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Characterizing the post-recolonization of *Antechinus flavipes* and its genetic implications in a production forest landscape

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Abstract

Production landscapes, where activities such as timber harvesting, grazing and resource extraction take place, have considerably reduced the extent of natural habitats. The ecological restoration of these landscapes is, in many cases, the best remaining option to protect biodiversity. However, it is unclear whether restoration designed to avert biodiversity loss in restored landscapes can also maintain genetic diversity in recolonising faunal populations. We employed core concepts in the field of population genetics to address questions of genetic diversity and gene flow in recolonising faunal populations, using a small and vagile marsupial (*Antechinus flavipes*) inhabiting a mined landscape under restoration as a model. We did not detect a disruption of gene flow that led to genetic sub-structuring, suggesting adequate levels of gene flow across the landscape. Parameters of neutral genetic diversity were high in groups of individuals sampled in both restored and unmined sites. Our results are encouraging as they indicate that ecological restoration has the potential to not just increase available habitat, but also to maintain genetic diversity. However, there is evidence that past anthropogenic disturbances affected the genetics of the population at the regional level. Even though restoration at the local level may seem to be successful, is necessary to manage populations at larger spatial scales than where restoration is conducted, and over long temporal scales, if genetic diversity is to be maintained in restored landscapes. The field of population genetics is an underused tool in ecological restoration yet can provide important insights into how well restoration achieves its goals.

Key words: ecological restoration, landscape connectivity, founder effect, mining.

Implications for Practice

- Restoration outcomes are influenced not just by management actions at local level but also by pre-existing conditions and by conditions occurring at regional level.
- Areas surrounding production landscapes should be managed and improved to buffer against production related disturbances, with the aim of increasing the carrying capacity at a regional level.
- Implementing a genetic monitoring program, instead of assuming that genetic diversity will be naturally "restored", will improve restoration practices and increase the credibility of the restoration process.
- Sex ratio of recolonizing individuals should be monitored, especially in philopatric species, to avoid detrimental consequences of reduced effective population sizes.
- Restoration practitioners should consider incorporating genetic goals into the design of restoration projects to increase the likelihood of species persistence.

Introduction

The conversion of natural landscapes into production landscapes, where activities such as agriculture, grazing, logging and mining take place, is the main driver of biodiversity loss (Vitousek et al. 1997). For instance, it was estimated that croplands and pastures alone occupy approximately 40% of Earth's surface (Foley et al. 2005). Ecological restoration of these production landscapes is emerging as a promising and effective activity to contribute to biodiversity conservation and the provision of ecosystem services (Benayas et al. 2009; Bullock et al. 2011). At present, fauna restoration success is measured primarily using species richness and abundance (Ruiz-Jaen & Aide 2005), however, these parameters do not mean that restored ecosystems are also maintaining faunal genetic diversity. Therefore, is unclear whether restoration designed to avert biodiversity loss in restored ecosystems can also maintain and conserve genetic diversity in recolonising faunal populations. Increasing attention has been put into the conservation of genetic diversity in natural populations, as it is the raw material upon which natural selection acts to bring about adaptive evolutionary change (Frankham et al. 2009). Its loss will reduce the ability of populations to respond and adapt to long and short term environmental changes (Burger & Lynch 1995) and reduce population fitness due to the exposure and accumulation of deleterious mutations and loss of heterozygosity in overdominant loci (i.e. inbreeding depression; Keller & Waller 2002). Thus, if restoration is to help conserve biodiversity into the future, it is critical that it helps conserves genetic variability, as well as populations, of fauna.

The level of gene flow across a landscape, which is critical for maintaining genetic diversity, is commonly associated with landscape connectivity, and it has been defined as the degree to which the landscape facilitates or impedes movement between resource patches (Taylor et al. 1993), however, it is important to differentiate structural from functional connectivity. While areas of suitable habitat within a landscape might be structurally connected (e.g. by corridors), they might not be functionally connected, as the species of concern might not be able to disperse or immigrate

between structurally connected habitat. Therefore, restoration needs to ensure functional, as well as structural, connectivity if it is to effectively maintain genetic diversity in production landscapes.

Genetic drift refers to the random sampling of alleles being transmitted from generation to generation with the result, within a population, that rare alleles are prone to disappear and common alleles to become fixed. The effective population size (N_e) is closely related to genetic drift and it is inversely related to: the loss of neutral genetic variation, the probability of fixation of deleterious alleles and the increase in inbreeding experienced by a population (England et al. 2006). Accordingly, ecological restoration may contribute in maintaining genetic diversity of faunal populations in two ways: 1) re-establishing landscape connectivity (Dixon et al. 2006) and promoting gene flow; and 2) increasing the area of suitable habitat in a landscape (Huxel & Hastings 1999) and, consequently, increasing effective population size and decreasing the negative effects of genetic drift. However, dispersal, recolonization and establishment patterns, such as founder effects (e.g. Vandepitte et al. 2012), high-density blocking - whereby secondary dispersers arrive in an already colonized, densely occupied habitat and consequently fail to reproduce or to establish themselves (Waters et al. 2013) – or an unequal sex ratio (Allendorf et al. 2010) of recolonising individuals, might reduce the value of restored areas in maintaining genetic diversity.

Most restoration projects are focused at a local level where production occurs and they often fail to take into account broader dynamics at larger spatial and temporal scales (Brudvig 2011). These broader dynamics may concurrently affect restored ecosystems, or have synergistic effects (Holl et al. 2003; Brudvig 2011) and are, likely, important to consider when making decisions on genetic issues in restoration. Additionally, from a genetic perspective, management actions directed to a whole population may be more efficient, in some circumstances, than those actions directed to just a subset of the population (Abdelkrim et al. 2010; Funk et al. 2012). To determine whether ecological restoration is able to maintain and conserve genetic diversity of recolonising populations, we used as a study species a small and vagile marsupial (the yellow footed Antechinus; *Antechinus flavipes*)

inhabiting a multiple-use production landscape under restoration, the northern jarrah (*Eucalyptus marginata*) forest. We first reconstructed the long-term demographic history of *A. flavipes* to evaluate our results across broad spatial and temporal scales. Subsequently, we evaluated whether, at a more local level, restored sites provided functional landscape connectivity and whether genetic diversity was influenced negatively by recolonization patterns in this species.

The northern jarrah forest is a multiple-use landscape that has been subjected to a range of disturbances, including fire, the plant pathogen *Phytophthora cinnamomi* and bauxite mining. Alcoa of Australia (hereafter 'Alcoa') currently clear, mine and restore ~550 ha of jarrah forest annually and, up until 2007, ~13 000 ha had been restored (Koch 2007). As disturbed areas are so extensive in the northern jarrah forest, they have a high potential to disrupt landscape connectivity, which makes it an excellent landscape to study issues related to restoration and the maintenance of genetic diversity. Specifically we asked: (1) What is the spatial scale at which a population can be discerned? (2) What is the demographic history of the population inhabiting this landscape? (3) Does post-mining restoration provide functional landscape connectivity? and (4) Do dispersal, recolonization or establishment patterns of the species represent a limitation for restoration to maintain and conserve its genetic diversity? With the first two questions, we aimed to provide a genetic context for where the restoration occurs while, with the last two questions, we aimed to test whether the restoration was successful in maintaining genetic diversity.

Methods

Study sites

Our study was conducted in the northern jarrah forest of south-western Australia. The jarrah forest is a type of dry sclerophyll forest whose canopy consists almost entirely of jarrah and marri (*Corymbia calophylla*). The study area has a Mediterranean climate with hot, dry summers and cool, wet winters. At Dwellingup, in the center of our study area, rainfall averages approximately

1240 mm annually (Bureau of Meteorology; www.bom.gov.au), with >75% falling between May and September.

We trapped *Antechinus flavipes* individuals at three different and contiguous landscapes covering a north/south distance of ~30 km, Huntly, Dwellingup and Willowdale (Fig. 1), to investigate its demographic history and estimated the spatial scale at which we could discern its population genetic structure. We restricted our evaluation of the genetic consequences of the restoration process entirely to Huntly, as that was the only landscape where we had large sample sizes in both the large areas of post-mining restoration and the original unmined forest. Huntly is a large Alcoa bauxite minesite (32° 36' S, 116° 06' E), where mining/restoration activities have been performed since 1976 and ~41% of the landscape is restored bauxite mine-pits (Triska et al. 2016), with the remainder being unmined forest. In contrast, Dwellingup (32° 42' S, 116° 03' E) is an area that has not been mined but ~40% of the area is infested by *Phytophthora cinnamomi,* a soil-borne plant pathogen that kills many native jarrah forest plant species (Shearer & Dillon 1995) and changes the forest structure (McDougall et al. 2002). Willowdale (32° 53' S, 116° 03' E) is another large Alcoa bauxite minesite where mining/restoration activities have been performed since 1984 and is also extensively infested (~ 62% of the mine area) with *P. cinnamomi*, but less than 9% had been mined before 2002 when samples were collected.

Bauxite strip-mining takes place in pods of one to tens of hectares on the hillsides, but not in valley floors, swamps and streams, resulting in a post-mining landscape that is a mosaic of restored and unmined forest (Koch 2007). Restoration practices include landscaping after mining, soil return, methods for selecting and propagating appropriate plant species and techniques to encourage faunal recolonization (Koch 2007). Restoration management includes fertilizing, thinning and burning, and control of invasive species (e.g. red fox, *Vulpes vulpes*; Grant & Koch 2007). These sophisticated practices have been shown to be largely successful in restoring the post-mining ecosystem (Koch & Hobbs 2007).

Phytophthora cinnamomi is an invasive plant pathogen that kills many plant species in south-western Australia with over half of all indigenous plant species considered either susceptible or highly susceptible to the pathogen (Shearer et al. 2004), including hundreds of jarrah forest species (Shearer & Dillon 1995). Mostly notably, the prominent mid-storey species, *Banksia grandis*, is considered highly susceptible and the dominant canopy species, jarrah, is considered susceptible (Shearer & Dillon 1995). Deaths of these species, along with a range of mid and understory species, means that sites infested with *P. cinnamomi* are very different structurally from uninfested sites with infested sites having less litter cover, less tree and shrub cover and greater cover of annuals than uninfested sites (McDougall et al. 2002).

Trapping

At Huntly, trapping grids (Fig. 2) were randomly installed in unmined forest (n = 22) and restored mined sites of different post-mining ages, ranging from 3 to 21 years, and management prescriptions (n = 17). The mean distance between neighboring trapping grids (1095 ± 134 m) was greater than both the home range size (a radius of ~56 m; Coates 1995) and average dispersal distance (~350 m; Marchesan & Carthew 2008) of *A. flavipes*. All grids were >70 m from other habitat types to maximize the probability of trapping individuals whose home ranges were entirely, or largely, in the sampled habitat. Each grid consisted of pit, Elliott and cage traps (see Fig. 1 in Craig et al. 2009). Trapping sessions were performed from 2005 to 2012. Trapping grids were opened over four periods of two weeks each in spring (October-November), summer (December), autumn (March) and winter (May) in every year except winter 2011 and 2012. At Dwellingup, trapping grids were installed in six sites with varying levels of *P. cinnamomi* infestation. The mean distance between neighboring trapping grids was 982 ± 355 m. Each grid consisted of 25 Elliott traps spaced 20 m apart and arranged in a 5 x 5 grid. Surveys were carried out monthly from May 2002 to April 2004 over four consecutive nights. Trapping surveys in December 2003, February and March 2004 were cancelled due to inclement weather. Trapping grids at Willowdale were

installed in four sites within a 100 x 100 m sampling grid. Five lines of five Elliot traps were placed, 25 meters apart within each site. Traps were opened during four nights in April, July and August 2002. Ear tissue from trapped individuals at all three landscapes was collected for genetic analyses and placed into tubes containing salt-saturated 20% DMSO solution until processing.

Laboratory work

DNA was extracted with the DNeasy Blood & Tissue Kit (Qiagen). Sixteen microsatellites, initially developed for *A. agilis* and described in Banks et al. (2005a), were tested through the polymerase chain reaction (PCR), using a fluorescently labeled (6-FAM, VIC or NED) M13 primer/probe (Schuelke 2000). Non-template and control samples (i.e. samples with known high quality DNA) were used in all PCR reactions. Each reaction contained: 5-50 ng of DNA template, 400 µM dNTPs, 2 mM $MgCl_2$, 1X reaction buffer, 0.1 mg/mL BSA (bovine serum albumin), 0.06 µM forward M13 tagged primer, 0.3 µM reverse primer, 0.3 µM fluorescently labeled M13 primer/probe, 0.825 U of *Taq* polymerase (Fisher Biotec) in a total reaction volume of 15 µL. We followed the cycling conditions described in Banks et al. (2005a; an initial step of 94 °C for 2 min, followed by 30 cycles of 94 °C for 20 s, 30 s at the annealing temperature and 45 s at 72 °C with a final extension step of 72 °C for 3 min). For fragment analysis, 2 µL of the PCR products were combined with Hi-Di formamide (Applied Biosystems, Foster City, California) and 0.3 µL of Genescan LIZ-500 size standard (Applied Biosystems, Foster City, California) by batch and separated by capillary electrophoresis on an ABI Prism 3737xl DNA Sequencer. Fragments were screened using the program GENEMARKER (v1.91, Soft Genetics LLC, State College, PA).

We also sequenced a 565-bp fragment of the mitochondrial control region from a total of 39 individuals from Huntly (n = 13), Dwellingup (n = 15) and Willowdale (n = 11). Amplifications were performed using primers L15999M and H16498M using the conditions described by Fumagalli et al. (1997). PCR products were purified using QIAquick PCR purification kit

(QIAGEN) as per manufacturer instructions. Sequences were aligned in Geneious v.6 (Biomatters, Auckland, New Zealand).

Data analyses

Deviations from Hardy-Weinberg Equilibrium (HWE), using all the samples, were verified through an exact test using GENEPOP 4.01. Tests of linkage disequilibrium were performed in FSTAT (Goudet 1995). The presence of genotyping errors was verified using Micro-checker (Van Oosterhout et al. 2004). As samples were collected over several years at Huntly, before pooling all samples from this location for genetic analyses, we confirmed that each year cohort did not differ significantly from each other, by testing genic and genotypic differentiation for all pairs of cohorts in GENEPOP 4.01 (Rousset 2008) with 10,000 dememorisations, 1,000 batches and 5,000 iterations per batch.

Current population boundaries and landscape connectivity

To estimate the spatial scale at which *A. flavipes* populations should be managed, we determined the number of populations occurring in the study area, using the whole dataset, by using a Bayesian clustering model implemented in STRUCTURE v2.3 (Pritchard et al. 2000). Bayesian clustering assigns individuals to each simulated population, so that every subpopulation would be approximately at Hardy-Weinberg and linkage equilibriums between loci. We used the admixture model (Pritchard et al. 2000) and correlated allele frequencies (Falush et al. 2003) and modeled the number of populations (K) from one to eight, with 20 replications each and a burn-in period of 100,000 followed by 1,000,000 Markov Chain Monte Carlo (MCMC) iterations. To determine *K*, we used STRUCTURE HARVESTER (Earl & vonHoldt 2012) to inspect the mean loglikelihood averaged across the 20 replications and the second order statistic method described by Evanno et al. (2005). We used the software FSTAT (Goudet 1995) to determine if there are pairwise F_{ST} differences between the populations, indicating any population differentiation.

We further hypothesized that, if restored mine sites acted as barriers to gene flow, we would find a significant correlation between the mean genetic distance between individuals and the proportion of surrounding area that has been mined/restored. We performed a Spearman's Rho test using SPSS v.21 to determine the correlation between the mean genetic distance between individuals (genetic distance/Euclidean distance) within a radius of 3 km for each sample and the proportion of mined area surrounding each sample in a radius of 1 km. We used only samples with more than four comparisons.

To examine the partitioning of genetic variation of mtDNA within and among the three landscapes, we performed an AMOVA test (Excoffier et al. 1992), as implemented by ARLEQUIN 3.5 (Excoffier & Lischer 2010). Haplotype frequency and sequence divergence was used to calculate Φ st (Excoffier et al. 1992)

Historical demography

To investigate changes in the demography and connectivity between the three landscapes, we compared migration models of different levels of complexity using coalescent-based analyses, implemented in the software Migrate-n (Beerli 2006). Migrate-n carries out a Bayesian MCMC analysis to estimate Theta, the population mutation parameter ($xN_e\mu$, where x is a multiplier that depends on the ploidy and inheritance of the gene being analysed, N_e is the effective population size and μ is the mutation rate) and M the mutation-scaled immigration rate (m/μ where m is the migration rate). We started with a stepping stone migration model where each sampling location was modeled as a separate population. We then considered two two-population models: Huntly and Dwellingup considered as a single population and then Dwellingup and Willowdale. Finally, we simulated all three as a panmictic system. For the analyses with microsatellites data, we used truncated exponential priors (Theta: mean = 200; max = 900; M: mean = 600; max = 1000) and ran four replicates, each of four heated chains (using a static heating scheme) with a MCMC of 800 million steps using the slice sampler (recording the genealogies every 5000 steps and discarding the

first 10% of the trees as burn-in). For the mtDNA sequences, we used truncated gamma priors (Theta: mean = 0.001; max = 0.01; *M*: mean = 300; max = 8000) and ran four replicates, each of four heated chains (using a static heating scheme) with a MCMC of 160 million steps using the slice sampler (recording the genealogies every 2000 steps and discarding the first 25% of the trees as burn-in). Convergence and adequate Effective Sample Size were assessed with the R package mtraceR (https://github.com/carlopacioni/mtraceR), which was also used to generate the final plots. Model comparisons were carried out using the log Bayes Factor (LBF) calculated with the Bezier marginal likelihoods obtained by thermodynamic integration (Beerli & Palczewski 2010). Using the most supported migration model, we then estimated demographic changes over time with the Bayesian Skyline Plot. Finally, following Pacioni et al. (2015), we calculated the percentage demographic change using the mean theta estimation divided by the theta estimation at 2 x N_e (1 X N_e for mtDNA) before present. The possible range of demographic change was calculated by adding or subtracting 1.96 standard deviations to the mean theta estimation for each time point. We used 2 x N_e (1 X N_e for mtDNA) as a reference point because coalescent events become sparse close to the root of the tree and therefore the parameter estimation is less accurate.

Conservation of genetic diversity

We calculated various measures of genetic diversity including: the mean number of alleles, fixation index (*F*), observed (H_o) and expected heterozygosity (H_e) using GENALEX 6 (Peakall & Smouse 2006). Private allelic richness and allelic richness were calculated with the software HP-RARE (Kalinowski 2005). We also calculated haplotype frequencies, haplotype diversity, and nucleotide diversity using ARLEQUIN 3.5 (Excoffier & Lischer 2010). In addition, a number of genetic parameters were calculated at the individual level: proportion of heterozygous loci in an individual, standardized heterozygosity based on the mean observed and expected heterozygosity (Coltman et al. 1999), internal relatedness (Amos et al. 2001) and homozygosity by locus (Aparicio et al. 2006), using the R-function GENHET (Coulon 2010). We tested the correlation between these genetic

parameters and three different categories according to the habitat type where individuals were sampled (<15 and >15 years post-restoration and unmined forest) with a Spearman's Rho test using SPSS v. 21. These habitat categories were chosen because the abundance of *A. flavipes* in 15-year old restored sites is approximately the same, or slightly higher, than in unmined forest (Craig et al. 2012). We also compared genetic diversity between unmined and restored sites through Mann-Whitney U tests for H_e and Wilcoxon tests for allelic richness, using SPSS v21. No comparison was carried out for mtDNA diversity indices due to small sample size for this dataset. Finally, we determined whether there was a difference in sex ratio between individuals (a factor reducing N_e) trapped at restored and unmined sites at Huntly using a Pearson's chi-squared test in SPSS v21. For this analysis we used the total number of trapped individuals (n = 120) and not the number of sampled individuals (57). The data for this analysis were collected immediately after postnatal juvenile dispersal and territory establishment (January) and were completed prior to male die-off (August).

Results

We collected tissue samples from 122 individuals trapped between 2002 and 2012 across all three landscapes (Table 1). At Huntly, twenty-four individuals were sampled at restored sites and thirty-three at unmined sites. At Dwellingup we sampled forty-two individuals and at Willowdale twenty-three individuals. We tested 16 microsatellite loci of which 11 successfully amplified a PCR product and were polymorphic. The loci 7H and 4A deviated from HWE (after sequential Bonferroni corrections; Rice 1989) and showed null alleles, so they were removed from all analyses.

Current population boundaries and landscape connectivity

In STRUCTURE, K=1 and K=2 had very similar mean ln-likelihood (Fig. 3). Because the second order statistic method does not permit discrimination between K=1 and K=2, we visually examined

estimated membership coefficients for each individual and in each run. We found that, when K=2, all individuals were approximately symmetrically assigned among populations and concluded that K=1 was the most likely number of clusters. F_{ST} pairwise analysis showed a low genetic differentiation between populations (Table 2). No correlation was found between the mean genetic distance between individuals and the proportion of surrounding area that has been mined/restored (r_{21} = 0.03, p = 0.882; see Table S1).

Historical demography

The coalescent-based analyses of mtDNA suggested that the best-supported model (probability of 0.68: log-likelihood = -916.95) was a two-population migration model with Huntly being a separate population from Dwellingup and Willowdale, with a migration rate from Huntly to Dwellingup and Willowdale of 0.45 (95% CI=0.06-1.23); and from Dwellingup and Willowdale to Huntly of 5.07 (95% CI=1.15-17.68). The second best supported model (with a probability of 28%, log-likelihood = -917.81) suggested the presence of three different populations. Conversely, the only supported migration model in the analyses of microsatellite data was a panmictic model; the same scenario that the Structure analysis suggested. Demographic analyses of microsatellite data demonstrated a population decline of 74% (range from 73 to 75%; Fig. 4a). In line with these results, the mtDNA demographic analysis, where Dwellingup and Willowdale were modeled together, showed a population decline of 29% (range from 20 to 36%; Fig. 4b). Demographic changes based on mtDNA were unclear for Huntly (range from -2 to an increase of = 50%; Fig. 4c) and we were not able to say effectively whether this population had declined or increased in size.

Conservation of genetic diversity

Nuclear genetic diversity parameters were relatively high (Table 1). None of the genetic diversity parameters calculated was correlated with any habitat or differed significantly between samples from unmined and restored sites (data not shown), suggesting that environmental conditions are not an important factor influencing genetic diversity. Eight mtDNA sequences were identified (Table

S2). Huntly had the largest number of unique haplotypes. AMOVA test of mtDNA data (Table S3) shows a high fixation index and that partitioning of genetic variation is higher within locations than among locations. At Huntly, analyses revealed the sex ratio was male biased at restored sites and female biased at unmined sites ($p = 0.007$, Table 3).

Discussion

The benefits and potential limitations of restoration efforts to maintain genetic diversity of recolonising fauna have rarely been examined (but see Baker et al. 2008 and Bonin et al. 2013). Our results suggested that restoration practices have been effective in maintaining landscape connectivity and that the restoration process did not influence the distribution of genetic diversity of *Antechinus flavipes* across the landscape. However, there were several lines of evidence (among which, most importantly, the substantial decline), that anthropogenic disturbances affected the genetics of the *A. flavipes* population at the regional level and these should be considered when assessing the risk of further disturbances and any post-disturbance restoration. The detected decline is reason of concern for the species conservation. We argue that it is possibly associated with the numerous anthropogenic disturbances, such as logging and dieback, and recommend further monitoring of the species' demographic and genetics trends to ensure that the decline has been halted and that genetic variability continues to remain high.

Patterns of dispersal, recolonization and establishment in faunal populations are highly complex but not random. Dispersal may be influenced by several factors such as inbreeding risk, female abundance, patch size, patch quality and matrix permeability (e.g. Banks & Lindenmayer 2013). Specifically in the study area, *A. flavipes*, despite its relatively specific habitat requirements (Nichols & Grant 2007; Swinburn et al. 2007), has recolonized restored areas successfully (as soon as two years post-restoration; Nichols & Grant 2007), and its abundance in 12 and 17-year old restoration is the same or slightly higher than in unmined forest (Craig et al. 2012). The relatively

high vagility of this species certainly plays an important role in its recolonization success. This trait has been documented by Lada et al. (2007), showing that gene flow between populations is not completely restricted by rivers. Our study did not demonstrate directly that ecological restoration improved landscape connectivity, however other studies have shown that, at finer scales, dispersal patterns can be influenced, for instance, by roads (Burnett 1992) or by a plantation of an exotic species in a closely related species (*A. agilis*; Banks et al. 2005a). Therefore, we believe that if restoration of mined sites were not performed, landscape connectivity may have been compromised, as exemplified by the above studies.

Lack of genetic structure at the local scale in our study supports the idea that, even during the early years following restoration, restored areas do not represent dispersal barriers. Similarly, a non-significant correlation between the distribution of individual heterozygosity across habitats subject to different levels of disturbance suggests that restored areas do not have any negative influence on the spatial distribution of genetic diversity. We found relatively high levels of neutral genetic diversity, in agreement with previous studies using the same set of microsatellites in *Antechinus* spp.. However, the genetic diversity reported here is within the range of those populations inhabiting fragmented habitats (*He* = 0.844; Banks et al. 2005a; and *He* = 0.771-0.833; Lada et al. 2008) and lower than those reported in continuous forests (*He* = 0.860; Banks et al. 2005a; and *He* = 0.886; Kraaijeveld-Smit et al. 2007). There have been many intense disturbances that have occurred and are still occurring in the jarrah forest (i.e. mining activities, *P. cinnamomi* infestation, altered fire regimes, invasive species, clearing and logging). We argue that cumulatively these changes have probably been responsible for the detected decline. On the other hand, *Antechinus* spp. have developed a series of inbreeding avoidance mechanisms whereby males disperse large distances after weaning, females are philopatric (Lada et al. 2007), individuals avoid sharing nests with opposite-sex relatives (Banks et al. 2005b) and multiple paternity within litters is common (Kraaijeveld-Smit et al. 2002). The relatively high vagility of this species, along with its short

generation time, aids in maintaining genetic diversity even when disturbances and habitat loss may affect the population size.

The availability of an undisturbed landscape would have been ideal to directly compare our data. Unfortunately, there are no pristine landscapes from where we could obtain *A. flavipes* samples, due to the long history of anthropogenic disturbance in the jarrah forest (Bartle & Slessar 1989; Dell & Malajczuk 1989). Therefore, we resolved to test the correlation between the mean genetic distance between individuals and the proportion of surrounding area that has been mined/restored in accordance with the general concept of the degree of isolation being proportional to genetic differentiation (Segelbacher et al. 2003). The failure of restoration goals can manifest itself with the fragmentation of suitable habitat throughout the study area and, as a result, there would be isolated pockets of suitable habitat where small isolated populations reside. Under this scenario, we expected that individuals surrounded by a larger mined/restored area would be more genetically distant from those individuals surrounded by a smaller mined/restored area. Our results did not support this hypothesis.

Analyses of the microsatellite data with STRUCTURE, FSTAT and Migrate-n resulted in the identification of a panmictic system, suggesting that the residential population boundaries are well beyond the local area where mining (and subsequent restoration) occurs. This is especially relevant as evolutionary processes, such as adaptation and speciation, and detrimental genetic issues, such as inbreeding depression, operate at a population level. Therefore, events outside this area may affect the restoration outcome as much as these inside i.e. the whole population loses genetic diversity at a rate of $1/N_e$ per generation (Frankham et al. 2009). If carrying capacity decreases in any area where the population in question is resident, that means that N_e becomes smaller and the rate of genetic diversity loss increases.

A skewed sex ratio is a known factor reducing the effective population size (Allendorf et al. 2013). The sex ratio in restored sites was male biased (female:male ratio 0.3:1) whereas in unmined forest

it was female biased (female:male ratio 2:1). These results are probably due to the fact that males are responsible for most dispersal events and consequently constitute the majority of recolonizers migrating to restored areas. In a previous study, in the closely related species *A. agilis*, the sex ratio was male biased in unfragmented habitat and female biased in fragmented habitat (Banks et al. 2005a). The male-biased sex ratio was believed to be an effect of a higher dispersal-associated mortality within the fragmented forest than in the unfragmented forest, which does not appear to be an issue here. This finding is encouraging as it may indicate that dispersing individuals do not incur increased mortality. However, if sex ratio biases in restored areas remain for a longer period, it could be detrimental for N_e in the long term.

MtDNA is maternally inherited and is thus an indicator of female, rather than male, dispersal patterns (Roffler et al. 2014). In accordance with the male-biased dispersal and female philopatry of the species, the support of Migrate-n analysis of the mtDNA data for a partially structured scenario, is not surprising. In *A. flavipes*, mtDNA diversity is probably strictly linked to the geographic distribution of haplotypes. Interestingly, mtDNA Migrate-n analysis resulted in a much larger number of migrants to Huntly. We argue that this result is possibly related to an increased displacement of females from the southern landscapes (i.e. Dwellingup and Willowdale) due to habitat degradation and it may explain the lack of a clear signal of decline in the mtDNA demographic analysis from Huntly.

We found that verifying the extent of the population boundaries and understanding the demographic history of the population were very informative as these provided a context through which to interpret the consequences of restoration. Clearly, when planning restoration, both temporal and spatial scales are important to consider. Even though restoration efforts of mining sites at local level may seem to be adequate, it does not necessarily mean that they are effective at larger spatial and/or longer temporal scales because other factors (i.e. past disturbances and environmental conditions at a regional level) may act synergistically and limit or prevent the recovery or maintenance of the

species' population size and/or genetic diversity. This is especially true when the species is affected by a relatively long term decline, where relatively minor perturbations may be significant, because small populations are more susceptible to go extinct as a result of factors that would not normally be cause for concern (Caughley et al. 1996).

We recommend continued monitoring of the restored sites to verify that the declining trend we detected is addressed and, most importantly, does not result in future loss of genetic diversity. To this end, we also recommend that areas of sub-optimal habitats are managed and improved to increase their carrying capacity and consequently maximize the maintenance of genetic diversity over time. The inclusion of genetic approaches in restoration science will help to achieve the ultimate goal of restoration ecology to re-establish self-sustaining ecosystems that will resist future perturbation without additional human input (Urbanska et al. 1997).

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The authors declare no conflict of interest.

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Supporting Information

The following information may be found in the online version of this article:

Table S1. Correlation between mined area and genetic distance.

Table S2. Distribution and frequency of the eight mtDNA *Antechinus flavipes* haplotypes found in each of the three landscapes sampled in Western Australia.

Table S3. Analysis of molecular variation of *Antechinus flavipes* showing the partitioning of genetic mtDNA variation among and within landscapes (Huntly, Dwellingup and Willowdale) sampled in Western Australia.

Tables

Table 1. Descriptive statistics of groups of individuals of *Antechinus flavipes* sampled in three different trapping landscapes in the south west of Western Australia. Numbers in parentheses indicate standard error.

	All	Huntly	Huntly unmined sites	Huntly restored sites	Dwellingup	Willowdale
Microsatellites						
No. samples	122	57	33	24	42	23
Average no. alleles/locus	9.59 (0.637)	11.00 (1.106)	10.2 (0.9)	9.6 (1.0)	10.10 (1.148)	7.667 (0.816)
Effective no. alleles	6.11 (0.470)	6.730 (0.856)	6.3 (0.8)	6.1 (0.8)	6.491 (0.960)	5.108 (0.555)
Observed heterozygosity	0.813 (0.020)	0.848 (0.022)	0.837 (0.021)	0.822 (0.051)	0.778 (0.047)	0.814 (0.031)
Expected heterozygosity	0.807 (0.016)	0.832 (0.028)	0.829 (0.026)	0.817 (0.039)	0.820 (0.033)	0.803 (0.023)
Fixation index	-0.008 (0.017)	-0.032 (0.015)	-0.029 (0.022)	-0.025 (0.027)	0.046 (0.029)	-0.039 (0.035)
Private allelic richness	-	1.23	0.61	0.52	0.76	0.28
Allelic richness	-	9.62	9.14	9.11	9.07	7.62
mtDNA						
Sample size	39	13	10	3	15	11
Number of haplotypes	8	6	4	3	4	3
Haplotype diversity	0.772 (0.035)	0.821 (0.082)	0.778 (0.091)	1 (0.272)	0.667 (0.099)	0.473 (0.162)
Nucleotide diversity	0.015 (0.008)	0.019 (0.011)	0.015 (0.002)	0.022 (0.010)	0.015 (0.008)	0.007 (0.004)

Table 2. Pairwise analysis of F_{ST} differences between three sampling locations from Dwellingup, Huntly and Willowdale, showing low levels of differentiation.

Locations	F_{ST}
Dwellingup/Huntly	*0.0155*
Willowdale/Huntly	*0.0275*
Dwellingup/Willowdale	*0.0083*

Table 3. Sex ratio of trapped *Antechinus flavipes* individuals at Huntly, Western Australia. The sites were categorized as restored and unmined. Number of trapped individuals differs from the number of individuals analysed because not all individuals were sampled.

	Females	Males	Ratio (females:males)
Unmined	22	11	2:1
Restored	20	67	0.3:1

Figures

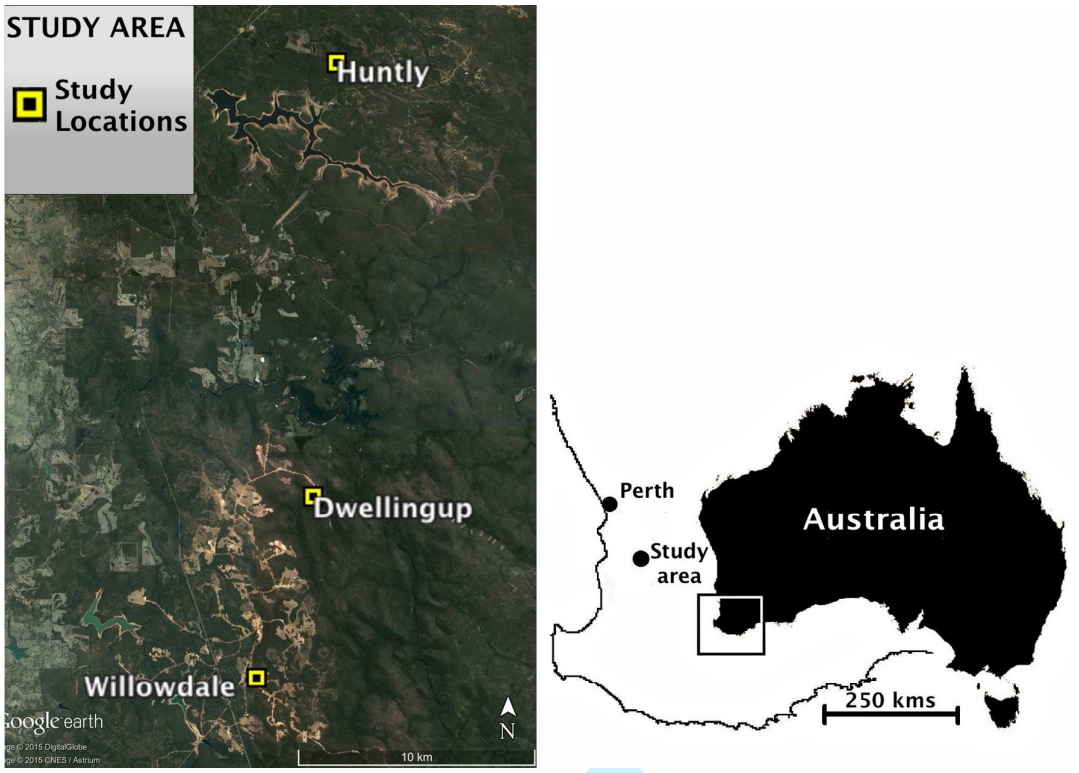

Figure 1. Location of studies landscapes within Australia.

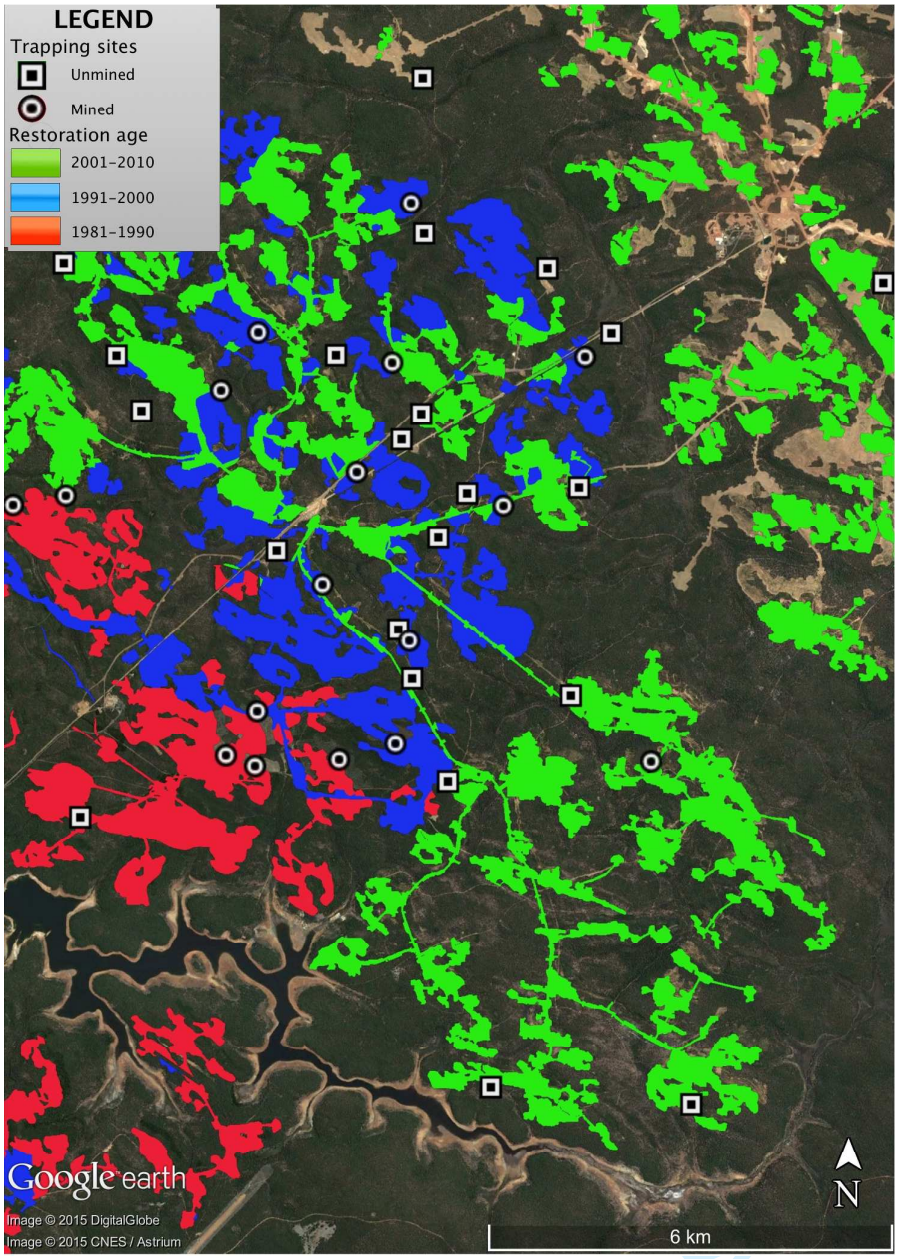

Figure 2. Location of trapping sites for *Antechinus flavipes* at Huntly (area = approximately 16,000 ha), Western Australia. Circles represent traps installed in mined/restored sites and squares those installed in unmined forest. Red color represents areas that were restored between 1981-1990, blue between 1991-2000 and green between 2001-2010.

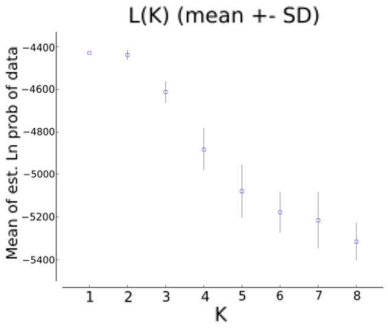

Figure 3. Mean likelihoods (± SD) across 20 replications for each K value of an 11 microsatellite loci analysis in STRUCTURE v2.3 (Pritchard et al. 2000).

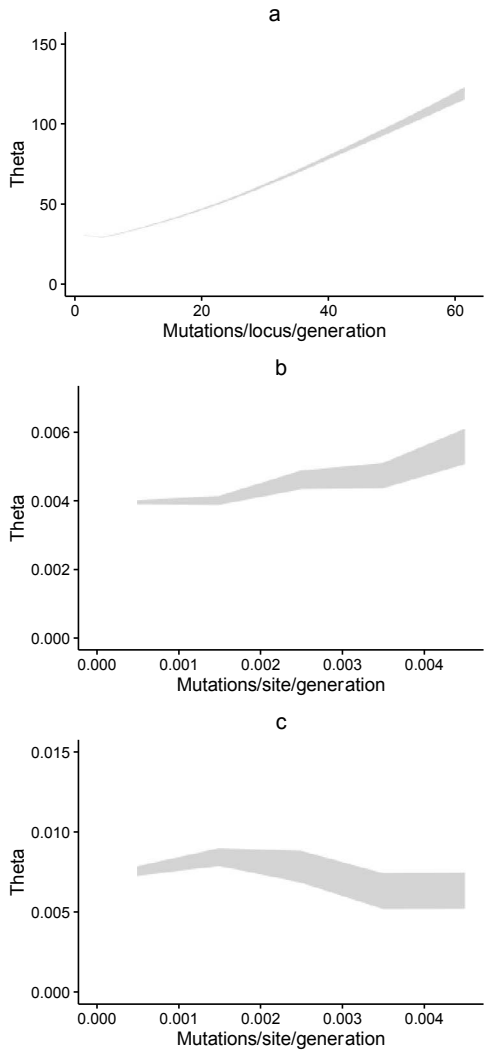

Figure 4. Demographic history reconstruction of *Antechinus flavipes* using Bayesian Skyline Plots in Migrate-n (Beerli 2006). Present time on the *x*-axis (expressed in mutation rates) is on the left hand side and past is on the right hand side. Shaded areas are 1.96 x standard deviation. a) Microsatellite based plot of theta for all three landscapes combined (see text for detail); b) MtDNA based plot of theta for Dwellingup/Willowdale population; c) MtDNA based plot of theta for Huntly population.

Supporting information

Table S1. Correlation between mined area and genetic distance.

ID	Proportion of area mined (%)	Genetic distance	Number of comparisons
11226	24	9.0	5
11216	34	14.5	6
11230	34	8.0	6
11244	35	12.1	8
11135	35	15.5	12
11181	35	10.1	12
12018	37	13.4	6
12021	37	18.0	6
12029	38	20.2	5
12249	38	11.4	5
12022	40	16.7	9
12025	40	14.7	9
11157	40	16.2	11
11194	40	15.4	11
12037	42	16.4	11
12050	42	16.1	11
11282	43	15.1	10
12019	43	12.4	14
11265	45	17.0	4
12016	45	20.2	4
12017	48	15.8	11
11188	53	8.1	11
11189	53	7.0	11

Table S2. Distribution and frequency of the eight mtDNA *Antechinus flavipes* haplotypes found in each of the three landscapes sampled in Western Australia.

Haplotype	Huntly	Dwellingup	Willowdale
1	0.231	0.133	0.727
2	-	0.533	0.182
3	0.385	0.267	0.09
4	-	0.066	-
5	0.076	-	-
6	0.076	-	-
7	0.154	-	-
8	0.076	-	-

Table S3. Analysis of molecular variation of *Antechinus flavipes* showing the partitioning of genetic mtDNA variation among and within locations (Huntly, Dwellingup and Willowdale) sampled in Western Australia.

Source of variation	Degrees of freedom	Sum of squares	Variance components	Percentage of variation
Among locations	2	2.713	0.07944	19.31
Within locations	36	11.953	0.33204	80.69
Total	38	14.667	0.41148	
Fixation index (FST)	0.193			

P-value<0.0001