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ORIGINAL ARTICLE

Pollen flow in fragmented landscapes maintains genetic diversity following stand-replacing disturbance in a neotropical pioneer tree, *Vochysia ferruginea* Mart

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In forests with gap disturbance regimes, pioneer tree regeneration is typically abundant following stand-replacing disturbances, whether natural or anthropogenic. Differences in pioneer tree density linked to disturbance regime can influence pollinator behaviour and impact on mating patterns and genetic diversity of pioneer populations. Such mating pattern shifts can manifest as higher selfing rates and lower pollen diversity in old growth forest populations. In secondary forest, where more closely related pollen donors occur, an increase in biparental inbreeding is a potential problem. Here, we investigate the consequences of secondary forest colonisation on the mating patterns and genetic diversity of open-pollinated progeny arrays for the long-lived, self-compatible pioneer tree, *Vochysia ferruginea,* at two Costa Rican sites. Five microsatellite loci were screened across adult and seed cohorts from old growth forest with lower density, secondary forest with higher density, and isolated individual trees in pasture. Progeny from both old growth and secondary forest contexts were predominantly outcrossed ($t_m = 1.00$) and experienced low levels of biparental inbreeding ($t_m - t_s = 0.00$–0.04). In contrast to predictions, our results indicated that the mating patterns of *V. ferruginea* are relatively robust to density differences between old growth and secondary forest stands. In addition, we observed that pollen-mediated gene flow possibly maintained the genetic diversity of open-pollinated progeny arrays in stands of secondary forest adults. As part of a natural resource management strategy, we suggest that primary forest remnants should be prioritised for conservation to promote restoration of genetic diversity during forest regeneration.
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INTRODUCTION

Widespread deforestation over the last 50 years has led to substantial, changes in tropical forest habitats (Ghazoul and McLeish, 2001; Pearce, 2001; Putz *et al.*, 2001). However, with the recent increase in urban migration and abandonment of agricultural land, secondary forest now covers a greater area than old growth forest in many tropical countries (Aide and Grau, 2004). Furthermore, secondary forests are rapidly becoming important economic resources and ecosystems in their own right (Finegan, 1992; Aide and Grau, 2004; Piotto *et al.*, 2004).

Pioneer species tend to be dominant in forests with 'stand-replacing' disturbance regimes, where cyclic removal of most or all trees is followed by colonisation. These species tend to occur at lower density in old growth forest but at very high densities in recently colonised sites (Noss, 1999). The colonisation process that follows stand-replacing disturbance typically drives changes to tree mating patterns and population genetic diversity, genetic structure and gene flow (Lowe *et al.*, 2005; Davies *et al.*, 2010). These patterns distinguish forest established under 'stand-replacing' disturbance regimes from old growth forests (Davies *et al.*, 2010). Consequently, devising effective management strategies to maintain genetic diversity in landscapes harbouring a significant secondary forest component requires an understanding of how gene flow and mating patterns respond to colonisation dynamics following stand-replacing disturbance.

Some of the genetic changes that arise during colonisation are predictable. For example, where progeny of only a few individuals make up the colonising population a genetic bottleneck can result. Genetic diversity in these populations is typically lower than the source population (Aldrich and Hamrick, 1998; Sezen *et al.*, 2005; Dlugosch and Parker, 2008; Davies *et al.*, 2010). In addition, multiple independent colonisation bottlenecks can result in secondary forests exhibiting stronger genetic structure than populations in old growth forest (Sezen *et al.*, 2005; Davies *et al.*, 2010). Furthermore, although tropical tree species predominantly outcross (Ward *et al.*, 2005), many species have flexible mating systems (Dick *et al.*, 2008; Breed *et al.*, 2012). Such flexibility may be advantageous at the low population densities typical of tropical forest (Pitman *et al.*, 2001), as it provides reproductive assurance against low or no pollen flow between isolated individuals (Eckert *et al.*, 2010; Breed *et al.*, 2012). Indeed, density appears to be an important factor in determining mating patterns for many tropical tree species (Breed *et al.*, 2015). For example, in *Helicteres brevispira*, outcrossing rates were observed to be greatest in

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high-density populations (Franceschinelli and Bawa, 2000). Outcrossing rates were observed to decline considerably in lower density and selectively logged populations of *Shorea curtisii* (Obayashi *et al.*, 2002) and low-density populations of *Neobalanocarpus heimii* (Naito *et al.*, 2005). Therefore, mixed mating is likely to be advantageous to pioneer species as density may vary from low to very high, and good examples have been documented (for example, *Senna multijuga*, Ribeiro and Lovato, 2004).

Far-reaching pollen flow is likely to be an adaptive strategy for tropical canopy trees as it achieves reproductive assurance in low-density populations, a pattern typical of many pioneer species in old growth forests (Nason *et al.*, 1998; Ward *et al.*, 2005; Cloutier *et al.*, 2007; Dick *et al.*, 2008). However, pollen flow may be particularly sensitive to density changes (Franceschinelli and Bawa, 2000; Degen *et al.*, 2004; Kenta *et al.*, 2004; Jones and Hubbell, 2006; Breed *et al.*, 2015). For example, increasing density has been found to decrease pollen flow distances in tropical tree species (Degen *et al.*, 2004), and where populations are fragmented or occur at low density, pollen flow distances may increase (White *et al.*, 2002; Dick *et al.*, 2008; Latouche-Halle *et al.*, 2004; Lowe *et al.*, 2005; Jha and Dick, 2010). In addition, long-distance seed dispersal is an expected characteristic of pioneer tree species, as it allows them to take advantage of disturbance opportunities (for example, gaps in the canopy, forest clear-felling and cleared agricultural pastures) (Kaufman *et al.*, 1998).

Together, genetic bottlenecks, mating patterns and gene flow will interact to shape patterns of genetic diversity in secondary forest populations. For example, where a dense secondary population is formed from a few related founders, aggregation of genotypes can lead to biparental inbreeding (matings among related individuals; Ennos and Clegg, 1982; Vekemans and Hardy, 2004). This effect was observed in *Dryobalanops aromatica*, where higher values of correlated and biparental matings occurred in disturbed and high-density populations (Lim *et al.*, 2002). Where there is limited seed or pollen dispersal, biparental inbreeding may increase genetic structuring, resulting in a feedback that maintains the clustering of related individuals and further increases the potential for inbreeding depression within populations (Zhao *et al.*, 2009; Dubreuil *et al.*, 2010). For pioneer species, these synergies are likely to result in a trade-off between rapid colonisation of forest gaps and inbreeding depression costs arising from colonisation by many related individuals. However, the risk of high biparental inbreeding should be dampened by far-reaching gene flow.

For a pioneer species that occurs at low-density within old growth forest and forms dense, even-aged stands following stand-replacing disturbance, several predictions can be made. If mating patterns are flexible, low-density old growth populations should show higher levels of selfing and lower levels of pollen diversity due to greater isolation. In contrast, recently established, high-density populations should have higher outcrossing rates, but accompanied by higher levels of biparental inbreeding due to spatial genetic structuring.

Here, we tested these predictions in a study of *Vochysia ferruginea*, a self-compatible, long-lived neotropical pioneer tree species (*sensu* Finegan, 1996). It forms dense, even-aged stands following both natural (Boucher *et al.*, 1994; Boucher and Mallona, 1997) and human (Finegan and Delgado, 2000) stand-replacing disturbance. In a previous analysis of this species, genetic diversity was observed to decrease and spatial structure increase markedly across an age cohort gradient from low-density old growth forest to high-density secondary forest, to very high-density seedling cohorts (Davies *et al.*, 2010).

Here, we assess mating patterns and genetic diversity shifts in open-pollinated progeny arrays sampled from four populations: two in low-density old growth and two in high-density secondary forest stands.

MATERIALS AND METHODS

Species and study site

V. ferruginea (Vochysiaceae) is widely distributed in the neotropics from Nicaragua to the Amazon basin. It is a potentially important reforestation and timber species as it is very fast growing and tolerant of low nutrient and high aluminium soils (Finegan, 1992; Herrera and Finegan, 1997). Stands of *V. ferruginea* often flower synchronously, where flowers are hermaphroditic and visited by a variety of pollinators: primarily bees, with occasional visits by moths, butterflies and hummingbirds (Oliveira and Gibbs, 1994; Arnáez and Moreira, 1995). In controlled pollination trials, Bawa *et al.* (1985a) reported *V. ferruginea* as largely self-compatible and self-pollinated flowers did not differ in fruit set relative to cross-pollinated flowers (Bawa *et al.*, 1985b). The seeds of *V. ferruginea* appear to be dispersed mainly by wind, although occasional bird dispersal is possible.

Samples were collected from two lowland sites in northern Costa Rica's San Juan-La Selva Biological Corridor (see Sesnie *et al.*, 2009). Both sites were tropical wet forest with mean annual precipitation of 3864 mm and mean annual temperature of 24.5 °C (Finegan and Camacho, 1999). The first site, Tirimbina Biological Reserve (Tirimbina), is a privately owned conservation area that consists mainly of old growth forest with small patches of secondary forest of ages currently in the range of 25 to 45 years old. Areas of permanent crops are also present on Tirimbina. The second site, Ladrillera, is a privately owned cattle farm with secondary forest ~25 years old that is adjacent to a large old growth forest fragment (see Schedlbauer *et al.*, 2007). *V. ferruginea* dominates the secondary forest that extends into pasture to the southwest of the old growth fragment at Ladrillera. Ladrillera pasture is regularly cut to prevent tree colonisation, except on steep ground where cutting is less frequent and *V. ferruginea* can colonise.

Sampling, DNA isolation and genotyping

Twenty seeds were collected from 12 to 20 mother trees in each of the four populations (old growth and secondary forest stands at both Tirimbina and Ladrillera). These mother trees were previously sampled as described by Davies *et al.* (2010). An additional two remnant trees situated in abandoned plantation adjacent to the Tirimbina site also had 20 seeds collected (Table 1).

DNA was extracted from leaf tissue, germinated seeds and cambial material using a DNeasy 96 Plant Kit (Qiagen, Hilden, Germany). All progeny were screened at five nuclear microsatellite loci previously developed by Lowe *et al.* (2002) for *V. ferruginea*. These loci included A1-5, A1-10, A1-15, A1-20 and A1-35. Amplification was performed in 15 µl PCR containing the following materials: 1–5 ng DNA, polymerase buffer (1 × volume, Promega, Madison, WI, USA), 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.2 µM of each primer, 2% dimethyl sulfoxide (DMSO, Anachem, Luton, UK), 0.5 unit Taq DNA polymerase (Promega), and made up to volume using dH_20. A touchdown PCR protocol was used with the following conditions: initial denaturation at 95 °C for 3 min; 11 cycles of 95 °C for 15 s, 65 °C for 25 s, 72 °C for 35 s with annealing temperatures decreasing 1 °C per cycle; followed by 25 cycles with the annealing temperature at 55 °C; a final extension step at 72 °C for 15 min.

Mating patterns and genetic diversity parameters

We estimated the following mating parameters for each population using the maximum likelihood procedures using the mixed mating

Table 1 Estimates of mating patterns using MTLR (Ritland 2002)

	Density (trees per ha)	*Progeny array size*	t_m	$t_m - t_s$	r_p
Tirimbina old growth forest	6.4	19.75 (0.63)	1.00 (0.05)[a]	0.00 (0.04)[a]	0.24 (0.05)[a]
Tirimbina secondary forest	40.6	19.08 (1.89)	1.00 (0.06)[a]	0.00 (0.05)[a]	0.27 (0.07)[a]
Tirimbina pasture trees		20.00 (0.00)	1.00 (0.00)[a]	0.00 (0.04)[a]	0.01 (0.12)[b]
Ladrillera primary forest	2.5	19.85 (0.37)	1.00 (0.02)[a]	0.04 (0.02)[a]	0.22 (0.04)[a]
Ladrillera secondary forest	241.5	19.05 (2.63)	0.93 (0.05)[a]	0.04 (0.02)[a]	0.21 (0.03)[a]

t_m, multilocus outcrossing rate; $t_m - t_s$, biparental inbreeding; r_p, multilocus correlated paternity; standard deviation in parentheses; 95% confidence interval homogeneous subgroups of mating patterns are indicated by 'a' and 'b'.

model in MLTR (Ritland 2002): multilocus outcrossing rate (t_m), biparental inbreeding ($t_m - t_s$) and multilocus correlated paternity (r_p). To calculate variance estimates, we bootstrapped families within each population 1000 times. To investigate the exclusion power of these loci, we estimated the per locus probability of paternity exclusion (Q) and combined probability of paternity exclusion (QC) in GENALEX (Peakall and Smouse, 2006).

We analysed the genetic diversity of all progeny (groups as described above) and adult trees ($n = 99$–120) using the same microsatellite loci. These adult trees were previously investigated in Davies *et al.* (2010). For all these data, we estimated Nei's unbiased expected heterozygosity (H_E; Nei, 1973) in GENALEX (Peakall and Smouse, 2006) and, to account for differences in sample size, we adjusted the mean number of alleles per locus (AR) by rarefaction, using the software HP-RARE (Kalinowski, 2005).

To investigate how fine-scale mating system dynamics might be affected by population density, outcrossing rate and the effective number of pollen donors were correlated with the density of potential pollen donors (all adult trees within the plot) around mother trees. Multilocus outcrossing (t_m) and number of pollen donors ($1/r_p$) were estimated within families using MLTR (Ritland 1990, 2002) and plotted against the number of trees within 100 m of the mother trees.

RESULTS

Mating patterns

The combined probability of paternity exclusion if neither parent is known indicates good resolution for the genetic markers used in this study (QC = 0.99). Results from both sites showed that *V. ferruginea* was predominantly outcrossed in both old growth and secondary forest populations, and also when found as isolated remnant trees ($t_m = 0.93$ to 1.00; Table 1). All populations and isolated remnant trees experienced very low biparental inbreeding ($t_m - t_s = 0.00$–0.04). Old growth and secondary forest populations from both sites had similar levels of correlated paternity ($r_p = 0.21 - 0.27$), but the isolated pasture trees had significantly lower levels of correlated paternity ($r_p = 0.01$). Levels of outcrossing and correlated paternity did not change with density in any population (r^2 all < 0.12, *P*-values all > 0.05; see Appendix S1 in Supplementary Information for table of correlations).

Genetic diversity of open-pollinated progeny arrays

Old growth forest populations had higher allelic richness and expected heterozygosity than secondary forest populations, and these differences were significant in the Ladrillera population and marginally not significant in Tirimbina (old growth vs secondary forest: Tirimibina AR = 3.22 vs 3.05, $H_E = 0.75$ vs 0.72; Ladrillera AR = 2.97 vs 2.33, $H_E = 0.70$ vs 0.53; Table 2). There were no significant differences in allelic richness and expected heterozygosity in progeny collected in old growth forests compared with adults in the same population (progeny vs adults: Tirimibina old growth AR = 3.33 vs 3.22, $H_E = 0.77$ vs 0.75; Ladrillera old growth AR = 2.92 vs 2.97, $H_E = 0.69$ vs 0.70). Genetic diversity was higher in progeny compared with adults in the secondary forest populations, and these differences were significant in the Ladrillera population and marginally not significant in Tirimbina (progeny vs adults: Tirimibina secondary forest AR = 3.12 vs 3.05, $H_E = 0.73$ vs 0.72; Ladrillera secondary forest AR = 2.83 vs 2.33, $H_E = 0.65$ vs 0.53).

Table 2 Genetic diversity and allelic richness of adult, seedling and progeny arrays

Site	*Population*	n	*AR*	H_E
Tirimbina	Old growth forest[d]	117	3.22 (0.76)[a,b]	0.75 (0.14)[a,b]
	Progeny old growth	237 (15 arrays)	3.33 (0.50)[a]	0.77 (0.10)[a]
	Secondary forest[d]	107	3.05 (0.39)[b,c]	0.72 (0.08)[b,c]
	Progeny secondary	248 (12 arrays)	3.12 (0.44)[b]	0.73 (0.09)[b]
Ladrillera	Old growth forest[d]	99	2.97 (0.62)[a]	0.70 (0.14)[a]
	Progeny old growth	397 (20 arrays)	2.92 (0.43)[a]	0.69 (0.09)[a]
	Secondary forest[d]	120	2.33 (0.55)[b]	0.53 (0.20)[b]
	Progeny secondary	381 (20 arrays)	2.83 (0.54)[a]	0.65 (0.14)[a]

Abbreviations: AR, alleles per locus; H_E, unbiased expected heterozygosity
n, sample size; 95% confidence interval homogeneous subgroups of genetic diversity metrics are indicated by 'a', 'b' and 'c'.
[d]Based on data published by Davies *et al.* (2010).

DISCUSSION

Deforestation and rural-to-urban migration in the neotropics have caused a considerable increase in the area of secondary forest. This increase in secondary forest has occurred to such an extent that it is becoming an increasingly important economic and environmental resource (Finegan, 1992; Aide and Grau, 2004). Where conditions are right, abandonment of deforested land can allow pioneer species to colonise in very large numbers. However, if these recolonised populations are founded by limited numbers of individuals, there is potential for genetic bottlenecks in regenerated stands (Davies *et al.*, 2010). Furthermore, there is a risk of a downward spiral of genetic diversity loss in secondary forest landscapes if recolonisation occurs sequentially from recently established low-diversity sources.

In contrast to predictions of density effects on mating patterns and the genetic diversity of open-pollinated progeny arrays (Eckert *et al.*, 2010; Breed *et al.*, 2012; Breed *et al.*, 2015), our results demonstrate that the mating patterns of *V. ferruginea* were robust to density differences between old growth and secondary forest stands. Furthermore, long-distance pollen flow seems to be maintaining or even increasing the genetic diversity of open-pollinated progeny arrays sired in secondary forest relative to their parental generation.

In our study, trees in old growth forests were exclusively outcrossing and there was little biparental inbreeding, suggesting extensive pollen flow is occurring between spatially isolated trees. A previous analysis of spatial genetic structure (Davies *et al.*, 2010) indicated little population sub-structuring in old growth forest, suggesting that pollen donors nearest to our observed seed trees were mostly unrelated. Our correlated paternity estimate indicates that approximately four unrelated adult trees contribute pollen to each progeny array ($r_p = 0.21$–0.27 and $1/r_p = 4.8$–$3.7 =$ effective number of pollen donors; Sork and Smouse, 2006), thus it appears that old growth populations maintain an effective network of gene flow between spatially dispersed adults.

Despite their isolation, both remnant pasture trees sampled at Tirimbina were also strongly outcrossed ($t_m = 1.00$). However, these highly isolated trees had significantly lower correlated paternity than observed in the populations from old growth and secondary forest (isolated trees: $r_p = 0.01$; forest populations: $r_p = 0.21$–0.27). This lower correlated paternity is potentially due to higher frequency of pollinator visits to isolated trees located in open landscapes or enforced diversity resulting from more pollinator visits from different places and/or unrelated individuals (*cf. S. humilis*, White *et al.*, 2002), both of which would result in more unrelated pollen sources being received into the isolated tree canopies.

The density of *V. ferruginea* in secondary forest populations sampled at both sites was approaching an order of magnitude higher than for adjacent old growth forest plots (see Table 1). Secondary forest stands were predominantly composed of even-aged individuals (Finegan, 1996), assumed to result from a small number of seed donors and composed of a large proportion of half siblings: a founder effect resulting from the initial colonisation event (Davies *et al.*, 2010). In a dense population of synchronously flowering trees, insects may fly shorter distances. Where populations exhibit spatial genetic structuring, matings occur more often between related individuals. For example, in *Symphonia globulifera*, Degen *et al.* (2004) found low selfing rates but high levels of biparental inbreeding ($t_m - t_s = 0.156$) in dense secondary populations. Although a positive correlation between density and inbreeding was found in three of the four populations of *V. ferruginea*, biparental inbreeding was not higher in secondary compared with old growth forest (old growth and secondary forest: $t_m - t_s = 0.00$–0.04). This suggests that significant pollen-mediated gene flow from unrelated individuals may be limiting biparental inbreeding, which may act to break down genetic structuring in secondary forest.

We showed higher levels of genetic diversity in seeds sired in secondary forest, where genetic diversity (both rarefied allelic richness and expected heterozygosity; Table 2) was highest in progeny compared with adult and seedling cohorts in secondary forest.

CONCLUSIONS

In the neotropical pioneer tree, *V. ferruginea*, genetic diversity declines in recolonised stands due to seed dispersal from limited sources (Davies *et al.*, 2010). However, we show here that the genetic diversity of subsequent generations of open-pollinated progeny arrays can be maintained by long-distance pollen flow even in fragmented landscapes. This pollen was not coming from near-neighbours but, rather, was most likely coming from diverse sources across heterogeneous forest and agricultural landscapes. We suggest that natural resource management should prioritise primary remnants for conservation, because they harbour valuable genetic diversity. Populations that maintain this genetic variation through gene flow in fragmented landscapes will benefit seed quality of trees in agricultural landscapes and promote resilience of populations in regenerating landscapes (Breed *et al.*, 2011).

DATA ARCHIVING

Data deposited in the Dryad repository: doi:10.5061/dryad.n06g2.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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