and indirect (pollination graphs and mating system characterisation) methods. Restored populations had greater allelic diversity ($A_r = 8.08; 8.34$) than remnant populations (A_r range = $6.49 - 7.41$). Genetic differentiation was greater between restored and adjacent remnants ($F_{ST} = 0.03$ and 0.10) than all other pairwise comparisons of remnant populations (mean $F_{ST} = 0.01 \pm 0.01$; $n = 16$ $P = 0.001$). All populations displayed low correlated paternity ($r_p = 0.06 - 0.16$) with wide-ranging realised pollen dispersal distances (<1.7 km) and well-connected pollen networks. Here, we demonstrate reproductive and connective functionality of old and new populations of <i>B. menziesii</i> within a fragmented landscape. Due to long distance pollination events, the physical size of these sites underestimates their effective population size. Thus, they are functionally equivalent to large populations, integrated into a larger landscape matrix.</p>	

Wide outcrossing provides functional connectivity for new and old *Banksia* populations within a fragmented landscape

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Running Title: Reproductive functionality retained in fragmented *Banksia* woodlands.

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This study is one of the first to examine plant functional connectivity, measuring effective pollen dispersal using new methods in landscape genetics. We found that new populations have integrated with old through long-distance pollination events. Retaining remnant populations in the urban matrix is vital for maintaining reproductive functionality at the landscape scale.

Declaration of Authorship

Author Contribution statement ALR, PGN, EAS and SLK conceived and designed the research. ALR performed the study and analysed the data. Popgraph analysis was performed by ALR and RJD. All authors contributed to writing the manuscript.

Abstract

Habitat fragmentation affects landscape connectivity, the extent of which is influenced by the movement capacity of the vectors of seed and pollen dispersal for plants. Negative impacts of reduced connectivity can include reduced fecundity, increased inbreeding, genetic erosion and decreased long-term viability. These are issues for not only old (remnant) populations, but also new (restored) populations. We assessed reproductive and connective functionality within and among remnant and restored populations of a common tree, *Banksia menziesii* R.Br. (Proteaceae), in a fragmented urban landscape, utilizing a genetic and graph theoretical approach. Adult trees and seed cohorts from five remnants and two restored populations were genotyped using microsatellite markers. Genetic variation and pollen dispersal were assessed using direct (paternity assignment) and indirect (pollination graphs and mating system characterisation) methods. Restored populations had greater allelic diversity ($A_r = 8.08$; 8.34) than remnant populations (A_r range = $6.49 - 7.41$). Genetic differentiation was greater between restored and adjacent remnants ($F_{ST} = 0.03$ and 0.10) than all other pairwise comparisons of remnant populations (mean $F_{ST} = 0.01 \pm 0.01$; $n = 16$ $P = 0.001$). All populations displayed low correlated paternity ($r_p = 0.06 - 0.16$) with wide-ranging realised pollen dispersal distances (< 1.7 km) and well-connected pollen networks. Here, we demonstrate reproductive and connective functionality of old and new populations of *B. menziesii* within a fragmented landscape. Due to long distance pollination events, the physical size of these sites underestimates their effective population size. Thus, they are functionally equivalent to large populations, integrated into a larger landscape matrix.

Keywords *Banksia menziesii*, restoration, mating system, pollinator services, popgraph.

Introduction

Globally, one of the major processes threatening natural ecosystem function is anthropogenic landscape change (Eckert et al. 2010; Valiente-Banuet et al. 2015), which often results in increased spatial fragmentation and potential loss of native taxa (Tulloch et al. 2015). For plant populations, these changes alter the effectiveness of pollen and seed dispersal mechanisms (Lamont et al. 1993; Coates et al. 2007; Aizen et al. 2016). Alteration of dispersal vectors can lead to genetic isolation, increased drift and inbreeding, reductions in fitness, and potentially reproductive failure and local extinction (Cunningham 2000; Aguilar et al. 2006; Eckert et al. 2010; Vranckx et al. 2012). Ecological restoration has the potential to mitigate these pressures, by restoring biodiversity and recovering ecosystem services such as pollination and seed dispersal (Wortley et al. 2013; Perring et al. 2015). The practice of ecological restoration is extensive and growing worldwide, with major commitments that include reinstating 350 million hectares of forest by 2030 (Suding et al. 2015) and the landscape-scale restoration of highly fragmented systems (Menz et al. 2013). A key goal of ecological restoration is to re-establish self-sustaining ecosystems, ensuring that restored populations are able to persist in a dynamic setting in the short-term and have the capacity to undergo adaptive evolutionary change in the long term (Kettenring et al. 2014; Miller et al. 2017). Re-establishment of pollinator communities and genetic connectivity is critical for the long-term success of ecological restoration (Dixon 2009; Menz et al. 2011; Aavik and Helm 2017).

To date, ecological restoration focusing on plant dispersal vectors have documented a wide range of responses. For example, the response of insect communities have ranged from no recolonization (Forup and Memmott 2005) to complete recolonization of a restored habitat (Forup et al. 2008). Bird pollinator communities have shown aggressive territorial behaviour (Comer and Wooller 2002) and reduced community diversity (Munro et al. 2011). While

these responses may be context specific, the overall goal of restoring pollination services must be to quantify standing patterns of genetic connectivity to an extent such that the robustness of ecological resilience can be estimated (Wortley et al. 2013; Proft et al. 2018).

The most common approach to quantifying genetic connectivity is through the application of hypervariable genetic markers (Kettenring et al. 2014; Williams et al. 2014; Mijangos et al. 2015; Krauss et al. 2017), which can address both localized as well as landscape level dispersal processes (e.g., Byrne et al. 2007, 2008; Krauss et al 2009; Ritchie and Krauss 2012; Aavik et al 2013). The spatial distribution of genetic variation within existing populations and within single offspring cohorts can be used to gain both direct estimates (e.g., through paternity analysis; Marshall et al. 1998; Kalinowski et al. 2007) and indirect estimates (e.g., using pollination graphs, mating system analyses, and 2-generational approaches; Smouse et al. 2001; Ritland 2002; Dyer et al. 2012) of dispersal capacity and effectiveness. When applied across sampling locations, these approaches can further capture the extent to which local heterogeneity interacts with dispersal potential (Dyer 2015a).

In this study, we assessed reproductive and connective functionality within and among remnant and restored populations of *Banksia menziesii* (Proteaceae), a common, animal-pollinated, self-incompatible tree native to Western Australia (Scott 1980). *Banksia* woodlands are becoming increasingly fragmented as the Perth metropolitan area of Western Australia continues to expand along the coastal corridor. Less than 28% of the original woodland area remains around Perth and as a consequence, *Banksia* woodlands were recently listed by the Australian government as an ‘*Endangered Ecological Community*’ under the *EPBC Act 1999* and therefore the species is a priority for ecological restoration (Stevens et al. 2016). Focusing on these fragmented and restored populations, we addressed the following questions:

- 73 1. Are these restored (new) populations genetically depauperate and differentiated from the
74 local natural (old) populations?
- 75 2. What is the extent of contemporary genetic connectivity via pollen dispersal among
76 natural (old) urban remnants?
- 77 3. How do the mating patterns of *B. menziesii* vary across natural (old) urban remnants?
- 78 4. Has reproductive functionality, as measured by mating patterns and connectivity through
79 pollen dispersal, been established in the restored (new) populations?
- 80

81 **Material and methods**

82 **Study system**

83 *Banksia menziesii* is a dominant tree or woody shrub species within these endangered
84 woodlands which are distributed across the deep sandy soils of the Swan Coastal Plain of
85 Western Australia (George and Gardner 1981; Corrick and Fuhrer 2002). The species is self-
86 incompatible, with a long flowering season during the Austral autumn/winter (April-
87 September). It is a major source of floral nectar for pollinating honeyeaters (Meliphagidae),
88 bees (Ramsey 1988; Ramsey 1989) and the marsupial Honey Possum (*Tarsipes rostratus*)
89 (Wooller and Woller 2013) during these months. The woody infructescences are weakly
90 serotinous, with viable seeds being retained within the follicles (fruits) for ca. 9 months prior
91 to release following summer heat or fire (Lamont et al. 2007). Dispersal is predominantly by
92 gravity, with almost all seeds falling within 10 m of the maternal plant, however, some long-
93 distance dispersal can occur by wind-vortices and large parrots (Krauss et al. 2009). Fine-
94 scale spatial genetic structure has been recorded up to 10 m within natural populations of
95 *Banksia* (Krauss et al. 2009; Ritchie and Krauss 2012; Ritchie et al. 2017). Adult plants
96 within the natural populations are likely to be up to 300 years old, surviving in the landscape
97 since well into the pre-European period (Lamont et al. 2007).

98

99 **Study populations and sampling**

100 The study contrasted plants within restored (new) and three types of natural (old)
101 populations: restored populations (RS1 and RS2), fragmented remnant populations that were
102 adjacent to restored populations (AFR1 and AFR2), geographically fragmented remnant
103 populations (FR1 and FR2), and one large non-fragmented remnant (LR; Table 1; Fig. 1).
104 The two restorations were established in 1995 (RS1) and 1996 (RS2) following sand mining
105 by Rocla Quarry Products (now Hanson Construction Materials). Therefore, at the time of
106 sampling, restored trees were between 14 and 15 years old. A previous genetic study showed
107 RS1 was restored with locally sourced seed (Ritchie and Krauss 2012), while the source of
108 seed for RS2 is unknown. The two remnant woodlands (FR1 and FR2) became
109 geographically fragmented between 42 and 51 years ago by urban expansion.

110

111 **Reproductive attributes**

112 Ten maternal trees from each population were assessed for reproductive attributes by
113 quantifying the number of inflorescences produced, follicles (fruits) produced post flowering,
114 seed produced per follicle and mean seed mass. Generalised linear models (GLM) were
115 employed to determine differences in inflorescence count, follicle count (with Poisson error
116 distribution) viable seeds per follicle and proportion of viable seeds (with binomial logit
117 transformation) between populations and tested for normality of residuals using the Shapiro-
118 Wilk test and variation homogeneity using the Bartlett's test. Pearson's correlation coefficient
119 was calculated to determine any correlation between inflorescence count and follicle
120 production. Furthermore, we employed ANOVAs (function aov) to determine the effects of
121 population on seed mass (log-transformed). In significant models, we assessed differences
122 between population responses with Tukey's HSD post-hoc tests using the glht function

within R multcomp package (Hothorn et al. 2008). All of the reproductive attribute data analyses were performed in the R statistical environment (R Development Core Team).

DNA sampling, extraction and PCR amplification

Approximately 100 trees were sampled from each population within an approximate area of 200m x 200m. Leaf samples were collected from reproductively mature *B. menziesii* trees at all populations between December 2010 and January 2011. A minimum of ten seeds were collected from multiple cones from the previous flowering season from each maternal tree selected for reproductive attributes. The position of each tree was recorded using a Global Positioning System (GPS). DNA was extracted from 0.8 g silica-dried leaf material using a CTAB (cetyltrimethylammonium bromide) method (He et al. 2004; Doyle and Doyle 1990). DNA was extracted from seeds using the extraction protocol of Jobes et al. (1995). Seed coats were separated from the embryo to avoid contamination of DNA by maternal tissues, then embryos were pulverized for 20 seconds in a FastPrep®-24 instrument (MP Biomedicals, Inc., Solon, OH). Microsatellite amplifications were performed for eleven polymorphic markers; *BmA1*, *BmB6*, *BmB102*, *BmB106*, *BmC2*, *BmD1*, *BmD4*, *BmD103* and *BmD105*, previously designed for *B. menziesii* (Supplementary Table S1) and two, *BaA3* and *BaB106*, previously designed for *B. attenuata* (He et al. 2004; He et al. 2007) (Supplementary Table S1). Amplifications were performed with the following PCR conditions: 96°C for 2 mins (1 cycle), followed by 30 cycles of 94°C for 1 min, 52 to 57°C for 1 min (depending on the primer pair), 72°C for 1 min and final extension time of 72°C for 7 mins. PCR products were separated by capillary electrophoresis and alleles scored using a Beckman Coulter CEQ 8800 Genetic Analysis System (internal size standard SS400).

Molecular data analysis

Genetic diversity within and among populations

Population genetic diversity measures were estimated for adult populations and offspring (herein referred to as seed cohorts) using GenAlEx (version 6.502, Peakall and Smouse 2012). Genetic diversity estimates included the number of alleles (N_a), effective number of alleles (N_e), frequency of private alleles (P_r), expected and observed heterozygosity (H_e and H_o) and fixation index (F). The program FSTAT (Goudet 2001) estimated allelic richness (A_r) and corrected for different samples sizes, allowing comparison among populations. Significant differences between all parameter means were defined where 95% confidence intervals did not overlap.

Pairwise population differentiation (F_{ST}) was estimated among all pairs of adult remnant populations, as well as between restored population and their adjacent remnant population using GenAlEx (Supplementary Table S2). Genetic structure of the restored populations relative to all the other sampled populations was visualised using a Principal Component Analysis (PCA) and performed using allele frequency data in gstudio 1.3 (Dyer 2015b) in R version 3.2.4 (R Core Team 2016) (Supplementary Fig. S1).

Spatial genetic structure and connectivity

Adult genetic structure was analyzed using Population Graphs (Dyer & Nason 2004), a graph-theoretic analysis designed to quantify the spatial distribution of conditional genetic covariance. Conditional covariance has been shown to capture underlying demographic processes more precisely than methods based upon pairwise estimates of genetic structure (such as those derived from F_{ST} or its derivatives) or various genetic distance metrics (Dyer et al. 2010). In the Population Graph, sample locations are represented as nodes within the network, whose size is proportional to their within-population components of genetic

covariance. Nodes are connected by edges, the presence of which are defined by conditional genetic covariance (see Dyer 2015a for full description).

There are several benefits to this approach salient to this work. First, overall population genetic structure is represented by the pattern of connectivity in the network itself representing genetic covariance. Covariance is primarily created by both gene flow and shared ancestry. If the nodes in the topology represent standing adult structure, then the connectivity represented is based upon a combination of historical and ongoing demographic processes. However, if the nodes are based upon pollen pools (e.g., the male genetic contribution to a half-sibling seed cohort, see Dyer & Sork 2001) then reconstructed connectivity is based upon contemporary gene flow. This allows a single analytical framework to be used to examine processes generating genetic structure across a wide range of temporal scales from a single reproductive event (Dyer et al. 2012) up to regional estimates of phylogeographic history (Garrick et al. 2009; Baker & Dyer 2011).

Finally, the distances between nodes in the network, based upon genetic covariance, can be evaluated in relation to physical separation of nodes on the landscape under a model of isolation by (graph) distance (IBGD; Dyer & Nason 2004). If dispersal is uniform (e.g., the heterogeneity in the intervening habitat has no differential impact on connectivity) then the relative distances between nodes in Population Graph space should be proportional to physical (Euclidean) separation. Parts of the network with deviations from these expectations can identified when either the sample locales are further apart spatially than expected by genetic covariance or sample locales are in closer proximity than expected by genetic covariance. In the former case, this pattern is consistent with long-distance dispersal, whereas the latter case represents a situation where intervening habitat is preventing direct connectivity (see Dyer 2015a).

For these analyses, both Population Graphs (from adult structure) and Pollination Graphs (from pollen pool structure) were constructed using the R packages *popgraph* and *gstudio* (Dyer 2015b, c). Topological analyses were performed using the *igraph* package (Csardi and Nepusz 2006) in R. Specific parameters measured were centrality (measures of node location with the network) and betweenness (measures of information-theoretic flow through the network).

Mating patterns, paternity assignment and contemporary gene flow

Mating system parameters were estimated for each population using the program MLTR version 3.4 (Ritland 2002) include maximum-likelihood single (t_s) and multilocus (t_m) outcrossing rates, bi-parental inbreeding ($t_s - t_m$), correlated paternity (r_p) and the effective number of pollen donors per family ($1/r_p$). Confidence intervals (CI at 95%) were generated for all estimates by bootstrap resampling 1,000 times.

Likelihood-based paternity analysis was conducted using the program CERVUS 3.0 (Marshall et al. 1998; Kalinowski et al. 2007). The simulation parameters used to assign paternity to the most-likely individuals with a known level of statistical confidence were set at 10,000 cycles of simulation, all trees were potential candidate parents (sampled reproductive adults from within site or all sites, see Table 2) and 0.01 as the proportion of loci mistyped (genotyping error rate). Confidence in assignments was measured as the difference in critical logarithm of odds (LOD) scores between the candidate sire with the highest score and the candidate sire with the second highest score under strict (95%) and relaxed (80%) confidence levels. A LOD of ≤ 3.0 indicates a father is 20 times more likely than not to be the true father (Slate et al. 2000). Pollen dispersal distances (PDD) were calculated from paternal assignments made under relaxed analysis conditions (80%

confidence) by using the GPS location of the maternal tree for a particular seed and the paternal tree identified as the most likely pollen donor.

Differentiation in maternal tree pollen pools (Φ_{FT}) and effective density (d) across populations were estimated with TwoGener analysis using GenAlEx 6.502. Overall genetic differentiation between pollen pools (Φ_{FT}) and its variance was calculated following (Austerlitz and Smouse 2002) using known tree densities. Maternal trees sampling overlapping sets of pollen donors will have pollen pools with low among-mother genetic variance (e.g., Φ_{FT}). Large estimates of pollen pool structure (Φ_{FT}) are consistent with reduction in pollen dispersal distance whereby maternal trees sample from increasingly differentiated pollen donor populations. The maximum pollen dispersal distance (δ), effective number of pollen donors per family ($N_{ep} = 1/(2\Phi_{FT})$), and effective pollination neighbourhood area ($A_{ep} = N_{ep}/d$ (ha)), where d is the density of reproductive plants, were also estimated (Austerlitz and Smouse 2001a, b).

Spatial differentiation of pollen pools was assessed to examine connectivity within and among populations by constructing Pollination Graphs (PG; Dyer and Nason 2004; Dyer et al. 2012) an extension of the Population Graph approach whereby the sampling location is based upon the pollen pool sampled by each maternal individual (estimated identically as is done for the TwoGener analysis) rather than adult structure. Pollination Graphs and their topological structure were analysed as described for Population Graphs above.

Results

Reproductive attributes

Inflorescence production was variable across populations being significantly higher in RS2 ($RS2 = 21.7 \pm 0.8$; GLM, $\chi^2 = 154.7$, $df = 6$, $P < 0.001$) in comparison to all populations (range 5.9 - 10.4 inflorescences per tree, Table 1). Follicle production was correspondingly

(Pearson's correlation coefficient = 0.52, $P < 0.001$) significantly different between populations (GLM, $\chi^2 = 653.7$, $df = 6$, $P < 0.001$), with adjacent population AFR1 and both restored populations having the highest number of follicles (mean = < 50 follicles per maternal) in comparison to all other natural populations (mean = > 38 follicles per maternal) (Table 1). These patterns of higher inflorescence and follicles production however, did not convert to higher numbers of viable seed produced per follicle, percentage of viable seed per plant, or mean seed mass as they did not differ between populations (Table 1).

Diversity, Structure and Connectivity

Adult diversity and structure varied widely across sites (Fig. 2). Genetic diversity (H_e) ranged from 0.53 (AFR1) up to 0.67 (RS1). Adult inbreeding was low overall but most pronounced in populations FR1, FR2, & RS2 with population RS1 showed the lowest level of inbreeding of all populations. Populations RS1 and RS2 generally had higher levels of allelic diversity (N_a and A_r) and higher frequencies of private alleles (P_r) (Supplementary Fig. S1). The Population Graph (Fig. 3) of adult locales was highly connected with populations LR, AFR1, AFR2, and RS2 were the only ones without edges connecting all other sites. This pattern suggests broadly covarying genetic structure among all locations.

The distribution of spatial and genetic structure in adult populations suggested restricted historical gene flow under a model of isolation by graph distance (IBGD; Mantel $Z=0.72$, $P = 0.003$). Among individual populations, physical separation was greater than expected from the genetic covariance (extended edges; a pattern consistent with long-distance dispersal) for population pairs LR/RS2, FR/AFR1, and AFR1/AFR2 (Fig. 3). The potential for intervening barriers to direct connectivity (edge compression) are also identified for connections between RS1/FR1, RS1/FR2, and RS2/AFR2 (Fig. 3).

The pattern of diversity within offspring cohorts was similar to that of adults (Fig. 2). Heterozygosity was largest in RS1 and RS2. Overall allelic richness (N_a) was most depauperate in FR1 and diverse in AFR2. Corrected for richness (A_r), RS1, AFR2, and RS2 were the most diverse, however, unlike the adult populations only RS2 showed increased frequency of rare alleles in the offspring pollen pool with RS1 having frequencies of rare alleles similar to the remaining locations.

We found significant, though low levels, of genetic structure among all adult remnant populations ($F_{ST\ remnant} = 0.047$, $P = 0.001$). Both adult and seed cohorts showed significant differentiation from adjacent remnant populations, though RS1 was not as pronounced ($F_{ST; adults} = 0.031$, $P = 0.001$ vs. $F_{ST; seed} = 0.029$, $P = 0.001$) as observed in RS2 ($F_{ST; adults} = 0.102$, $P = 0.001$ vs. $F_{ST; seed} = 0.036$, $P = 0.001$) suggesting some degree of isolation between restored and remnant populations. Across all pairs of populations, the restored site RS2 had the highest average structure (mean $F_{ST} = 0.093$) (Supplementary Table S2).

Mating system, paternity assignment and contemporary gene flow

Single and multilocus outcrossing rates were not significantly different from 1.0 and biparental inbreeding estimates were low for the restored populations and four of the five remnants (Table 2). The adjacent fragmented remnant 1 exhibited higher biparental inbreeding ($t_m - t_s$ AFR1 = 0.231). All estimates of correlated paternity were low (r_p range = 0.06 - 0.16) and estimates of the effective number of pollen donors ranged from 6.3 – 16.4 (Table 2). Differentiation among sampled pollen pools (Φ_{FT}) showed no significance between restored populations (RS1: $\Phi_{FT} = 0.08$; RS2: $\Phi_{FT} = 0.043$, $\Phi_{FT\ remnants}$ mean = 0.07 ± 0.01) indicating similar pollen pool structure among populations. Estimates of effective number of pollen donors were not significantly different between restored and natural populations (RS1: MLTR $N_{ep} = 7.9$, TwoGener $N_{ep} = 6.2$; RS2: MLTR $N_{ep} = 16.4$, TwoGener $N_{ep} = 11.9$; and

MLTR $N_{ep; \text{remnants}}$ mean = 8.8 ± 1.3 , TwoGener $N_{ep \text{ remnants}}$ mean = 7.9 ± 0.9). The effective neighbourhood area of the pollinators in RS1 and RS2 (14.7 m and 22.0 m radius around maternal trees, respectively; $A_{ep \text{ restored}}$ = 0.07 ha and 0.15 ha) was lower than the average for natural populations ($24.5 \text{ m} \pm 5.8$ radius; $A_{ep \text{ remnants}}$ mean = $0.23 \text{ ha} \pm 0.10$). From pollen pool structure, the TwoGener analysis suggest that restored populations had shorter pollen dispersal distance estimates (δ_{restored} = 9.2 m and 13.6 m) in comparison to the natural populations (δ_{remnants} mean = $27.9 \text{ m} \pm 10.9$; Table 2).

While the TwoGener analysis documents the magnitude of genetic structure in sampled pollen pools (via Φ_{FT}), Pollination Graphs depict how covariation is spatially distributed across the landscape. Edges among maternal locations indicate significant genetic covariance between sampled pollen pools (Fig. 4). In general, the amount of edge connectivity (e.g., the fraction of potential edges in the network) was greatest in LR (Fig. 4a; $p = 0.27$), roughly half as much for the FR1/FR2 and AFR1/RS1 sites (Fig. 4b & Fig. 4c; $p = 0.13$ each), and lowest in the AFR2/RS2 (Fig. 4d; $p = 0.08$). For locations with dual sampling sites, the FR1/FR2 complex had the highest amount of among-fragment connectivity (23% of the edges connect nodes between fragmented sites; Fig. 4b) across the urban matrix. Both AFR/RS sites (Fig. 4c & 4d) had lower inter-site connectivity (11% for AFR1/RS1 and 13% for AFR2/RS2 respectively), however do indicate some degree of restored site integration. Not all sites had completely connected components, FR1/FR2 had three isolated nodes (e.g., no connections whatsoever) and the AFR2/RS2 locale has several disconnected subgraphs. Disconnected pollen pools suggest independence in the set of pollen donors are contributing to maternal individuals, for which we have not captured

Direct analysis of paternity in these populations yielded low assignment likelihoods (< 5% of 681 seed, restored = 9 seed, 4.5%; natural = 28 seed, 5.8%) as critical LOD scores were high, 8.04 for 95% and 6.24 for 80%. Sampled plants were excluded as sires for 55 (8%)

seeds, from which we inferred these to be the product of long-distance dispersal of pollen, from beyond the local population. Fragmented populations had greater maximum pollen dispersal distances than restored populations (CERVUS: PDD_{remnants} mean = 614.8 m; PDD_{restored} mean = 185.5 m) (Table 2). The pollen dispersal distance estimates from assignments (CERVUS) ranged from 4.0 m to 1.7 km, with a mean and median of 391.0 m and 146.4 m.

Discussion

Habitat fragmentation may result in fewer and potentially more isolated plant populations with reduced dispersal at local, landscape and regional scales predicted (Young and Clarke 2000). The exchange of pollen offers a way by which genes can be moved between these remnant patches, mitigating potential negative genetic impacts and ensuring plant functional connectivity (Kramer et al. 2008; Auffret et al. 2017). For remnant (old) and restored (new) populations this requires that individuals not only survive to produce viable seed (following effective pollen dispersal) but result in the establishment and survival of a new adult plant for genetic integration.

Our assessment of functional connectivity across new and old populations of *Banksia menziesii* within a highly fragmented urban landscape has shown that seed of both remnant and restored populations retain high genetic diversity through wide outcrossing. We found no evidence to suggest habitat fragmentation and the associated spatial isolation of natural remnants or restored *B. menziesii* populations is a barrier to pollinator movement into and among remnants within an urban landscape at present. All study populations were equally fecund (once adjusted for flowering), similarly genetically diverse, completely outcrossed, with low correlated paternity, multiple shared paternal assignments, and wide genetic connectivity. Natural remnant populations adjacent to restored populations were shown to provide trans-boundary pollinator services, indicating a vital role in establishing reproductive

functionality. Genetic covariance among populations displayed in the popgraph network analysis confirms extensive historical genetic connectivity. There are (unmeasured) and likely were (historically) intermediary remnants facilitating gene flow between populations and it is also likely that these populations are not yet on their own evolutionary trajectories.

There was no measurable effect of fragmentation on genetic diversity or effective population size estimates (N_e) in this study, which is likely due to a combination of large effective population size and time since fragmentation (42-51 years) for these long-lived trees. Longevity of these slow maturing trees (many may be decades old) provides the capacity for populations to withstand short-term physical fragmentation effects (Vranckx et al. 2012), and therefore have some resilience as long as pollinators are present (Breed et al. 2015).

Connectivity among old remnant populations

This study complements others (Coates et al. 2007; Krauss et al. 2009; Ritchie and Krauss 2012; Krauss et al. 2013; Frick et al. 2014; Ritchie et al. 2017) to show that banksias typically contain high allelic diversity, low levels of population genetic differentiation, and a capacity for long-distance pollen dispersal due to their highly mobile nectarivorous honeyeater bird pollinators. For plant species that depend on biotic pollination to produce seeds, functional connectivity depends to a large extent on the type of pollinator, their behaviour and how their movement is influenced by landscape factors (Auffret et al. 2017). Some honeyeaters, such as Western Spinebills (*Acanthorhynchus superciliosus*) have a recorded home range of 0.2 – 0.5 ha within nature reserves (Newland and Wooller 1985), whereas Red Wattlebirds (*Anthochaera carunculata*) and Singing Honeyeaters (*Gavicalis virescens*) do well in the urban environment (Davis et al. 2013) and can fly much larger distances during their foraging routes (Davis and Wilcox 2013). This is likely why we see

differences in the topologies of our Pollination Graph networks. These pollinators may use intermediate remnants, road verges or urban gardens to forage, helping maintain connectivity across fragmented urban landscapes (Shanahan et al. 2011). Our results suggest that they can effectively transfer pollen grains at least 1.7 km between fragments.

Connectivity of new restored populations and the importance of remnant vegetation

Successful recruitment, maintenance and survival of restored populations is influenced by the surrounding landscape (Aavik and Helm 2017; Proft et al. 2018), and our study demonstrates the role that natural adjacent remnants play in delivering trans-boundary pollinator services to restored populations. The increased allelic richness, reduced differentiation between the adult and restored seed cohorts (F_{ST}), and paternity assignments confirms high genetic exchange between restored and adjacent natural *B. menziesii* populations even though restored populations displayed shorter pollen dispersal distance estimates. These shorter distances in comparison to natural populations was likely due to the significantly higher inflorescence production in the restored populations leading to higher pollinator attraction and higher follicle production. However, these pollination events and follicle production did not result in higher seed production, most likely due to a combination of resource allocation by the plant and geitonogamy of a self-incompatible species with prezygotic and/or postzygotic mechanisms stopping the production of selfed seed.

Pollen pool structure analysis in this study showed low to comparable levels of differentiation among pollen pool families and high number of effective pollen donors, similar to that of the large non-fragmented remnant. This implies a high level of gene flow between these restored and remnant adjacent populations and successful integration of restored plants has been achieved.

Adjacent land use has been proven to play a key role in the maintenance of genetic

diversity of habitats (Aavik et al. 2013; Helsen et al. 2013). A recent study by Crouzeilles and Curran (2016) showed that restoration success of forests, assessed through changes in species diversity, was directly associated with undisturbed forest cover within a 10 km radius. Conservation actions have largely focused on retaining large fragmented patches, disregarding the increasingly crucial role that smaller remnant patches play in the conservation and community persistence of remaining and restored vegetation (Tulloch et al. 2015).

Genetic integration

Higher genetic diversity (allelic richness and frequency of private alleles) was found in restored populations compared to remnant populations. The degree of genetic differentiation among adults of the (new) restored populations was 3.5 times greater in magnitude than those at (old) remnant populations, indicating a mixed provenance seed source was used. The Population network also supports this conclusion, displaying compressed edges due to higher than expected genetic diversity in the restored population with respect to distance, between all the populations sampled. The genetic structure within restored populations differed due to the random mixing and sowing of seed, rather than limited natural dispersal (Krauss et al. 2009; Ritchie and Krauss 2012; Ritchie et al. 2017) by gravity and thus specifically, the gene pool and effective pollination neighbourhood area of Restored Site 2 was greater than in the natural remnant populations sampled.

The introduction and mixing of non-local alleles with a local gene pool could be beneficial (heterosis), detrimental (outbreeding depression) or neutral (McKay et al. 2005), depending on the level of inbreeding and the length of time gene pools have been isolated (Frankham et al. 2011). Experimental outcrossing by hand of *B. ilicifolia* flowers on plants separated by 30 km led to heterosis, expressed as improved germination of seed and early growth of seedlings

compared to local crosses (Heliyanto et al. 2006). A similar result may be expected in *B. menziesii* and suggests that wide outcrossing stemming from the introduction of non-local provenance genotypes may be beneficial in highly outcrossed banksias, but does require further investigation. These restoration sites offer unique opportunities for on-going assessment, through a facilitation of wide outcrossing between co-occurring plants by hand pollination.

Conclusions

Despite the recognized importance of pollination and functional connectivity in restoration (Dixon 2009; Menz et al. 2011), few studies have addressed these issues (Thomas et al. 2014; Aavik and Helm 2017; Proft et al. 2018). Recent methodological advances in landscape genetics provides powerful new tools for assessing the impacts of landscape change through the ecological functioning of old and new populations (Hufford and Mazer 2003; Williams et al. 2014; Mijangos et al. 2015; Proft et al. 2018) of longer-lived species that have now matured to reproductive age. We have demonstrated a capacity for reproductive functionality in new *B. menziesii* populations that is only possible through functional connectivity within and among remnant and restored populations, as a consequence of highly mobile bird pollinators.

Landscape genetics and functional connectivity needs to be taken into account for ensuring the long-term survival of old and new populations, with consideration of how populations are integrated into the larger ecological matrix (Elliott et al. 2012; Aavik and Helm 2017). Most (87.5%) flowering plant species are dependent on animals for pollination (Ollerton et al. 2011). Plant functional connectivity therefore can change, not only as a result of a landscape modification but also with changes in the type, characteristics, behaviour, diversity or abundance of pollinators, or the plants' ability to use these pollinator services

(Auffret et al. 2017). Conservation and restoration of pollinator services and functional connectivity requires a consideration of all stages of the dispersal process with the presence, community (multiple pollinator species) and movement of pollinators in the broader landscape (Aguilar et al. 2006; Menz et al. 2011). Conservation of remnants irrespective of their size needs consideration, especially in this threatened ecological system as they are shown to contain high genetic diversity, and display differing mating system metrics. The distances at which inter-patch connectivity dissolves for different pollination syndromes needs to be identified (Aizen et al. 2016) for the benefit of conservation and restoration success.

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Conflict of Interest The authors declare that they have no conflict of interest.

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687 **Table 1** Mean values (± 1 SE) for reproductive attributes (inflorescences, follicles and seeds) of 10 trees within five remnants (old) and two
688 restored (new) populations of *Banksia menziesii*. Different row subscripts indicate significant differences between those populations.

	Non-fragmented	Fragmented		Adjacent		Restored		<i>P</i> -value
	Large remnant (LR)	Fragmented remnant 1 (FR1)	Fragmented remnant 2 (FR2)	Adjacent fragmented remnant 1 (AFR1)	Adjacent fragmented remnant 2 (AFR2)	Restored Site 1 (RS1)	Restored Site 2 (RS2)	
Latitude, Longitude	31 40'28"S, 115 53' 44"E	31 49'09"S, 115 52' 29"E	31 49'38"S, 115 51' 31"E	31 47'07"S, 115 56' 23"E	32 06'18"S, 115 52' 07"E	31 47'09"S, 115 56' 32"E	32 06'28"S, 115 52' 01"E	
Reproductive attribute								
No. inflorescences tree ⁻¹	9.2 $\pm$ 1.4 ^b	5.9 $\pm$ 1.3 ^a	10.4 $\pm$ 1.2 ^b	7.5 $\pm$ 0.9 ^{ab}	8.8 $\pm$ 1.5 ^b	7.7 $\pm$ 1.1 ^{ab}	21.7 $\pm$ 0.8 ^c	< 0.001
No. follicles tree ⁻¹	31.6 $\pm$ 8.4 ^{ab}	38.6 $\pm$ 15.1 ^c	36.1 $\pm$ 7.7 ^{bc}	59.9 $\pm$ 16.3 ^d	35.4 $\pm$ 12.8 ^a	52.9 $\pm$ 16.7 ^e	99.9 $\pm$ 16.7 ^f	< 0.001
No. of viable seeds/follicle ⁻¹	1.0 $\pm$ 0.1	1.2 $\pm$ 0.2	1.2 $\pm$ 0.1	0.9 $\pm$ 0.1	1.0 $\pm$ 0.2	1.1 $\pm$ 0.1	0.8 $\pm$ 0.2	n.s.
Viable seed set (%)	75.5 $\pm$ 5.6	72.3 $\pm$ 5.3	76.7 $\pm$ 6.0	75.7 $\pm$ 5.4	70.1 $\pm$ 0.8	79.7 $\pm$ 6.5	80.8 $\pm$ 2.9	n.s.
Seed weight (g)	0.047	0.047	0.034	0.044	0.034	0.048	0.034	n.s.

690 **Table 2** Mating system, pollen gene pool, spatial genetic structure measures and simulation of paternity assignment (father given known mother)
 691 at each site under strict (95%) and relaxed (80%) confidence levels for seven sites* of *Banksia menziesii*. Different lower-case letters indicate
 692 statistically significant differences at $P = 0.05$. *Population codes listed in Table 1.

Parameter	LR		FR1		FR2		AFR1		AFR2		RS1		RS2	
<i>N</i>	98		98		100		98		95		100		98	
<i>MLTR Mating system</i>														
Multilocus outcrossing rate (<i>t</i> _m)	1.09 ^{ab}		0.98 ^a		0.96 ^a		1.20 ^b		0.99 ^a		1.20 ^b		1.03 ^a	
Singlelocus outcrossing rate (<i>t</i> _s)	0.96		0.97		0.94		0.97		1.01		1.02		0.92	
Biparental inbreeding (<i>t</i> _m − <i>t</i> _s)	0.13 ^{abc}		0.01 ^{ac}		0.02 ^a		0.23 ^b		-0.03 ^a		0.19 ^{bc}		0.11 ^{abc}	
Correlated paternity (<i>r</i> _p)	0.08		0.15		0.09		0.16		0.14		0.13		0.06	
Effective number of pollen donors <i>N</i> _{ep} = (1/ <i>r</i> _p)	12.5		6.5		11.5		6.3		7.4		7.9		16.4	
Effective pollination neighbourhood area <i>A</i> _{ep} = <i>N</i> _{ep} / <i>d</i> (ha)	0.65		0.06		0.27		0.33		0.04		0.09		0.21	
<i>TwoGener analysis</i>														
Differentiation in pollen gene pool among families <i>Φ</i> _{FT}	0.05		0.07		0.07		0.09		0.06		0.08		0.04	
Number of pollen donors <i>N</i> _{ep} = (1/ 2 <i>Φ</i> _{FT})	11.1		7.3		7.2		5.7		8.3		6.2		11.9	
Effective pollination neighbourhood area <i>A</i> _{ep} = <i>N</i> _{ep} / <i>d</i> (ha)	0.58		0.06		0.17		0.30		0.04		0.07		0.15	
Estimated effective pollen area radius centred on each maternal tree <i>A</i> _{ep} radius (m)	26.96		8.77		14.58		19.44		69.96		9.20		13.63	
Maximum pollen dispersal distance <i>δ</i> (m)	26.96		8.77		14.58		19.44		69.96		9.20		13.63	
Neighbourhood size <i>Nb</i>	361		285		156		144		286		401		undefined	
<i>CERVUS</i>														
Confidence (%)	95	80	95	80	95	80	95	80	95	80	95	80	95	80
Critical LOD	8.20	6.46	8.34	6.30	8.38	6.32	8.38	6.64	8.31	6.16	8.18	6.17	8.66	6.63
Observed assignment	2	5	5	9	0	5	1	7	1	2	4	3	1	5
Assignment rate	2%	5%	5%	9%		5%	1%	7%	1%	2%	4%	3%	1%	5%
Maximum pollen dispersal distance <i>PDD</i> (m)	112		1320		1331		210		101		212		159	

Figure Legends

Fig. 1 Location of study in Western Australia. a) the pre-European distribution (light grey) and the likely current distribution of *Banksia* woodlands (dark grey). b) the old and new populations sampled (in black) of *Banksia menziesii* for genetic diversity and mating system parameters assessment of; Large non-fragmented remnant (LR), Adjacent fragmented remnant 1 (AFR1) and Restored Site 1 (RS1), two fragmented remnants (FR1 and FR2) within an urban landscape, and Adjacent fragmented remnant 2 (AFR2) and Restored Site 2 (RS2). Perth CBD is Perth Central Business District. Figure modified from ERIN, Department of the Environment and Energy, Australian Government: <http://www.environment.gov.au/biodiversity/threatened/communities/pubs/131-indicative-distribution-map.pdf>

Fig. 2 Genetic diversity across adult (dark grey) and seed cohort (grey) cohorts ($\pm$ SE) for heterozygosity (observed H_o and expected H_e), inbreeding (F ; *n.b.*, not calculated for haploid offspring genotypes), average number of alleles (N_a), effective number of alleles, (N_e) private allele frequencies (P_r) and allelic richness (A_r). Different lower-case letters indicate statistically significant differences at $P = 0.05$.

Fig. 3 Population Graph representing both old (black) and new (white) sampling locations of *Banksia menziesii*. The size of the nodes represent within population genetic variance and connections reflect the topology of genetic covariance amongst all sampled populations. Edges were classified based upon the physical separation of sampling locations and the expectation given genetic covariance under a model of isolation by graph distance. Normal edges are shown in grey, extended edges (e.g., sample locations more spatially distant than expected by genetic covariance, indicating long-distance dispersal) are depicted with dashed

lines, and compressed edges (e.g., sample locations in closer proximity than expected by genetic covariance, short distance dispersal) are in dark bold lines. See Fig. 1 legend for population abbreviations.

Fig. 4 Conditional genetic covariance among pollen pools for a) Large non-fragmented remnant (LR), b) two fragmented remnants within an urban landscape (FR1 and FR2), c) Adjacent fragmented remnant 1 (AFR1) and Restored Site 1 (RS1) and d) Adjacent fragmented remnant 2 (AFR2) and Restored Site 2 (RS2), depicted as pollination graphs overlaid on satellite images of sites. Nodes represent the population of pollen haplotypes sampled by each maternal family. Edges represent significant statistical covariance among pollen pools. Colour version of this figure available online.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

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