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ARTICLE

Short distance pollen dispersal and low genetic diversity in a subcanopy tropical rainforest tree, *Fontainea picrosperma* (Euphorbiaceae)

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Abstract

Gene flow via pollen movement affects genetic variation in plant populations and is an important consideration in plant domestication. *Fontainea picrosperma* is a subcanopy rainforest tree that is of commercial interest because it is the source of tigilanol tiglate, a natural product used for the treatment of solid tumors. We identify patterns of pollen-mediated gene flow within natural populations of *F. picrosperma* and estimate genetic parameters and genetic structure between adult and juvenile groups using microsatellite markers. Our results show pollination events occur over much shorter distances than reported for tropical canopy species. At least 63% of seeds are sired by male trees located within 30 m of the mother. On average, 27% of the local male population contributed to successful reproduction of *F. picrosperma* with most fathers siring a single seed, however, the contributions to reproduction were uneven. Larger male trees with more flowers had greater reproductive success than those with less flowers ($P < 0.05$). There were comparatively low levels of genetic variation across the species ($H_E = 0.405$ for adult trees and 0.379 for juveniles) and we found no loss of genetic diversity between adult and juvenile trees. Short distance pollen flow and low genetic diversity is theoretically a prelude to genetic impoverishment, however *F. picrosperma* has persisted through multiple significant climatic oscillations. Nevertheless, the remaining low genetic diversity is of concern for domestication programs which require maximal genetic diversity to facilitate efficient selective breeding and genetic improvement of this commercially significant species.

Introduction

Genes move within and among plant populations through pollen and seed dispersal as well as physical movement of vegetative plant material. Plant mating patterns in tropical rainforests, mediated by gene movement of pollen, is an important determinant in the level of genetic variation within and among populations. Gene flow can counteract the potentially detrimental effects of genetic drift and may be a source of new alleles within populations (Burczyk et al. 2004). However, when gene flow is restricted, inbreeding or biparental inbreeding (mating with close relatives) can occur and ultimately lead to a loss of genetic diversity, directional selection and genetic drift (Ellstrand and Elam 1993). Tropical forest ecosystems are experiencing high rates of habitat destruction and forest fragmentation that can negatively impact on genetic variation within species (Bradshaw et al. 2009; Eckert et al. 2010). The modification of habitat can disrupt natural patterns of gene flow by creating environments that are stressful for pollinator survival and activity (Eckert et al. 2010). Though, the impacts to mating patterns vary between species and context (Hamrick 2004; Lowe et al. 2015) and can be affected by a species life history, reproductive biology and the mobility of pollinators (Breed et al. 2015; Rymer et al. 2015; Vinson et al. 2015).

Most woody tropical rainforest species are strongly outcrossed and rely on insects for pollination (Bawa 1992; Ollerton et al. 2011). The density of flowering conspecifics,

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including factors such as spatial distribution and the distance to the nearest pollen source can influence pollinator foraging behavior (House 1993; Ghazoul 2005). Commonly, pollinator flight distances tend to increase with lower plant densities and decrease when flowering plants exhibit a clumped distribution or occur at high densities (House 1992; Stacy et al. 1996; Hardy et al. 2006; Born et al. 2008; Ashley 2010; Silva et al. 2011; Naoki et al. 2012; Duminil et al. 2016). This is because near-neighbor mating increases the foraging economy of the pollinating insect by maximizing the net energy gained by the pollen or nectar source (Levin and Kerster 1974; Degen and Sebbenn 2016). There is strong empirical evidence that suggests that tropical canopy species with low population densities exhibit long distance pollen dispersal (Akihiro et al. 2000; Kenta et al. 2004; Ward et al. 2005; Hardesty et al. 2006; Born et al. 2008; Carneiro et al. 2009; Ottewell et al. 2012; Monthe et al. 2017). While there are relatively few studies describing gene flow within understorey species, pollen has been found to disperse shorter distances in tropical or subtropical woody taxa, particularly in species with high local densities (Lasso et al. 2011; Castilla et al. 2016; Hahn et al. 2017).

Pollen-mediated gene flow, measured in terms of successful reproduction, is affected by other density measures such as phenology and its synchronicity, as well as the size of the nearest pollen source (O'Connell et al. 2018). Variation in these factors influence pollination distances, pollen dispersal patterns and levels of genetic isolation (Castilla et al. 2017). For example, large male trees (measured by species specific differences in diameter at breast height) can contribute disproportionately to seed production (Hoebee et al. 2007; Naoki et al. 2012; Tambarussi et al. 2015; Monthe et al. 2017; Younginger et al. 2017). Thus, the size of nearest pollen source becomes important because a large but more distant male may contribute more pollen than a smaller, closer one. Determining variations in male fecundity within populations is important because mating partners can influence genetic diversity and population fitness (Breed et al. 2012a). Unfit combinations of pollen and ovules are more likely to occur in offspring when fewer males contribute to reproduction. With more mating partners, there is a smaller probability that recessive deleterious alleles will be involved in reproduction (Breed et al. 2012b).

Historical gene flow can be inferred through the distribution of genetic diversity between generations of individuals (Slatkin 1987; Young et al. 1996; Lowe et al. 2005). Genetic diversity and differentiation between age cohorts within populations are critical parameters that impact population genetics and structure, particularly in landscapes where habitat has been modified. This is because altering the number of reproductive individuals in the population can negatively impact genetic diversity and inbreeding levels in progeny due to disruptions in pollen diversity and pollinator mobility (Breed et al. 2015). Mating systems also contribute to the structure of genetic diversity (Hamrick and Godt 1996) and while most tropical trees are facultative outcrossers, bi-parental inbreeding can occur in species with limited gene dispersal capabilities (Ellstrand and Elam 1993; Collevatti et al. 2001a; Castilla et al. 2017). Genetic variation, measured in terms of heterozygosity, is often significantly correlated with fitness (Reed and Frankham 2003; Nutt et al. 2016). It is therefore important to assess both contemporary and historic gene flow patterns to identify the strength and spatial scale at which evolutionary forces act upon populations (Stockwell et al. 2003). This will aid management of species in the face of anthropogenic habitat modification and exploitation of forest resources.

Fontainea picrosperma C.T. White (Euphorbiaceae) is a dioecious, subcanopy tree endemic to upland tropical rainforests on the Atherton Tablelands, Queensland, Australia. The species is locally common but has a restricted natural range. The region has been subject to natural habitat fragmentation during the climatic fluctuations of the Plio-Pleiestocene that led to rainforest species retreating to moist refugia when climate conditions cooled and recolonized surrounding areas once climate conditions improved (Kershaw et al. 2007). *Fontainea picrosperma's* current discontinuous distribution is due to anthropogenic habitat fragmentation primarily as a result of agricultural expansion, but also due to urban settlements. *Fontainea picrosperma* is the source of tigilanol tiglate (Boyle et al. 2014; Linkliter et al. 2015), a small molecule, natural product used for the local treatment of solid tumors in humans and companion animals (Linkliter et al. 2015; Miller et al. 2019). Tigilanol tiglate is not synthetically tractable, so production of the drug on a commercial scale relies on raw material harvested from plantations of *F. picrosperma*. It is critical to understand the scale of realized gene flow across generations as well as the overall genetic diversity of the species throughout its natural range to optimize production through selective breeding and genetic improvement of planting stock.

Many studies have examined gene flow and population genetic structure in rainforest canopy species, however understorey species remain underrepresented. This study follows on from the work conducted by Lamont et al. (2016) who studied the population genetics of *F. picrosperma* across the species distribution. Here, we identified localized patterns of gene flow and estimated the population genetic structure between subpopulations of adults and juveniles in natural populations of *F. picrosperma* using microsatellite markers. Specifically, we asked (1) what is the distance of pollen-mediated gene flow and what proportion of seeds are sired by local males? (2) How many males contribute to progeny for each mother tree and how

Table 1 Sampling method used for each mother tree

Mother	Population	Year fruit collected	Number of males sampled	Number of seedlings
A156	Evelyn Highlands 1	2014/2015	32	13
A336	Evelyn Highlands 1	2015/2016	31	26
B27	Boonjie	2015/2016	43	45
B283	Boonjie	2014/2015	17	19
B595	Boonjie	2014/2015	34	17
B706	Boonjie	2014/2015	16	15
E17	Topaz	2014/2015	6	19
J15	Evelyn Highlands 2	2014/2015	40	20
J169	Evelyn Highlands 2	2014/2015	44	20
J424	Evelyn Highlands 2	2014/2015	37	20
Total			300	214

does male reproductive fitness relate to paternal tree characteristics including flowering effort and location (direction and distance) relative to the mother tree? (3) What are the levels of genetic diversity in adult trees and juveniles in the population and what are the levels of genetic differentiation between generations? (4) Are individuals growing near to each other more related than expected from mating two random individuals?

Materials and methods

Study species and site

Fontainea picrosperma is a subcanopy tree to 25 m (Jessup and Guymer 1985) endemic to the complex mesophyll and notophyll vine forests on the Atherton Tablelands, north Queensland, Australia. The species possesses small, white and fragrant flowers that have an unspecialized structure and an open access receptacle that are likely to be pollinated by small generalist insects (Grant et al. 2017). Flowering occurs simultaneously between individuals within subpopulations from September to November. The red drupaceous fruits (up to 3 cm diameter) ripen in December and January and are dispersed primarily by gravity. Secondary long-distance seed dispersal can occur either by hydrochory along drainage lines or zoochorous vectors (Cooper 2004; Lamont et al. 2016). Natural stands of *F. picrosperma* therefore are not uniformly distributed within appropriate habitat, but rather form small, but dense clumps or clusters (2–10 m inter-tree spacing) with ~50:50 male:female ratios (Lamont et al. 2016; Grant et al. 2017). These clumps or clusters are often isolated from neighboring clumps with no conspecific individuals found in between.

Data in this study were collected from trees in discrete populations from across the natural range of the species. The collection locations are labeled according to place names and are described in detail by Lamont et al. (2016). The populations included in this study were Boonjie, East Barron, Malanda, Topaz, Gadgarra, Towalla, and Evelyn Highlands. We use the term subpopulation when more than one site, representing one clump or cluster, was sampled within a population.

Sample collection

Pollen-mediated gene flow and male fitness

We estimated pollen movement by direct paternity analysis using seedling cohorts of selected mother trees from across the *F. picrosperma* geographic range. Mother trees were selected based on the number of fruit that had matured and fallen at the time of sampling as well as their physical location within the discrete clump or cluster. A representative sample of fruit were collected from the base of 10 female trees (mother trees) from four populations (A156; A336; B27; B283; B595; B706; E17; J15; J169; and J424) during the 2014/2015 reproductive season. Very few seedlings survived in the nursery from two female trees and therefore we re-collected from two mother trees (A336 and B27) during the 2015/2016 reproductive season (Table 1). The subpopulations of "Evelyn Highlands 1" and "Evelyn Highlands 2" are from the same refugial population approximately 3.5 km apart. Locations of all males within a 30 m radius of each of the mother trees were mapped using a compass and tape measure. This spatial range was selected because it represents the typical approximate scale of the local density estimates of discrete clumps or clusters of *F. picrosperma*. By sampling a 30 m radius around the mother tree, we estimated that we captured at least 90% of individuals located within the clump. We sampled to 35 m around one mother tree, B283, to capture three males tree located just outside the sampling radius. Mother tree E17 was from a small, isolated population, Topaz, where every male individual was sampled and mapped (16 m radius).

A leaf was sampled from each male and the mother tree for genetic analysis (Table 1). The height, diameter at breast height (dbh) and flowering effort were recorded for each male sampled. Flowering effort was determined by the number of inflorescences per tree and measured on a scale from 1 to 5 (1 ≤ 10; 2 = 10–20; 3 = 20–50; 4 = 50–75; 5 ≥ 75). Trees that were not flowering because they were juveniles were not considered as candidates and were not sampled. 20–60 seeds (according to permit limitations) from each mother tree were sown in the nursery at the University of the Sunshine Coast (USC, Sippy Downs, QLD, Australia), where 13–44 seeds per individual germinated (Table 1). Leaf tissue from each of the germinated seedlings was collected for genetic analysis.

Genetic diversity and differentiation in adult trees and juveniles

We examined the genetic diversity of *F. picrosperma* and genetic differentiation between age cohorts using 187 adult trees (height > 2.5 m) and 122 juveniles (height ≤ 2.5 m) from nine *F. picrosperma* populations in the 2012–2013 reproductive season (Table 2). For each sampling site, a focal point was randomly selected, and trees were sampled in an expanding radius circling the focal point. The radius expanded to a maximum of 50 m, more typically 30 m, which captured at least 90% of all trees within the clump or cluster. Each sampling site represents one subpopulation. The number of samples from each subpopulation were dependent on-site characteristics including the numbers and density of individuals present. Three subpopulations (focal points) were chosen from across the Boonjie population (Table 2) because Boonjie is the largest population of *F. picrosperma*. The subpopulations, "Boonjie 1 and Boonjie 2" are approximately 400 m apart. "Boonjie 3" is approximately a further 3.5 km east. The three subpopulations are within continuous rainforest but represent discrete clumps.

Table 2 Number of adult and juvenile *F. picrosperma* individuals sampled from each population

Population	A_i	J_i
Evelyn Highlands	24	4
Boonjie 1	26	17
Boonjie 2	38	27
Boonjie 3	17	14
Malanda	12	19
Topaz	13	6
Gadgarra	14	16
East Barron	24	6
Towalla	19	13
Total	163	118

A_i is the number of adults (>2.5 m) sampled, J_i is the number of juveniles (≤2.5 m) sampled in each population

DNA extraction and microsatellite analysis

Genomic DNA was extracted from silica-dried leaf tissue using the DNeasy™ 96-well kit or the DNeasy™ Plant Mini Kit (Qiagen, Valencia, California, USA, Hilden, Germany) following the manufacturer's instructions. Eleven polymorphic microsatellite loci (between 2 and 7 alleles per locus), previously developed and optimized for *F. picrosperma* (Agostini et al. 2013), were used to genotype all sampled individuals following the method detailed in Lamont et al. (2016). A total of 281 *F. picrosperma* individuals (adults and juveniles) were genotyped for the genetic diversity and differentiation study using the 11 loci. An additional six microsatellite loci with consistent PCR amplification, clear allelic variation, and clarity of electrophoretic signatures were developed for *F. picrosperma* and used in the paternity analysis (Table S1). These loci were developed to increase the discriminatory exclusion power of the paternity analysis. A total of 524 individuals comprised of mother trees, seedlings and candidate father trees were genotyped for the paternity analysis.

The forward primer of each locus was direct-labeled with a fluorescent dye (VIC, PET, FAM, NED). Two multiplex PCR pools (Pool 1: FP38, FP68, FP84; Pool 2: FP66, FP69, FP82) were amplified using the Multiplex PCR Plus Kit (Qiagen). Forward and reverse primers for each multiplex pool were combined in a 10× primer mix. Reactions, with volumes adjusted to 10 µL, each contained 1.25 µL of 10× primer premix, 6.25 µL of Qiagen Multiplex Buffer, 3 µL of ddH2O, and 2 µL of template gDNA. Where samples had to be repeated, single PCR reactions, with volumes adjusted to 10 µL, each contained 0.3 µL forward and 0.3 µL reverse primer, 8.425 µL of ddH_2O, 1.5 µL PCR reaction buffer, 1.2 µL dNTP, 1.2 µL $MgCl_2$, 0.075 µL Taq DNA Polymerase and 2.0 µL of template gDNA. Amplification for the microsatellite loci was performed using an Eppendorf Mastercycler (Hamburg, Germany) with cycling conditions as follows: initial denaturation at 95 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 45 s with a final extension at 72 °C for 10 min. PCR products were separated by capillary electrophoresis on an AB 3500 Genetic Analyser (Applied Biosystems). Fragment sizes were determined relative to an internal lane standard (GS-600 LIZ; Applied Biosystems) using GENEMARKER v. 2.4.0 (SoftGenetics LLC, PA, USA) and double-checked manually. A subset of samples were run a second time to assure accuracy of genotype reads and minimize the risk of non-amplifying alleles. Individual loci with low intensity or missing peaks were also amplified and genotyped a second time, after which, if they failed to amplify, they were included as missing data. 97.19 and 99.76% of all loci was successfully amplified and scored for the paternity analysis and for the genetic diversity and differentiation study, respectively.

Statistical analysis

Pollen-mediated gene flow and male fitness

We used CERVUS 3.0.3 (Kalinowski et al. 2007) to perform paternity analysis on seedling cohorts from 10 mother trees using 17 loci. CERVUS uses maximum likelihood for statistical evaluation of progeny-parent pairs (Oddou-Muratorio et al. 2003). Offspring genotypes that conflicted with the assumed mother tree genotypes were excluded before assigning paternal parents. These conflicts arose because seeds were collected from under the canopy of the presumed mother trees and in some instances, the presence of proximate female conspecifics led to field-based misallocation of maternal parents. We ran the program based on the multilocus genotypes of each mother tree and their associated seedlings and the candidate father trees. Simulations on paternity were run using the following parameters: 100,000 simulated offspring, the proportion of mistyped loci was set at 0.01, and the proportion of candidate fathers sampled was estimated at 0.90.

Critical Delta values were obtained from simulations and used as a criterion for parentage assignment. We compared trio Delta scores to assign the father with "strict" (>80%) and "most likely" (<80%) confidence levels. If the seedling received a negative LOD score, no paternal parent was sampled, and the seedling's father was left unassigned. Trio LOD scores were used to determine if there was more than one equally-likely male candidate (equal LOD scores). Two male candidates for each of two seedlings from the mother tree J15 received an equal LOD score and thus were assigned joint paternity. These fathers were allocated a score of 0.5 each for the sired seedling and allocated the "most likely" confidence level. We manually checked the CERVUS assignments and none of the paternity assignments ("strict" or "most likely") had greater than one mismatch between parent pairs and offspring genotypes (i.e., no greater than one trio mismatches).

Null alleles and allelic drop out are likely to occur in microsatellite studies (Ashley 2010). However, simulation studies have shown that parentage assignment using likelihood-based parentage techniques are robust against Type II errors, i.e., when a true parent is excluded due to mistyping at one or more loci (Oddou-Muratorio et al. 2003). Nevertheless, MICRO-CHECKER v2.2.3 (van Oosterhout et al. 2004) was used to check for scoring errors, homozygote excess, large allele dropout and potential null alleles based on 1000 bootstraps. There was no evidence of homozygote excess, scoring errors, large allele dropout or null alleles.

We calculated the maximum pollen immigration rate as the percent of progeny that could not be assigned a father at any confidence level (negative LOD score). We then calculated the conservative minimum pollen immigration rate as the percent of progeny that could not be assigned a father with "strict" (>80%) confidence. *Fontainea picrosperma* is a dioecious species and so the parentage analysis can determine the pollen dispersal distance for each mating event. The spatial position of all candidate fathers was recorded and used to estimate the average distance of pollen dispersal. We compared the frequency distribution of the distances among putative male parents with the frequency distribution of the realized pollination using the Kolmogorov-Smirnov test (K-S test; Sokal and Rohlf 1995) implemented in "R" (R Development Core Team 2013) to determine if mating success was a function of distance between male trees and mother trees.

We assessed the relationship between paternal tree characteristics and male (reproductive) fitness. Individual male fitness was determined by the proportion of seeds sired by a given male on a mother tree. We used both "strict" ($n = 135$) and all CERVUS assignments ($n = 176$) in two separate analyses. Paternal characteristics: height, dbh and flowering effort were significantly autocorrelated (Spearman's Rank correlation; Height × dbh r_s (309) = 0.747, $P = 0.001$; Height × Flower count r_s (309) = 0.729, $P = 0.001$; dbh × Flower count r_s (309) = 0.789, $P = 0.001$). Therefore, we tested for differences in number of seeds sired between categories of flowering effort using a Kruskal–Wallis H test with Bonferroni correction for multiple comparisons. The distances between the mother tree and candidate father trees were grouped into five meter intervals (0–5; 6–10; 11–15; 16–20; 21–25; 26–35 m). We then used a Spearman's Rank correlation to determine the relationship between male fitness and distance to the mother. To determine if the movement of pollinators is influenced by prevailing winds the direction of the candidate male to the mother tree was grouped into eight categories (representing 45°) and then analysed for each mother tree separately using a Spearman's Rank correlation. All inferential analyses were performed using SPSS (IBM SPSS Inc. Released (2016)).

Genetic diversity and differentiation in adult trees and juveniles

The original 11 microsatellite loci reported by Lamont et al. (2016) were used in the genetic diversity and differentiation analysis of adult and juvenile subpopulations. GenAlex 6.5 (Peakall and Smouse 2012) was used to calculate the mean number of alleles per locus (N_A) and expected heterozygosity (H_E) at Hardy-Weinberg equilibrium for adults and juveniles from nine subpopulations. Allelic richness (A_R) and private allelic richness (PA_R) was estimated using HP RARE (Kalinowski 2005) using a minimum sample size of eight. The average pair-wise level of genetic differentiation (F_{ST}) was calculated using multilocus comparisons based on 999 permutations to quantify the partitioning of genetic differentiation

Table 3 Results of the CERVUS analysis of pollen dispersal for the sampled *F. picrosperma* mother trees, showing the number of offspring that had fathers assigned with "strict" (>80%) and "most likely" (<80%) confidence levels, and those that were unable to be assigned

Mother	*n*	Number of seedlings assigned parentage			Number of fathers	
		Strict (%)	Most likely (%)	Unassigned (%)	Strict	All assignments
A156	13	6 (46.1)	3 (23.1)	4 (30.8)	5	7
A336	26	19 (73.1)	3 (11.5)	4 (15.4)	13	16
B27	45	34 (75.6)	0	11 (24.4)	18	18
B283	19	13 (68.4)	0	6 (31.6)	7	7
B595	17	15 (88.2)	0	2 (11.8)	10	10
B706	15	15 (100)	0	0	5	5
E17	19	13 (68.4)	0	6 (31.6)	4	4
J15	20	7 (35)	12 (60)	1 (5)	7	16
J169	20	4 (20)	16 (80)	0	4	14
J424	20	9 (45)	7 (35)	4 (20)	8	14
Total	214	135 (63.1)	41 (19.2)	38 (17.7)	81	111

Percentages of each category in relation to the total number of seedlings sampled from each mother tree are shown in parentheses. Number of fathers for assigned seedlings of each *F. picrosperma* mother tree showing the number of male candidates which could be assigned paternity under conditions of "strict" confidence and the total number able to be assigned ("strict" and "most likely")

n is the number of seedlings

between adult trees and juveniles in each subpopulation using GenAlex 6.5 (Peakall and Smouse 2012).

Statistical comparisons were carried out in IBM SPSS 24 (IBM SPSS Inc. Released 2016) to determine whether there were significant differences in diversity (H_E, A_R, PA_R) between the adult trees and juveniles for each subpopulation. All data sets did not meet the assumptions of parametric tests and were subsequently compared using Mann–Whitney *U*-tests.

We calculated mean within group pairwise genetic relatedness (*r*) for each subpopulation of adults and juveniles sampled using Lynch and Ritland (1999) (l_{xy}) estimator in GenAlex (Peakall and Smouse 2012). Significant differences in mean relatedness were tested using 9999 permutations and 9999 bootstrap resamplings to calculate the upper and lower 95% confidence intervals for the expected range of l_{xy} based on the sampled population and within subpopulation estimates of mean relatedness. Subpopulation l_{xy} values that fall above the 95% expected values from permutations indicate a higher degree of relatedness that expected from random mating across the sampled population.

Results

Pollen-mediated gene flow and male fitness

From the sampled population, 135 (63.1%) of the 214 individual seedlings tested could be assigned paternity with "strict" confidence (Table 3). An additional 41 (19.2%) seedlings were assigned a father when considering the "most likely" CERVUS assignments (Table 3). The males assigned as the "most likely" father had zero (87%) or one (13%) loci mismatch. The "most likely" father could not be assigned paternity with "strict" confidence because the second (or more) "most likely" father also had zero or one mismatches and thus the delta score was close to zero. Therefore, we believe that false mismatches have occurred and up to 82.3% of fathers could be assigned to offspring when accounting for all CERVUS assignments ("strict and most likely"). However, our sampling method of a 30 m radius surrounding the mother tree can potentially downwardly bias the results of the "most likely" father assignments towards short distance pollen flow. As such, the results of the "most likely" CERVUS assignments must be received with caution.

The total mean pollen immigration rate from greater than 30 m was 36.9% (maximum pollen immigration rate; $n = 79$; Fig. 1a) when considering fathers that could be assigned with "strict" confidence and 17.7% (minimum pollen immigration rate; $n = 38$) when considering all CERVUS assignments (Fig. 1b).

We found that 27% ($n = 81$) of the 300 candidate fathers sampled, sired seedlings from the 10 mother trees tested (Table 3), when using "strict" CERVUS assignments (cf. 37% of candidate fathers for "all" CERVUS assignments). Mother tree E17 had one of the lowest number of fathers for the seedling cohort sampled due to the low number of available candidate fathers in the small, isolated population. Most of the assigned fathers sired only a single seed (Fig. 2), and these seeds represented 74.1% of the total assigned offspring (cf. 74.3% for "all" CERVUS assignments). In contrast, one or two males sired greater than 10% of the total progeny for each mother tree (Fig. 2).

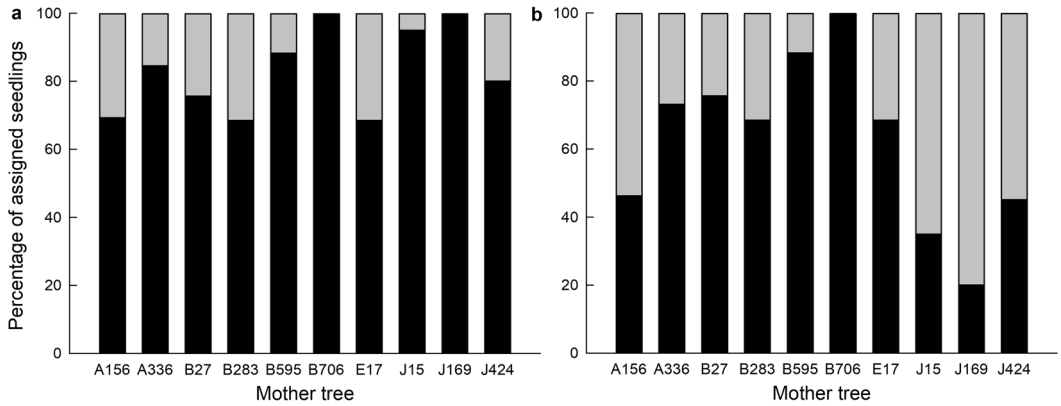

Fig. 1 Percentage of local and immigrant pollinations for each mother tree (number of seedlings per tree: A156 = 13; A336 = 26; B27 = 45; B283 = 19; B595 = 17; B706 = 15; E17 = 19; J15 = 20; J169 = 20; J424 = 20). **a** All assignments ('strict and most likely') are shown in black and unassigned seedlings (minimum pollen immigration rates) are shown in gray. **b** "Strict" assignments (seedlings assigned with >80% confidence) are shown in black and seedlings assigned with <80 % confidence (maximum pollen immigration rates) are shown in grey

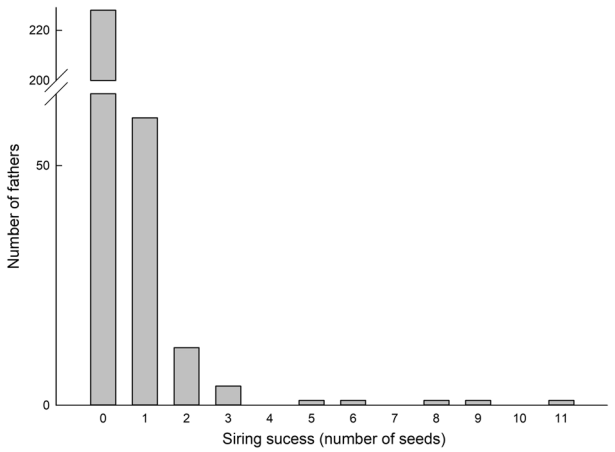

Fig. 2 Total number of candidate fathers vs. number of seedlings assigned with "strict" confidence

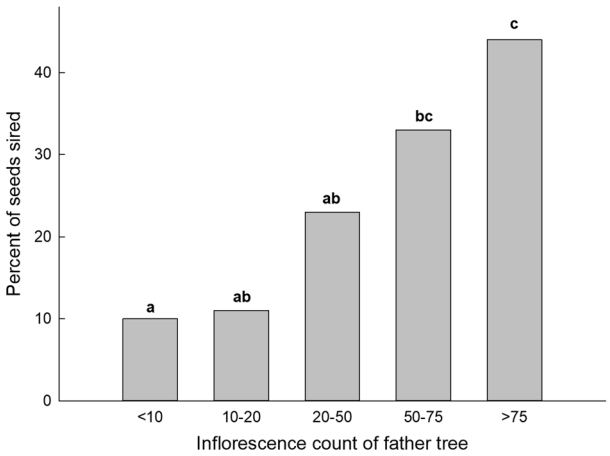

Fig. 3 Percentage of seeds per mother tree sired by fathers relative to male inflorescence number (assigned with "strict" confidence). Categories with different letters are significantly different ($P < 0.05$, Kruskal–Wallis H-test, Stepwise step-down Bonferroni correction)

Siring success was significantly higher for candidate fathers with more flowers (X^2 (4) = 16.74, $P = 0.002$, Fig. 3). No significant relationship existed between the direction of the assigned fathers to the mother trees ($P > 0.05$) for all but one mother tree, J424 (r_s (37) = −0.351, $P = 0.033$), thus wind direction is unlikely to influence the movement of pollinators that results in successful reproduction. The relationship between the number of offspring sired by pollen donors and the distance between the maternal and paternal trees was not significant (r_s (363) = −0.080, $P = 0.127$) for "strict" assignments. The frequency curve of pollen dispersal was significantly different to the frequency curve of distance measured among all male trees relative to the respective 10 mother trees (Kolmogorov–Smirnov test ($D = 0.14743$, $P =$ 0.03367) suggesting a non-random distribution of pollen distances (Fig. 4). The median pollen distance was 15 m for both "strict" and "all" CERVUS paternity assignments (Fig. 4).

Genetic diversity and differentiation in adult trees and juveniles

Moderately low levels of genetic diversity were detected, with a total of 43 and 41 alleles resolved across the 11 microsatellite loci used in the analysis of the 187 adults and 122 juveniles, respectively. Mean number of alleles per locus per subpopulation was 2.687 for adult trees and 2.384 for juveniles (Table 4). Mean expected heterozygosity (H_E) for adult trees was 0.405 and 0.379 for juveniles (Table 4).

No significant differences were found in the mean expected heterozygosity (H_E), allelic richness (A_R) and private allelic richness (PA_R) between adult and juvenile subpopulations ($P > 0.05$). Pairwise population F_{ST} values

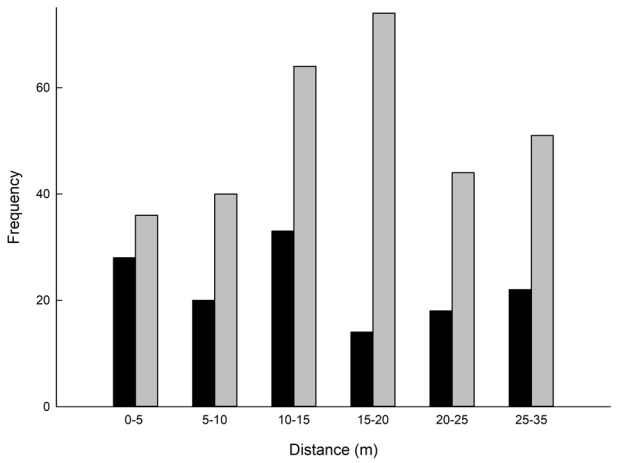

Fig. 4 Frequency distributions of pollen dispersal distances for seeds assigned with "strict" (>80%) paternity. Black bars represent inter-tree distances between the mother tree and male trees with successful pollination events. Grey bars represent the distance between the mother tree and all candidate male trees that were sampled

also displayed negligible genetic differentiation between generations (Table 4).

We found that the mean pairwise relatedness (r) of individuals within adult and juvenile subpopulations were significantly higher ($P > 0.05$) than the simulated confidence intervals for all subpopulations. This indicates that individuals within subpopulations have significantly higher measures of relatedness than expected from mating from two random individuals.

Discussion

Pollen-mediated gene flow and male fitness

Our study has detected short distance pollen flow in the subcanopy rainforest tree, *F. picrosperma*. We observed that many males contributed to reproduction and most fathers sired a single seed on the mother tree. Large males with high flowering intensity had a disproportionally higher reproductive success and at least two thirds of successful

Table 4 Comparison of summary genetic measures between 187 adult and 122 juvenile *F. picrosperma* sampled from nine subpopulations

Subpopulation	n	N_A	H_E	A_R	PA_R	F_{ST}	r
Adult subpopulations							
Evelyn Highlands 1	24	2.636 (0.31)	0.367 (0.06)	2.1	0.05	0.023	0.179
Boonjie 1	29	3.182 (0.35)	0.517(0.04)	2.52	0.03	0.018	0.042
Boonjie 2	38	3.000 (0.27)	0.476 (0.03)	2.29	0.1	0.015	0.059
Boonjie 3	17	2.727 (0.20)	0.424 (0.04)	2.2	0.01	0.025	0.140
East Baron	24	2.818 (0.35)	0.405 (0.06)	2.14	0.09	0.001	0.155
Malanda	12	2.545 (0.28)	0.448 (0.05)	2.26	0	0	0.120
Topaz	13	2.818 (0.38)	0.373 (0.05)	2.2	0.02	0.011	0.159
Gadgarra	14	2.091 (0.21)	0.257 (0.06)	1.73	0	0.003	0.285
Towalla	19	2.364 (0.28)	0.377 (0.05)	2.03	0	0	0.133
Mean		2.687 (0.11)	0.405 (0.02)	2.16	0.03	0.011	–
Juvenile subpopulations							
Evelyn Highlands 1	4	1.818 (0.24)	0.307 (0.08)	1.82	0	–	0.224
Boonjie 1	17	2.545 (0.21)	0.447 (0.03)	2.15	0.03	–	0.160
Boonjie 2	27	2.909 (0.25)	0.453 (0.04)	2.26	0.04	–	0.061
Boonjie 3	14	2.545 (0.21)	0.386 (0.05)	2.09	0	–	0.119
East Baron	6	2.000 (0.19)	0.340 (0.06)	1.91	0	–	0.189
Malanda	19	2.545 (0.25)	0.436 (0.06)	2.23	0.01	–	0.092
Topaz	6	2.455 (0.34)	0.360 (0.07)	2.17	0.05	–	0.164
Gadgarra	16	2.364 (0.24)	0.326 (0.06)	1.9	0.02	–	0.211
Towalla	13	2.273 (0.19)	0.359 (0.06)	1.96	0	–	0.166
Mean		2.384 (0.11)	0.379 (0.02)	2.05	0.02	–	–

Mean subpopulation values averaged across the 11 loci are shown with standard errors in parentheses where calculated

n is the number of individual plants; N_A is the mean number of alleles per locus, H_E is the expected heterozygosity, A_R is the allelic richness, PA_R is the private allelic richness, F_{ST} is the genetic differentiation among populations, r is the average pairwise relatedness within subpopulations

mating events occurred with male trees located within a 30 m radius of the mother tree.

Our findings demonstrate that pollen dispersal in *F. picrosperma* in the subcanopy occurs over short distances compared to many insect-pollinated canopy trees (Akihiro et al. 2000; Kenta et al. 2004; Ward et al. 2005; Hardesty et al. 2006; Born et al. 2008; Monthe et al. 2017). The long dispersal distances reported for canopy species are partly because these taxa generally occur at low population densities. If only distant trees are flowering, pollinators must travel long distances to locate resources. While the breakdown of nearest-neighbor mating can occur (Dick et al. 2008), pollen dispersal patterns of many insect-pollinated tropical trees are influenced by preferential visitations to close neighboring trees (Silva et al. 2011; Theim et al. 2014; Noreen et al. 2016). The short distance pollen dispersal we observed for *F. picrosperma* can be partly attributed to the species clumped distribution and synchronous flowering. Pollinators are preferentially visiting trees within the clump of *F. picrosperma* such that on average, two-thirds of the successful reproductive events occurred within a 30 m radius of the mother tree. Other studies of rainforest understorey and subcanopy species have also found a high proportion of short distance pollination events, for example, in *Piper* shrub spp. that have high density, aggregated populations (Lasso et al. 2011), and *Rhododendron simsii*, that have highly synchronous flowering events (Hahn et al. 2017). Many males contributed to successful reproduction of individual *F. picrosperma* females with approximately 75% of the assigned fathers siring a single seed. Together, these findings conform to the theory of density-dependent animal pollination, which assumes that tree species occurring at low densities receive pollen from fewer individuals than trees in denser populations, where dispersal distances are lower (Murawski and Hamrick 1991; Bianchi and Cunningham 2012; Castilla et al. 2017).

Spatial considerations as well as the attractiveness of floral displays of individual trees are important factors in determining pollinator-assisted gene flow (Barrett and Harder 1996; Degen and Roubik 2004; Duminil et al. 2016). Our results show that while many male trees sired seeds, the contributions to reproduction were uneven. Consistently, only one or two males were responsible for a proportionally greater number of successful fertilization events across all 10 mother trees. As expected, large males displaying high intensity flowering had significantly greater reproductive success than males with less flowers. Flower count was autocorrelated with tree stem diameter size (dbh) in our study, this finding is congruent with other studies of tropical trees where dbh size class was positively correlated with mean individual fecundity (Latouche-Hallé et al. 2004; Naoki et al. 2012; Monthe et al. 2017). Greater biomass has been found to result in greater male fitness in many plant species (Younginger et al. 2017), and may help to explain the skewed number of fathers found to be siring progeny in *F. picrosperma*.

In addition, plant-pollinator relationships can influence pollen dispersal distances. The floral structure of *F. picrosperma* suggests that it is likely to be pollinated by small generalist insects (Grant et al. 2017). The predominant floral visitors in Australian tropical forests are small insects, particularly beetles, flies, small bees, and thrips (Irvine and Armstrong 1991; Gross 2005). This class of generalist pollinators are known to visit unspecialized flowers and often move shorter distances compared to specialized insects, larger insects, or vertebrates (Dick et al. 2008). The 18–37% pollen immigration rate suggests that some pollinator's of *F. picrosperma* are able to transport pollen greater than 30 m. However, it remains unclear how far pollen can travel beyond this radius. We used direction to the mother tree as a proxy for prevailing wind conditions and did not find any significant correlation between mother trees and the direction of the pollen donors within the sampled plot area. This is presumably because of the lack of wind and/or turbulent wind patterns under the dense rainforest canopy. Gene flow in *F. picrosperma* is also limited by the transport of pollen by pollinators (Grant et al. 2017), which further reduces opportunities for long-distance pollen flow.

While the mean pollen dispersal distance herein is potentially underestimated due to our sampling method that aligned with the approximate clump size, we could confidently assign 63% of successful pollen donors from within the plot. Distance between the mother tree and the pollen source was not significantly correlated. This statistic is somewhat confounded by the fine (~30 m) scale of this study and given the localized pollination rate, distance, when analysed over a larger scale, is likely to be an important factor in reproductive success. Particularly given that *F. picrosperma* is dioecious and pollen-mediated gene flow is not restricted by self-pollination. Our results implying predominantly short distance pollen dispersal suggests that cultivation of the fruit will require a significant number of males within the carefully designed plantation to increase efficient pollination.

We believe the maximum pollen immigration rate (37% greater than 30 m) observed in this study is conservative due to the low genetic diversity of *F. picrosperma* and subsequently low discriminatory power of the microsatellite markers used in this study. False negatives are likely to have occurred as a great majority (87%) of the males assigned the "most likely" father had zero loci mismatches with the offspring when accounting for the mother's genotype. Thus, localized pollination events could be up to 82% when accounting for all paternity assignments. However, it is important to highlight that the fine scale of our study has the

potential to downwardly bias the calculated dispersal range estimates when accounting for fathers assigned as "most likely". Moreover, our results are reflective of a single reproductive year and we acknowledge that pollinator composition can change over space and time (Dick et al. 2008; Kenta et al. 2004) and that natural flower and seed production can be influenced by natural climatic variations between years, all of which can produce different patterns of gene flow.

Genetic diversity and differentiation in adult trees and juveniles

The genetic diversity measures reported in this study are congruent with similar populations of *F. picrosperma* reported by Lamont et al. (2016) and for the related species *F. rostrata* (Conroy et al. 2019). Summary measures of genetic diversity were almost identical for both *F. picrosperma* adult and juvenile cohorts (H_E, A_R, PA_R; $P > 0.05$). Measures of diversity such as overall allelic richness (2.16 in adult trees and in 2.05 juveniles) and expected hetrozygosity (H_E) (0.405 for adult trees and 0.379 for juveniles) are relatively low when compared to microsatellite-based studies on tropical rainforest tree species reported elsewhere. For example, reports of H_E in outcrossing rainforest taxa range between 0.732 and 0.907 (Collevatti et al. 2001b; Naito et al. 2005; Carneiro et al. 2009; Sebbenn et al. 2011; Melo and Franceschinelli 2016; Monthe et al. 2017) while self-compatible species ($H_E = 0.629–0.797$; Latouche-Hallé et al. 2004; Tani et al. 2009), and species surviving in highly fragmented populations ($H_E = 0.662–0.701$; Gaino et al. 2010; Wang et al. 2014) are still considerably higher than *F. picrosperma*. From the limited studies available, the genetic diversity of *F. picrosperma* is more akin to other Australian rainforest tree species including *Elaeocarpus angustifolius* ($H_E = 0.61$) and *E. largiflorens* ($H_E = 0.54$; Rossetto et al. 2007) and species with reported low genetic diversity due to mechanisms such as asexual reproduction (Rossetto et al. 2004; Rossetto and Kooyman 2005; Thurlby et al. 2012).

Genetic variation is often significantly related to population fitness and hence, the evolutionary potential of a species (Reed and Frankham 2003). Yet *F. picrosperma* has successfully persisted through historical environmental changes with low genetic diversity (Lamont et al. 2016). Euphorbiaceae is a family that exemplifies the post-Cretaceous diversification of the Australian rainforest flora and *Fontainea* first appears in the fossil record in the early Tertiary period (Williams and Adam 2010). Expansion and contraction of *F. picrosperma* populations during the climatic oscillations of the Pleistocene over the last 230,000 years (Kershaw et al. 2007) has likely reduced the level of genetic variation within the species, as found in this study and by Lamont et al. (2016), compared to other plant species in biomes with more diverse topography and greater elevation range (Broadhurst et al. 2017). These processes have led to low genetic diversity in other upland taxa in the Australian tropics, such as *Elaeocarpus* spp. (Rossetto et al. 2009). Species with natural restricted geographical range are also usually less genetically diverse than more widespread species (Arguilar et al. 2008). Habitat fragmentation and degradation that has occurred in the region since European settlement could also have contributed to a loss of genetic diversity due to a reduced number of local and immigrant pollen sources in some populations (Sork and Smouse 2006).

Spatially limited pollen and seed gene dispersal is known to increase the likelihood of similar genotypes mating with each other (Seidler and Plotkin 2006; Ellstrand 2014). We found evidence that individuals were significantly more related than is expected between two random individuals within the adult and juvenile subpopulations studied. These results are concordant with the predominantly short distance pollen flow indicated from our paternity analysis. Theoretically, selfing and mating between close relatives will increase differentiation among populations (F_{ST}) by increasing inbreeding (Duminil et al. 2009) and reducing genetic variation at the population level (Loveless and Hamrick 1984). However, we found a lack of genetic differentiation between *F. picrosperma* adults and juveniles (represented by a low F_{ST} value), despite the adult cohort representing a larger sample of the total available genetic diversity due to a greater number of overlapping generations present than in the juvenile group. The 18–37% pollen immigration rate (greater than 30 m) estimated by paternity assignments could contribute to the lack of differentiation between age cohorts. Only low levels of gene flow are necessary to counteract opposing mutation, drift and selection (Ellstrand 2014). Immigrant pollen can connect populations through gene flow and some empirical evidence has suggested that long distance pollination events attenuate genetic decline due to drift and inbreeding in isolated populations (Ashley 2010). While we do not know how far pollen can travel, Lamont et al. (2016) found recent bottlenecks with subsequent founder effects in two isolated populations of *F. picrosperma*, Malanda (centrally located) and Gadgarra (North-East). This implies that pollen may not travel long distances from the refugial populations of Boonjie and Evelyn Highlands, which are located in the east and west peripheries of the species natural distribution (Lamont et al. 2016).

The lack of genetic differentiation between adult and juvenile groups observed in this study remains consistent with Lamont et al. (2016) who found negligible levels of inbreeding within adult populations of *F. picrosperma*, which is expected in a dioecious species. Deleterious alleles may be purged through an increase in mortality in inbred

individuals and survivorship of those composed of half or unrelated siblings (Hufford et al. 2003; Naito et al. 2005; Tambarussi et al. 2017). This is a common pattern in long-lived species (Duminil et al. 2009). In addition, the large number of males contributing to reproduction of a single seed found in this study suggests that heterogenic pollen pools are received on flowering females. This can maintain variability, reduce the occurrence of full sibling progeny arrays and dilute the effects of kin mating (Breed et al. 2012b). This result may reflect the synchronous flowering and high density of available fathers surrounding the mother tree. However, not all candidate fathers sired offspring. We acknowledge that inbreeding depression can affect initial seed set as well as plant growth (references within Angeloni et al. 2011), which was not studied here.

More than half of the mating events in *F. picrosperma* occur over very limited spatial scales. Population genetic theory suggests that restricted gene flow among populations results in population differentiation and allows populations to evolve independently in response to genetic drift or local natural selection (Slatkin 1987; Ellstrand 1992), which has been demonstrated empirically in some tropical understorey species (Lasso et al. 2011; Theim et al. 2014). In comparison, low genetic differentiation among tropical tree populations has been interpreted as evidence of continuous long-distance, historical gene flow (Dick et al. 2008). *Fontainea picrosperma* is a species with low interpopulation F_{ST} (Lamont et al. 2016), however, in the context of low genetic diversity, the short distance pollen dispersal is likely to have contributed to the significant, albeit weak population structuring across *F. pirosperma's* natural distribution. The observed low levels of genetic differentiation and genetic diversity between adult and juvenile cohorts, and the determination that proximate plants are significantly related to each other suggests that this species is highly adapted to its environment, short distance pollen flow does not affect the species capacity to persist in the environment, and there is sufficient long-distance gene flow to keep the level of genetic diversity stable across the species distribution. Future cultivation of the species however, may benefit from mixing genetically dissimilar stocks from across *F. picrosperma's* natural distribution as a means of increasing allelic diversity.

Conclusion

Our results reveal spatially limited gene dispersal in the subcanopy species, *F. picrosperma*. Size and flowering effort are more important than distance in determining male fitness at the fine-scale focus in this study. The species' short-distance gene dispersal and skewed success rate of pollen donors is potentially a prelude to genetic impoverishment. However, adult and juvenile subpopulations have similar multilocus genotypes and there is no evidence of an intergenerational loss of diversity. Furthermore, *F. picrosperma* has survived several significant climatic oscillations through the Pleistocene, likely by persisting in refugia offering a more stable environment and it is likely that the low genetic diversity observed in *F. picrosperma* is an indication of its significant adaption to local environmental conditions. *Fontainea picrosperma* is geographically confined to a region that has been subjected to intense anthropogenic habitat fragmentation since European settlement. It may be that the species' natural clumped distribution coupled with predominantly short distance pollen dispersal helps to attenuate population genetic pressures due to habitat fragmentation. Despite the species' ongoing resilience, low overall genetic diversity may compromise *F. picrosperma*'s ability to adapt to changing environmental conditions and extreme stochastic events. Therefore, it is important to conserve the remaining populations of *F. picrosperma* to ensure that the current level of genetic diversity is maintained for both conservation and domestication of this commercially significant species.

Data archiving

Data have been deposited at Dryad: https://doi.org/10.5061/dryad.73pn293.

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Compliance with ethical standards

Conflict of interest EcoBiotics Ltd partly funded this research. S.M.O. is a director and shareholder of QBiotics Group Ltd. P.W.R. is a director, employee and shareholder of EcoBiotics Ltd and QBiotics Group Ltd. The remaining authors declare that they have no conflict of interest.

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