MINERAL RESOURCES

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J5 and Bungalbin East Iron Ore Proposal
Response to Submissions – Appendix B
Revised Flora Genetics Analysis

Assessment of population genetic variation and structure of priority and threatened taxa in the Helena-Aurora Range, South-Western Australia

Final report to:
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Executive summary

The banded iron formation (BIF) ranges of the Yilgarn Craton in the Midwest and Goldfields regions of Western Australia are isolated ancient ranges set in a predominantly flat landscape. They form a small proportion of the total land area of the region and provide unique habitat for flora and fauna, due to different geology, soils and landforms compared to the majority of land in the region.

Botanical survey and research shows that BIF ranges of the Yilgarn Craton provide island like environments with frequently high levels of endemism, rare and geographically restricted species. Exemplifying patterns of plant diversity on BIF ranges are three geographically restricted taxa, *Acacia adinophylla* (Conservation status P1), *Lepidosperma bungalbin* (Conservation status P1) and *Tetratheca aphylla* subsp. *aphylla* (Conservation status Threatened). The three taxa grow within the rocky and cliff environments or adjacent slopes, and each taxon is restricted to the Helena-Aurora Range, south-western Australia.

There are significant iron ore deposits throughout the BIF ranges, including the Helena-Aurora Range. In 2015, Curtin University was engaged by Polaris Metals Pty Ltd to undertake research into the population genetics of these three short-range endemic plant species and to assess the potential impact for mine development in the Helena-Aurora Range.

The objectives of the research program were to: (i) characterise population genetic variation, and its spatial structuring across the entire distribution of *A. adinophylla, L. bungalbin* and *T. aphylla* subsp. *aphylla*; (ii) determine the effect of landscape (i.e. habitat suitability, topographic complexity) and plant density on genetic connectivity and (iii) quantify any genetic variation that may be impacted by proposed mining.

Key Findings

Microsatellite loci were identified using shotgun sequencing, or cross-transferred from related taxon. Optimal primers and PCR conditions were identified and samples of *A. adinophylla* (n = 273, 13 sites), *L. bungalbin* (n = 236, 11 sites) and *T. aphylla* subsp. *aphylla* (n = 294, 13 sites) collected from the Helena-Aurora Range were scored for allelic variation at 12, 12 and 9 polymorphic microsatellite loci, respectively.

Acacia adinophylla

The 12 microsatellites used in this study were all polymorphic with 3–18 alleles per locus (an average of 8 alleles per locus; total 98 alleles). Observed heterozygosity (H_O) per site varied from 0.51 to 0.63 (overall mean = 0.56) and was lower than expected heterozygosity (H_E = 0.56 – 0.64; overall mean = 0.60). The inbreeding statistic F_{IS} was generally low to moderate (F_{IS} = -0.06 – 0.014; overall mean = 0.06). Allelic richness was similar among sites (AR = 4.48 – 5.39; overall mean = 4.88) and private alleles were found in very low frequencies at 5 of 13 sites. Evidence of genetic bottlenecks (mode shift) was detected in sites AA 4 and AA 11.

Pairwise F_{ST} comparisons ranged from 0.002 (sites AA 2 and AA 13) to 0.073 (sites AA 4 and AA

9) and were generally low with a global F_{ST} of 0.030 ± 0.005. A similar pattern was observed with D_{EST} (pairwise data not shown; overall D_{EST} = 0.042 ± 0.006). We detected a significant association between pairwise genetic distance and geographic distance among all pairs of sites and ordination of the genetic data supports the association, with geographically proximate sites clustering more closely than geographically distant ones. Geographic distance (IBRD) was a better explanatory variable of genetic differentiation among sites than habitat suitability (CH), topographic complexity (CTC) or plant density (CD). STRUCTURE analysis identified K = 2 as the optimal number of clusters to the data and comparable patterns were found with more mixed proportions of membership for centrally located sites suggesting a clinal association between genetic and geographic distance.

The genetic impact of proposed mining on *A. adinophylla* was assessed on the basis of repeating analyses of genetic variation after removal of all genotyped samples located within the disturbance area at J5 and Bungalbin East (impacted sites: AA 3, AA 4, AA 5, AA 7 and AA 12), including a 20 m buffer zone for indirect impacts, and comparing to the complete dataset of all individuals from 13 sites. The removal of genotyped samples from within the disturbance area and buffer zone resulted in the loss of two alleles (i.e. 96 of 98 alleles (98%) were recovered), one of which was private (found in only one population), from within the remaining sites. All other parameters of genetic variation (mean H_O, H_E, AR) were unaffected. Similarly, overall measures of genetic differentiation were not affected by removal of samples from impacted sites (F_{ST} = 0.030 and 0.028; D_{EST} = 0.050 and 0.047, for full and reduced dataset respectively). Consequently, while up to ~12% of all plants may be removed by mine development, 2% of alleles detected at 12 microsatellite markers will be lost, and other genetic parameters remain unaffected.

Lepidosperma bungalbin

The 12 microsatellites used in this study were all highly polymorphic with 9–29 alleles per locus (an average of 17 alleles per locus; total 207 alleles). Observed heterozygosity (H_O) per site varied from 0.63 to 0.82 (overall mean = 0.73) and was lower than expected heterozygosity (H_E = 0.71 – 0.82; overall mean = 0.78). Inbreeding was generally low (F_{IS} = 0 – 0.19; overall mean = 0.06) and allelic richness was similar among sites (AR = 6.78 – 9.08; overall mean = 8.11). Private alleles were found in very low frequencies at 9 of 13 sites and no evidence of genetic bottlenecks was detected ($p > 0.05$ and no mode shifts detected).

Pairwise F_{ST} comparisons ranged an order of magnitude from 0.005 to 0.111 but were generally low with a global F_{ST} of 0.068 ± 0.006. A similar pattern was observed with D_{EST} (pairwise data not shown; overall D_{EST} = 0.187 ± 0.045). A Mantel test showed that genetic distance was significantly correlated with geographic distance, among all pairs of sites. However, CH was a better predictor of genetic differentiation among sites than IBRD, CTC and CD. The ordination of genetic data clearly segregated central, south-western and north-eastern sites. The STRUCTURE analysis identified K = 3 as the optimal number of clusters and these three genetic groups were similar to those found in the ordination.

The genetic impact of proposed mining on *L. bungalbin* was assessed on the basis of repeating analyses of genetic variation after removal of all genotyped samples located within the

disturbance area, including a 20 m indirect buffer zone (impacted sites: LB 2, LB 7 and LB 8), and comparing to the complete dataset of 11 sites. The removal of genotyped samples from within the disturbance area and buffer zone resulted in the loss of 2 alleles (i.e. 205 of 207 alleles (99%) were recovered from within the remaining sites). These alleles were private and rare (lost allele frequencies all < 0.05). All other parameters of genetic variation (mean H_O, H_E, AR) were unaffected. Similarly, overall measures of genetic differentiation were not affected by removal of sites (F_{ST} = 0.068 and 0.067; D_{EST} = 0.187 and 0.190, for full and reduced dataset respectively). While up to ~8%% of all plants may be removed by mine development, 1% of alleles detected at 12 microsatellite markers will be lost, and other genetic parameters remain unaffected. However, the majority of plants in the distinct 'green' genetic cluster will be removed with mine development.

Tetratheca aphylla subsp. *aphylla*

The 9 microsatellites used in this study were polymorphic with 4–28 alleles per locus (an average of 13 alleles per locus; total alleles 114). Observed heterozygosity (H_O) per population varied from 0.62 to 0.73 (overall mean = 0.66) and was slightly lower than expected heterozygosity (H_E = 0.64 – 0.72; overall mean = 0.68). All populations deviated from Hardy-Weinberg proportions, however, the overall inbreeding statistic F_{IS} was low (F_{IS} = -0.07 – 0.012; overall mean = 0.03). Allelic richness was similar among populations (AR = 5.60 – 7.30; overall mean = 6.70) and private alleles were found in very low frequencies at 6 of 13 sites. Evidence of genetic bottlenecks ($p<0.05$) were detected in 4 populations and there was no geographic pattern to their distribution.

Pairwise F_{ST} comparisons ranged an order of magnitude from 0.002 to 0.104 (and were generally low with a global F_{ST} of only 0.031 ± 0.005. A similar pattern was observed with D_{EST} (pairwise data not shown; overall D_{EST} = 0.078 ± 0.027). We detected a significant association between pairwise genetic distance and geographic distance among all pairs of sites but CH was the most explanatory variable of genetic differentiation among sampling sites. STRUCTURE analysis identified K = 2 as the optimal number of clusters in *T. aphylla* subsp. *aphylla* and we found an east/west cline for proportions of membership in the two genetic clusters with more mixed proportions of membership for centrally located sites.

The genetic impact of proposed mining on *T. aphylla* subsp. *aphylla* was assessed on the basis of repeating analyses of genetic variation after removal of all genotyped samples (impacted sites: TA 1, TA 2, TA 3 and TA 10) located within the disturbance area and a 20 m indirect buffer zone and comparing to the complete dataset comprising 13 sites. The removal of impacted samples at these four sites resulted in the loss of 9 alleles (7 private) (i.e. 105 of 114 alleles (92%) were recovered from within the remaining sites). The lost alleles were rare (lost allele frequencies all < 0.05) and found at only a single site. All other parameters of genetic variation (mean H_O, H_E, AR) were unaffected. Similarly, overall measures of genetic differentiation were not affected by removal of sites (F_{ST} = 0.031 and 0.031; D_{EST} = 0.078 and 0.076, for full and reduced dataset respectively). Accordingly, while up to ~20% of all plants may be removed by mine development, 8% of alleles detected at 9 microsatellite markers will be lost, and other genetic parameters remain unaffected.

Conclusions

A comparison to population genetic studies of other short range BIF endemics in the region that are impacted by proposed or approved mining (e.g. *T. erubescens* and *T. paynterae* subsp. *paynterae*) supports a conclusion that the proposed mining at J5 and Bungalbin East in the Helena-Aurora Range will result in a relatively negligible assessable impact on extant genetic variation and spatial genetic structuring of *A. adinophylla*.

Whilst current genetic diversity and differentiation parameters assessed in *L. bungalbin* will remain largely unaffected by proposed mining, the majority of plants in the distinct 'green' genetic cluster will be either removed or indirectly impacted.

The detectable genetic impact of the removal of *T. aphylla* subsp. *aphylla* plants from the disturbance area was greater than that of proposed mining on *T. erubescens* but similar to that of *T. paynterae* subsp. *paynterae* (loss of private alleles not reported in that study). However, the loss of 8% of alleles and, in particular, 50% of private alleles in *T. aphylla* subsp. *aphylla*, represents a significant amount of the species genetic diversity.

The longer-term genetic consequences of reducing the number of individuals both overall and in particular genetic groups (i.e. 'green' cluster in *L. bungalbin*) is unclear, as is increasing the geographic isolation of remaining plants (e.g. *L. bungalbin* sites LB 1 and LB 10). An understanding of pollinators, direct estimates of seed/pollen dispersal, and reproductive success, across the range of the species', is required to properly understand the impacts of this proposal on genetic processes and structure in *A. adinophylla*, *L. bungalbin* and *T. aphylla* subsp. *aphylla*. This information would also prove critical in managing impacts should mining proceed. However, maintaining suitable habitat between populations appears important, particularly for *L. bungalbin* and *T. aphylla* subsp. *aphylla,* in the maintenance of genetic connectivity. Also, any genetic impacts of proposed mining could be minimized *in situ b*y maintaining plants from the distinct geographic clusters and particularly geographically adjacent sites that are genetically clustered but where one is to be removed as part of mine development. Similarly, the careful targeting and inclusion of sites in any *ex situ* seed collections needs to be guided by the genetic data.

Introduction

Background

The small ancient banded iron formation (BIF) ranges of the Southern Cross biogeographic sub-region are floristically distinct from the surrounding matrix and many have unique floristic communities strongly correlated with topography (Beard 1981; Gibson et al. 2011). These ranges occupy a relatively small proportion of the total land area of the sub-region and provide unique habitat for flora and fauna due to different geology, soils and landforms, compared to the majority of land in the region.

These ranges are considered to be repositories of taxa endemic to, or with distribution centred on these landforms (Gibson et al. 2007; 2010; 2011), and may have acted as refugia during drier climatic cycles, as well as centres of more recent speciation (Butcher et al. 2007). Ranges in the region have a particularly high number of ironstone specialists (Gibson et al. 2011), however, despite the importance of BIF ranges, genetic patterns of their endemic flora remain poorly understood.

In response to a request from Polaris Metals Pty Ltd as part of its ongoing biodiversity research of the Helena-Aurora Range, this report describes a population genetic study conducted on three short range, endemic, conservation priority taxa of the Helena-Aurora Range: *Acacia adinophylla* (Fabaceae), *Lepidosperma bungalbin* (Cyperaceae) and *Tetratheca aphylla* subsp. *aphylla* (Elaeocarpaceae)(Figure 1). This research addresses the key objective of an assessment of population genetic variation and its spatial genetic structure within the three species, which will enable a quantification of any genetic variation that may be impacted through proposed mining activity at the J5 and Bungalbin East iron ore deposits.

Due to their isolation, low population numbers, and proximity to mining activity, *A. adinophylla* (Conservation status P1), *L. bungalbin* (Conservation status P1) and *T. aphylla* subsp. *aphylla* (Conservation status Threatened) are protected under the *Wildlife Conservation Act 1950* (WA). *Tetratheca aphylla* subsp. *aphylla* is also protected under the Environment Protection and Biodiversity Conservation Act 1999 (Cth). Each of these species inhabit different niches within parts of the Helena-Aurora Range: *A. adinophylla* is the most broadly distributed and found on ironstone ridges and surrounding undulating plains (Maslin 1999); *L. bungalbin* is confined to upper slopes (Barrett 2007) and *T. aphylla* subsp. *aphylla* is confined to cliff tops and steep stony slopes (Butcher 2007) (Figure 2). *Tetratheca aphylla* subsp. *megacarpa*, is restricted to the Newdegate area, some 300 km south of the only known populations of *T. aphylla* subsp. *aphylla* and given the distance separating populations of these species, gene flow between them is extremely unlikely. Therefore, individuals of subsp. *megacarpa* were not included in this study. Total population sizes are currently (01/08/16) recorded at 10,529, 45,976, and 87,921 individuals, respectively for *A. adinophylla*, *L. bungalbin* and *T. aphylla* subsp. *aphylla,* based on targeted flora surveys undertaken by Polaris and others throughout the region. All three taxa have a narrow distribution limited to less than 30 linear kilometres on the Helena-Aurora Range. Although the reproductive biology of the three study species is unknown, based on previous studies of *Acacia*, *Lepidosperma* and *Tetratheca* taxa, seed is likely to be dispersed by ants and birds, and pollen by insects or wind (e.g. Davidson and

Morton 1984; Butcher et al. 2009; Millar et al. 2013; Binks et al. 2015; Millar et al. 2015)

Initial population genetic studies of plant species in the region show that patterns are complex and variable. For example, contrasting patterns of genetic diversity and population structure were found in two narrow range endemic sedges (Binks et al. 2015). The smaller, more isolated *Lepidosperma* sp. Parker Range populations were characterized by lower diversity and stronger divergence, relative to higher diversity and extensive connectivity among the geographically clustered *L.* sp. Mt Caudan populations. However, neither species exhibited low diversity, despite high inbreeding. Similarly complex patterns are suggested in studies of Acacia (Millar et al. 2013) and Tetratheca (Butcher et al. 2009). Therefore, given the difficulty in making predictions of genetic patterns, a study of the genetic impact of proposed mining on *A. adinophylla*, *L. bungalbin* and *T. aphylla* subsp. *aphylla* is warranted.

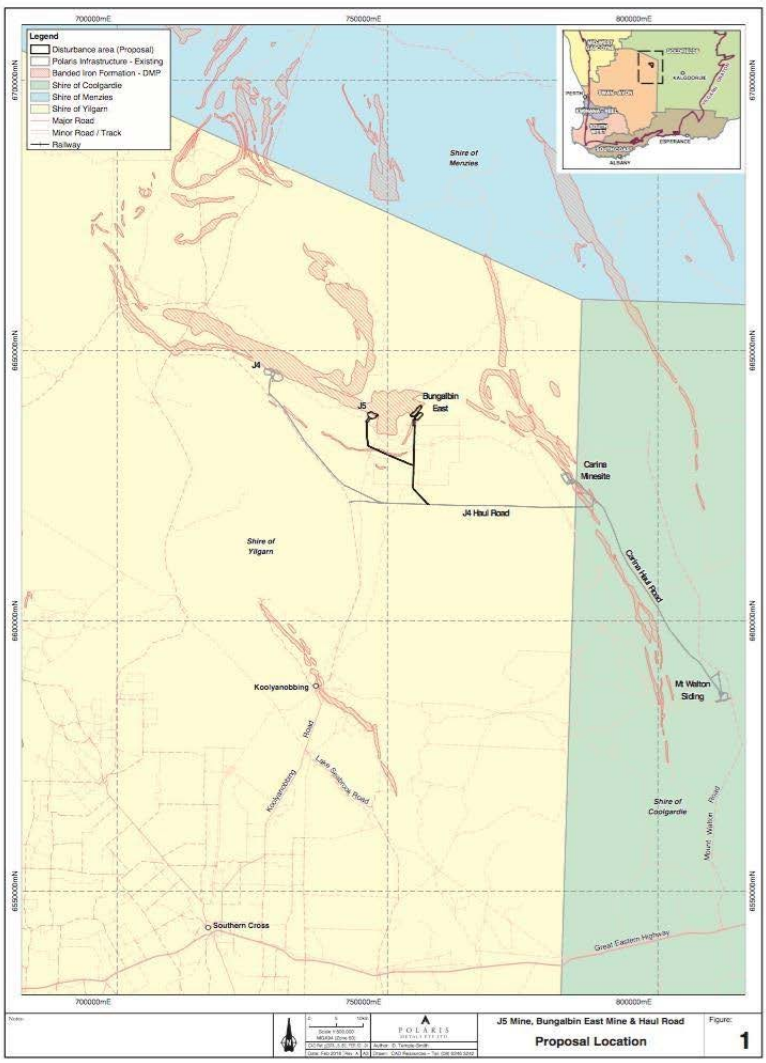


Figure 1 The study region and location of the Helena- Aurora Range where *A.* a*dinophylla*, *L. bungalbin* and *T. aphylla* subsp. *aphylla* occur

Figure 2 *T. aphylla* subsp. *aphylla* inhabiting cliffs of the southern Helena-Aurora Range

Research objectives

Key objectives were to:

- Characterise population genetic variation, and it's spatial structuring across the entire distribution of *A. adinophylla, L. bungalbin* and *T. aphylla* subsp. aphylla;
- Determine the effect of landscape and plant density on genetic connectivity;
- Quantify any genetic variation that may be impacted by proposed mining.

These analyses will enable the quantification of the potential impact of mining on genetic variation within these species, establish a baseline for future management of genetic variation and restoration, and expand our understanding of the genetics of narrow-range endemic flora on BIF.

Materials and Methods

DNA extraction

DNA extraction is a key step in molecular biology based studies of plants and can prove problematic. We tested different DNA extraction methods (e.g. QIAGEN DNeasy Plant Mini Kit; Carlson et al. 1991; high throughput Nucleospin 96 Plant II) on eight individuals of each species. We found that the Nucleospin 96 Plant II and the Nucleospin Plant II methods (both Macherey-Nagel GmbH and Co., Düren, Germany) consistently produced the highest yields and highest quality DNA for *A. adinophylla*/*T. aphylla* subsp. *aphylla* and *L. bungalbin,* respectively. Genomic DNA was extracted using these methods from fresh leaf, phyllode and stem material, following collection.

Microsatellite marker development

A combination of cross-transferal of loci previously developed for related taxon and identification of new loci using next-generation sequencing was employed to identify microsatellite markers. Following a review of the literature, we did not attempt cross transferal of microsatellite loci from other *Acacia* (e.g. Nevill et al. 2010) and *Lepidosperma* (e.g. Barrett et al. 2012; Binks et al. 2014) species as the evolutionary distinctiveness of *A. adinophylla* and *L. bungalbin* combined with published microsatellite loci means that this approach was unlikely to be successful. Instead, we developed markers for these species using a shotgun sequencing approach (e.g. Nevill et al. 2013). We extracted high molecular weight DNA from *A. adinophylla* and *L. bungalbin,* which was sent to the Australian Genome Research Facility node in Melbourne, Victoria, for shotgun sequencing and identification of DNA sequences containing microsatellites. Briefly, genomic DNA was sequenced on an Illumina MiSeq with Pippin Prep size selection sequencing and 250 bp paired end reads (*A. adinophylla* 6,372,575 paired reads; *L. bungalbin* 6,538,181 paired reads)

We then used QDD v.3.1.2 pipeline (Meglecz et al. 2014) to screen the raw sequences for ≥ 8 di-, tri, tetra- and penta-base repeats, remove redundant sequences. The resultant sequences were filtered to ensure that the primer is not overlapping the repeat sequence, there are no poly-'A' or poly-'T' runs for more than seven bp within the sequence, and there is only one repeat motif between the primers. QDD uses Primer3 (Rozen et al. 2000) to design primers for detected microsatellites.

Sixty potentially suitable microsatellite loci were identified for each species and screened using DNA from six individuals of *A. adinophylla* and *L. bungalbin*, with individuals selected from different populations. Of these microsatellite loci, 20 that amplified consistently were subsequently screened against 24 individuals of *A. adinophylla* and *L. bungalbin,* from a single population. On completion of this marker optimisation stage, we identified a set of 12 loci that were then screened against all individuals of *A. adinophylla* and *L. bungalbin.* The remaining loci were either monomorphic or difficult to score accurately.

Each marker was amplified in a 6 µl reaction volume containing PCR buffer, Bioline Immolase DNA polymerase and dNTPs based on the recommendations provided by Bioline, 1.5 mM $MgCl_2$, 0.06 µM of M13-labelled forward locus-specific primer, 0.13 µM of reverse locus-specific primer, 0.13 µM of fluorescently-labeled M13 primer and 15 ng gDNA. The following PCR conditions were used: 94°C for 5 min followed by 11 cycles at 94°C for 30 sec, 60°C for 45 sec (dropping 0.5°C per cycle), and 72°C for 45 sec; followed by 30 cycles at 94°C for 30 sec, 55°C for 45 sec, and 72°C for 45 sec; followed by 15 cycles at 94°C for 30 sec, 53°C for 45 sec, and 72°C for 45 sec; and a final elongation step at 72°C for 10 min. For a given panel, the markers were pooled together for each sample, 1 µl of pooled sample was then applied to 10 µl mixture of Applied Biosystems Hi-Di Formamide and LIZ 500 size standard. This was then heated at 95°C for 5 minutes. Capillary electrophoresis of the product was performed by an Applied Biosystems (AB®) 3730 DNA Analyser. Running time for a 96 well plate was approximately 1 hour (230V, 32amp). Allele sizes were determined using Geneious V 7.1 (Biomatters 2005-2014). Multiple replicate runs were performed to ensure the accuracy of the

final dataset.

For *T. aphylla* subsp. *aphylla,* 38 potentially suitable microsatellite loci (previously developed for *Tetratheca* species were identified (Butcher and Krauss 2009; Krauss and Anthony 2014; McPherson et al. 2008). Screening, optimization and amplification of primers followed the approach described above for *A. adinophylla* and *L. bungalbin*. On completion of this marker optimisation stage, we identified a set of nine loci that were then screened against all individuals of *T. aphylla* subsp. *aphylla.* The remaining 29 loci were either monomorphic or difficult to score accurately.

Range wide sampling

In total, we sampled 300 (ca. 24 at each of 13 sites, labeled 1- 13; Figure 3), 260 (ca. 24 at each of 11 sites, labelled 1- 11; Figure 4) and 313 (ca. 24 at each of 13 sites, labelled 1- 13; Figure 5) plants of *A. adinophylla*, *L. bungalbin* and *T. aphylla* subsp. *aphylla,* respectively.
Plants were sampled from across the range of each species and included outlier populations. To assess the total genetic diversity in each species and avoid sampling potential clone mates, we avoided sampling adjacent plants. Samples were taken under licenses/permits # SW017143, CE004960 and 6-1516 issued by the Department of Parks and Wildlife to Dr Paul Nevill. Sampling involved the collection of fresh green leaf, phyllode or stem material, which was stored in zip-locked bags, and the location of each sample determined by GPS and recorded. Sampling locations were based on a combination of spatially distinct plant groupings (i.e. where plants grouped together, separate from other plant groups, e.g. site AA 8) and by division of larger continuous plant groupings (e.g. sites AA 3 and AA 4). This sampling scheme will enable an assessment of the spatial structuring of genetic variation, and consequently, the assessment of any genetic impact with removal of plants within the proposed disturbance area. Sampling occurred on August 10th-13th and September 15th-19th. Collections were stored on ice in the field prior to storage at 4^{o}C in the genetics facility at Curtin University until DNA extraction.

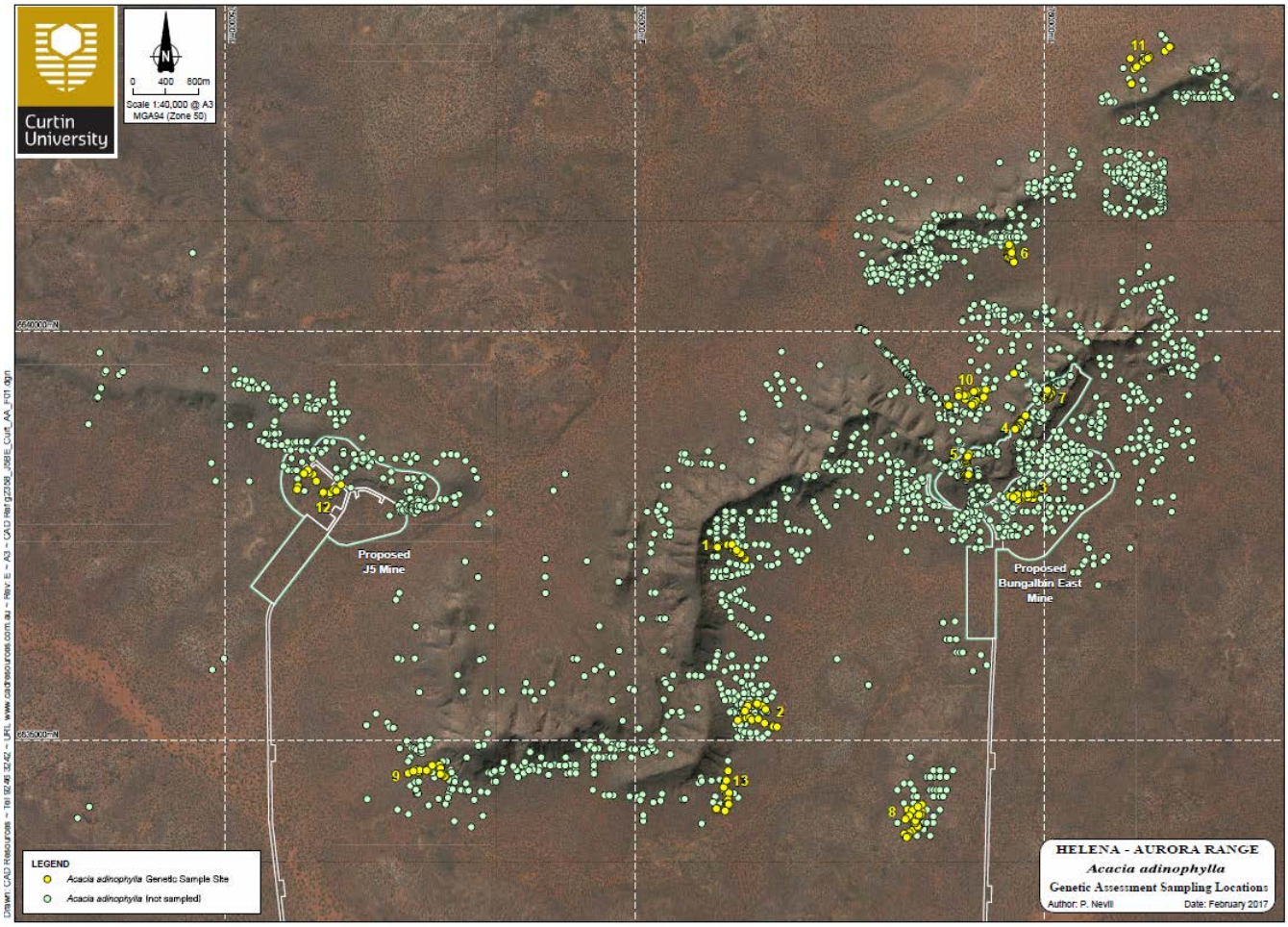


Figure 3 Map showing the location of all known plants of *Acacia adinophylla*. Yellow dots indicate the location of 300 plants sampled for genotyping. Green dots indicate the location of an un-sampled plant or aggregations of un-sampled plants. Numbers indicate site locations and the proposed disturbance area is outlined including the 20 m buffer zone

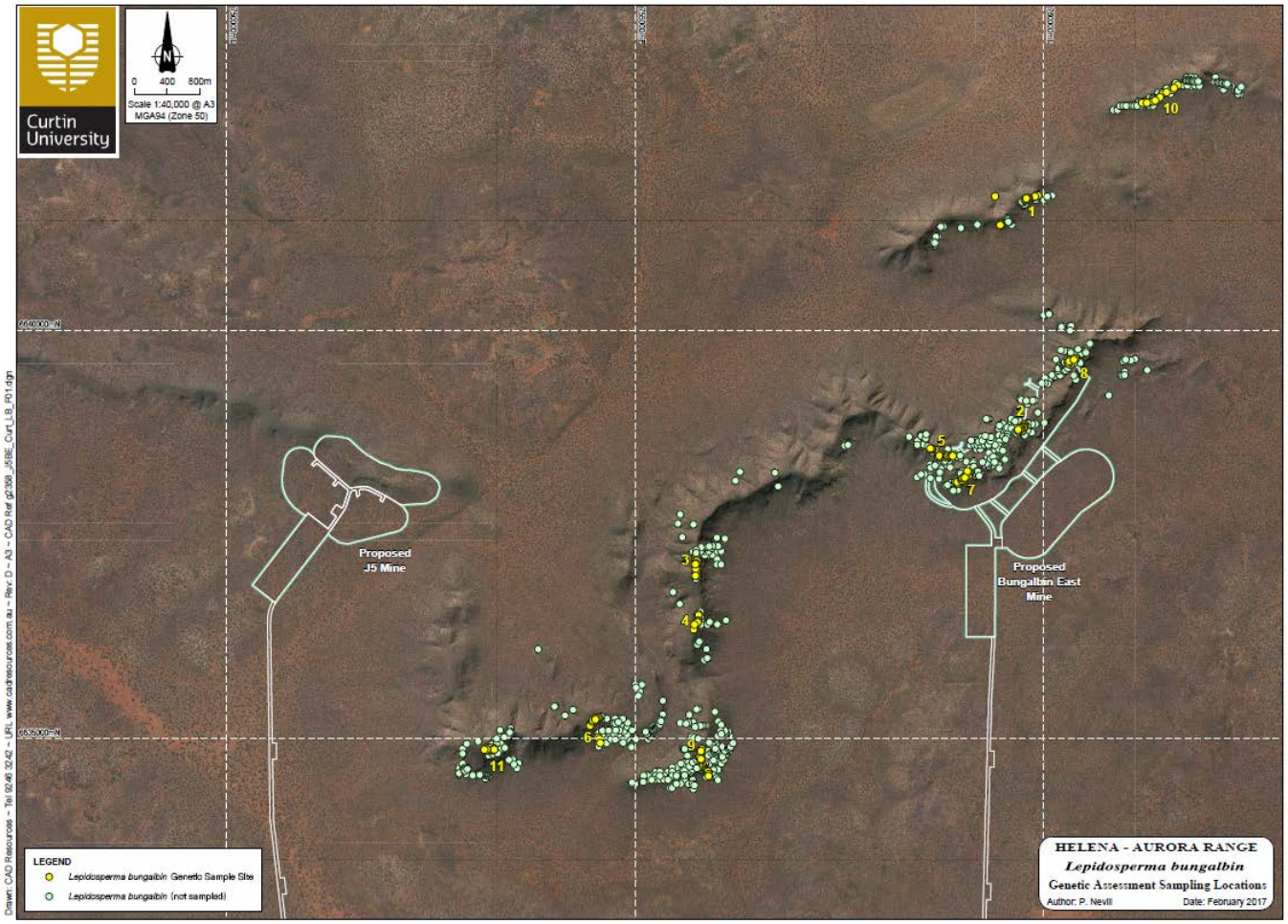


Figure 4 Map showing the location of all known plants of *Lepidosperma bungalbin*. Yellow dots indicate the location of 260 plants sampled for genotyping. Green dots indicate the location of an un-sampled plant or aggregations of un-sampled plants. Numbers indicate site locations and the proposed disturbance area is outlined including the 20 m buffer zone

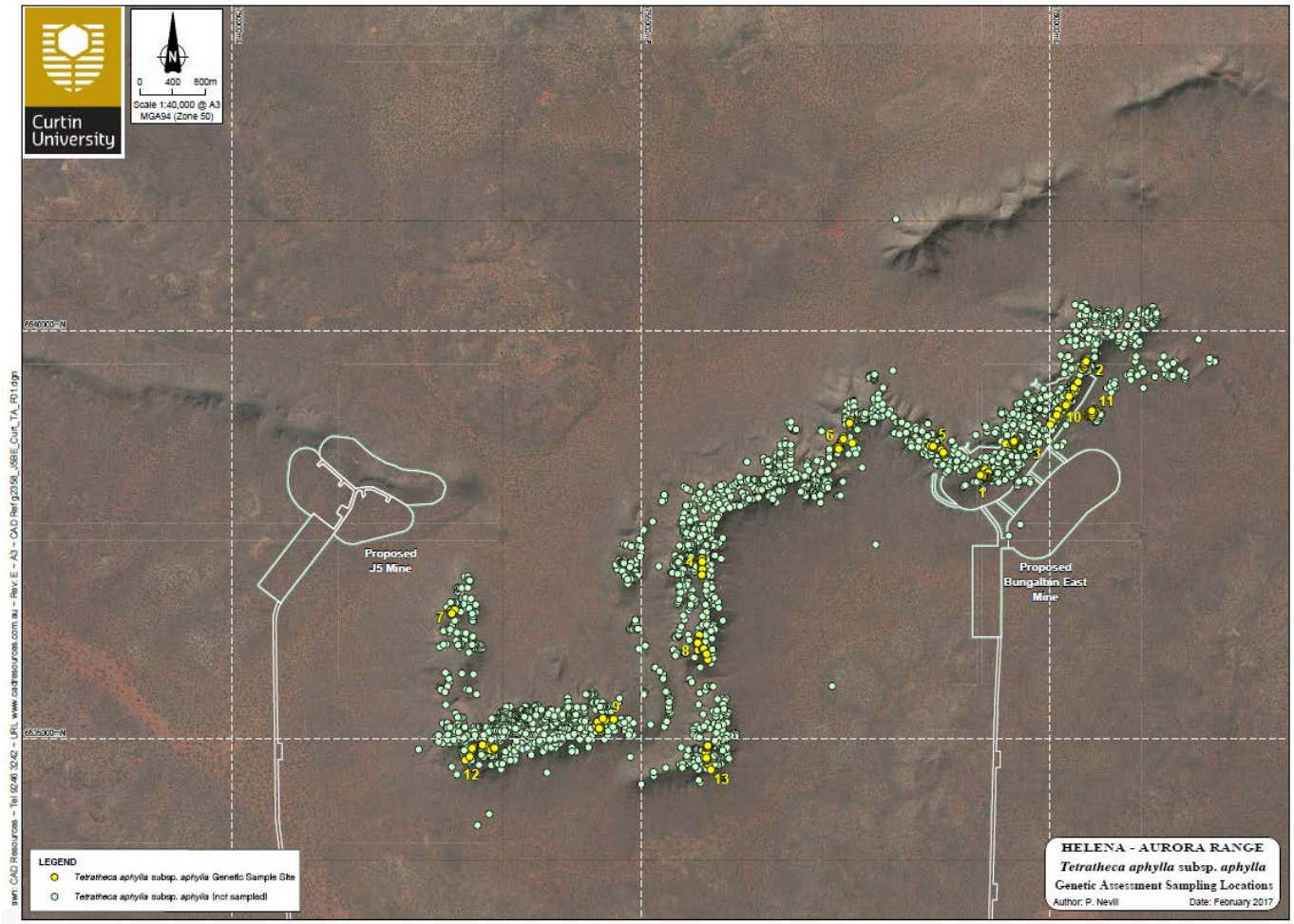


Figure 5 Map showing the location of all known plants of *Tetratheca aphylla* subsp. *aphylla*. Yellow dots indicate the location of 313 plants sampled for genotyping. Green dots indicate the location of an un-sampled plant or aggregations of un-sampled plants. Numbers indicate site locations and the proposed disturbance area is outlined including the 20 m buffer zone

Analysis of genetic diversity

Prior to analysis, the datasets were checked for clonality using GENCLONE v.2.0 (ArnaudHaond and Belkhir 2007), as per Millar et al. (2010). Replicate multilocus genotypes found likely to have arisen by asexual reproduction were removed from subsequent analyses. We tested for linkage disequilibrium (LD) among loci using FSTAT v.2.9.3.2 (Goudet 2002). LD means that two or more loci are located are located close together on the same chromosome, are inherited together and thus are not independent. Sequential Bonferroni corrections were applied to alpha values in the determination of significance to correct for multiple comparisons of LD (Rice 1989). Departure from Hardy-Weinberg equilibrium was assessed for each locus and site by chi-square tests in GenAlEx v.6.5 (Peakall and Smouse 2006), and the possibility of null alleles was checked using MICROCHECKER v.2.2.3 (Van Oosterhout et al. 2004). Standard measures of genetic variation including observed and expected heterozygosity (H_O, H_E), private alleles (*PA*) (alleles found in only one site) and Wrights *F* statistics were calculated using GenoDive (Merrimans and Van Tienderen 2004). Allelic richness (*AR*) was also calculated and rarefied to the smallest population size using HP-Rare (Kalinowski 2005)(n = 13 for *A. adinophylla*, n = 18 for *L. bungalbin* and n = 19 for *T. aphylla* subsp. *aphylla*).

Changes in population size

Changes in population size can have a strong influence on genetic patterns, particularly on rates of genetic drift. To examine whether past changes in population sizes, may account for any genetic differences between species, sites were assessed for past reductions in population size (over 10s to 1000s of years) using BOTTLENECK v.1.2.02 (Cornuet and Luikart 1997). Of the three available tests, the Wilcoxon sign-rank test was applied, because 1) the sign test has low statistical power; and 2) the standardized differences test requires data from 20 or more loci (Cornuet and Luikart 1997). We used the two-phase mutation model (TPM), which is intermediate between the step wise mutation model (SMM) and the infinite allele model (IAM), because few microsatellite loci follow the strict (one-step) SMM (Di Rienzo et al. 1994). We ran the TPM simulation as 90% one-step mutations and 10% multistep changes.

Genetic structure

The overall genetic structuring of microsatellite variation was investigated using measures of allelic differentiation, Mantel tests, principal coordinate analysis and Bayesian clustering. Genetic differentiation among populations in each species was estimated using F_{ST} (Weir and Cockerham 1984), the proportion of the total genetic variance contained in a population relative to the total genetic variance, with low values suggesting little genetic differentiation between populations. Population pairwise F_{ST} and F_{ST} across all populations were calculated using FSTAT v.2.9.3.2 (Goudet 2002). Measures of genetic differentiation among sites were also assessed by D_{EST} estimated in SMOGD v.1.2.5 (Crawford, 2010). D_{EST} is an alternative measure of allelic differentiation among populations that is not biased by the genetic diversity of the populations (Jost 2008).

Isolation by distance in the microsatellite data was evaluated using Mantel tests. Regression of population pairwise genetic (transformed to $F_{ST}/(1- F_{ST})$) and geographic distances (shortest distance between populations transformed using the natural logarithm) were conducted using

GenAlEx v.6.5 (Peakall and Smouse 2006) and significance values assessed by 9999 randomisations. There has been recent debate on the use of Mantel tests mainly focused on their reduced statistical power compared to recent methodologies (reviewed in Balkenhol et al. 2009). We used Mantel tests because they are widely used which aids in comparison between studies, they are relatively easy to interpret and are considered appropriate for distance data (Legendre and Fortin 2010).

To visualise microsatellite variation among populations we first used a principal co-ordinates analysis (PCoA) of genetic distance matrices generated in GenAlEx v.6.5 (Peakall and Smouse 2006). Genetic distance matrices were constructed by AMOVA on allele frequencies with 9999 randomisation steps and the first two principal coordinates were plotted in three-dimensional space by PCoA.

To further assess the structuring of genetic variation we used Bayesian clustering to assign individuals to populations. STRUCTURE v.2.0 (Falush et al. 2003; Pritchard et al. 2000) assigns individuals probabilistically to user defined K populations so as to achieve Hardy Weinberg and linkage disequilibrium within populations. STRUCTURE was run using the admixture model and assuming correlated allele frequencies. STRUCTURE was run with 1,000,000 Markov chain Monte Carlo iterations after a burn-in period of 500,000 iterations and modelled with K = 1 to K = 8, with 10 iterations of each K. Structure Harvester (Earl and vonHoldt 2012) was used to infer an optimal K based on the method of Evanno et al. (2005). The 10 runs of the optimal value of K were summarised using CLUMPP (Jakobsson and Rosenberg 2007) with the Greedy algorithm and graphically displayed.

Landscape Genetic Analysis-Resistance Surfaces

Isolation-by-resistance-distance (IBRD) was used to explore if nearby sampling units were genetically more similar than distant ones against the null hypothesis of randomness (Wagner and Fortin 2012; Willoughby et al. 2015). Pairwise resistance distances were calculated using Circuitscape 4.0 on a completely ‘flat’ landscape, simulated by using a constant raster layer (all pixels set to 1) following Noguerales et al. (2016). This is said to yield similar results to pairwise Euclidean distances between sites (e.g. Nowakowski et al. 2015), but is more appropriate for comparison between competing resistance surfaces (McRae 2006). We created an additional three resistance surfaces to examine their relationship with gene flow: (1) connectivity-by-habitat (CH); (2) connectivity-by-topographic-complexity (CTC); and (3) connectivity-by-density (CD). These variables were selected as potentially important determinants of genetic connectivity based on the findings of previous studies on BIF ranges (e.g. Butcher et al. 2009; Millar et al. 2013; Binks et al. 2015; Millar et al. 2015).

CH and CTC utilised an existing species distribution model and surface of topographic complexity, respectively, both developed by Di Virgilio (2015) from ca. 2 m resolution Lidar and derivatives. As coordinates of all individual plants of the three species were available, CD was calculated as the Euclidean distance to nearest plant. All three surfaces were rescaled to be between 1 and 100, where 1 indicates low resistance (i.e., highly suitable habitat, highly complex topography, low distance between plants) and 100 indicates high resistance (Mims et al. 2016).

While this range is somewhat arbitrary, inversion of the original range was required based on our hypotheses of these layers' influence on gene flow.

Circuitscape Modelling

We used Circuitscape 4.0 to estimate landscape resistances between focal nodes (McRae et al. 2013). Focal nodes were created as the convex hull of plants making up non-contiguous populations that had been sampled for genetic variation. We set all focal nodes to zero at initialisation to gauge the cumulative current flowing through them to give an idea of their individual importance for connecting other focal nodes in the network. For example, those that remain close to zero do not facilitate connection. All analyses were performed in the pairwise modelling mode, using eight neighbours to allow current flow from any direction. We output the cumulative current maps as ASCII rasters for visualisation in ArcGIS and utilised the pairwise resistance distances in statistical analyses of landscape genetic relationships.

Statistical Analyses: Multiple Regression Distance Matrices

The four resistance surfaces were individually tested against two genetic differentiation response variables (F_{ST} and D_{EST}) using multiple matrix regressions with randomisation (MMRR) using the ECODIST 1.2.2 package (Goslee and Urban 2007) in R 3.3.0 (R Core Team 2016). To test for multicollinearity, each resistance surface was regressed against one another (also using MMRR). Resistance surfaces with an $R^2 > 0.49$ ($r = 0.7$) were eliminated one at a time starting from the largest correlated pair (Mims et al. 2016). At each comparison the individual variable with the highest correlation to the genetic response variables was retained. Regressions were rerun with these filtered sets and non-significant variables were dropped at iteration until no insignificant variables remained (Nowakowski et al. 2015; Noguerales et al. 2016). As the pairwise distance values in the distance vectors fail to be independent of each other, the response matrix was permuted 10 000 times to determine the significance of the regression coefficients and R^2 (Lichstein 2006).

Genetic assessment of impact

The potential impact of proposed removal of plants on genetic diversity in *A. adinophylla*, *L. bungalbin* and *T. aphylla* subsp. *aphylla* was quantified by recalculating standard measures of genetic variation including the observed number of alleles (*A*), observed and expected heterozygosity (H_O, H_E), private alleles lost (*PA*) and allelic richness (*AR*), for a dataset that did not include genotyped plants that fell within the proposed direct disturbance area and a 20 metre buffer zone (buffer zone dimensions D. Temple-Smith pers. com). Impacted sites for *A. adinophylla* (3, 4, 5, 7 and 12), *L. bungalbin* (2, 7 and 8) and *T. aphylla* subsp. *aphylla* (1, 2, 3 and 10) were identified using maps provided by Polaris (Figures 3, 4 and 5). To assess genetic impact, these recalculated genetic diversity measures were compared to those estimated from the complete dataset. .

Measures of genetic differentiation F_{ST} and D_{EST} among sites were recalculated for the dataset that did not include the plants which fell within the disturbance area. These were compared to the

measures calculated on the full data set to assess any impact on genetic differentiation among sites.

Results

Acacia adinophylla *- Genetic variation and its spatial structure*

We did not find any identical multilocus genotypes and there was no evidence of linked loci after Bonferroni corrections. All loci were polymorphic in all populations and there was no consistent departure from Hardy-Weinberg equilibrium for any locus across all sites (results not shown). From MICROCHECKER, the average frequency of null alleles was < 5% across the whole data set but two loci (accur26 and accur5) had higher frequencies (10 and 8%, respectively). To determine the overall effect these loci had on the results, all analyses were performed with these loci both present and absent. Although excluding these loci reduced overall average null frequency to only 1.7%, F_{ST} and diversity metrics did not change greatly. As such, all subsequent analyses were performed on the observed allele frequencies.
The 12 microsatellites used in this study were all polymorphic with 3–18 alleles per locus (an average of 8 alleles per locus; Table 1). Observed heterozygosity (H_O) per population varied from 0.51 to 0.63 (overall mean = 0.56), and was lower than expected heterozygosity (H_E = 0.56 – 0.64; overall mean = 0.60; Table 2). All but one population (AA 4) deviated from Hardy-Weinberg proportions due to heterozygote deficits, however, the inbreeding statistic F_{IS} was generally low to moderate (F_{IS} = -0.06 – 0.014; overall mean = 0.06) (Table 2). Allelic richness was similar among populations (*AR* = 4.48 – 5.39; overall mean = 4.88) and private alleles were found in very low frequencies at 5 of 13 sites (Table 2). Evidence of genetic bottlenecks (mode shift) was detected in populations AA 4 and AA 11.

Pairwise F_{ST} comparisons ranged from 0.002 (sites AA 2 and AA 13) to 0.073 (sites AA 4 and AA 9) and were generally low (Table 3) with a global F_{ST} of 0.030 ± 0.005. A similar pattern was observed with D_{EST} (pairwise data not shown; overall D_{EST} = 0.042 ± 0.006). Mantel tests detected a significant association between pairwise genetic distance and geographic distance among all pairs of sites (Figure 6). Using a landscape genetics approach we found a significant ($p<0.01$) positive association with geographic distance (IBD), habitat (CH), topographic complexity (CTC) and plant density (CD), irrespective of genetic differentiation metric used as the response variable (F_{ST} or D_{EST}). Multiple matrix regression showed that all variables exhibited unacceptably high levels of multicollinearity to develop models with more than one variable and so the most parsimonious models were found from backward selection. This resulted in the choice of IBD as the most explanatory variable for *Acacia adinophylla* (R^2 = 0.22 F_{ST}; R^2 = 0.21 D_{EST}).The first two axes of the PCoA accounted for 58% of the total genetic variation (Figure 7) and the ordination of the genetic data supports the association between genetic and geographic distance, with geographically proximate populations clustering more closely than geographically distant ones. STRUCTURE analysis and the method of Evanno et al. (2005) identified K = 2 as the optimal number of clusters to the data (Figure 8) and comparable patterns were found with more mixed proportions of membership for centrally located sites suggesting a clinal association between genetic and geographic distance.

Table 1 The twelve nuclear microsatellite loci used to study *Acacia adinophylla* and their genetic diversity parameters

Name	*Size range (bp)*	A	H_O	H_E	F_{ST}
aacur5	129-137	6	0.41	0.54	0.06
aacur25	184-190	3	0.42	0.39	0.10
aacur4	120-146	11	0.76	0.82	0.05
aacur20	173-183	5	0.20	0.20	0.04
aacur11	144-172	10	0.80	0.75	0.06
aacur21	180-194	8	0.60	0.72	0.05
aacur29	192-204	4	0.27	0.28	0.04
aacur32	202-214	6	0.64	0.65	0.03
aacur52	255-285	18	0.74	0.75	0.06
aacur26	178-205	8	0.44	0.57	0.05
aacur58	254-275	9	0.68	0.70	0.04
aacur19	167-189	10	0.69	0.80	0.06
MEAN		8.2	0.56	0.60	0.05

Abbreviations: locus name; allele size range in base pairs; number of alleles observed per locus (*A*); observed heterozygosity (H_O); expected heterozygosity (H_E); and genetic differentiation indice F_{ST} per locus are shown

Table 2 Genetic diversity parameters for populations of *Acacia adinophylla*

Population	n	H_O	H_E	F_{IS}	AR	PA
AA 4	20	0.63	0.60	-0.06	4.48	1
AA 5	24	0.59	0.61	0.05	5.09	0
AA 6	20	0.59	0.62	0.02	4.81	0
AA 7	23	0.52	0.58	0.08	4.63	0
AA 1	22	0.54	0.64	0.14	5.39	1
AA 2	20	0.57	0.61	0.03	4.97	0
AA 3	21	0.52	0.59	0.12	4.96	1
AA 8	23	0.53	0.56	0.02	4.76	0
AA 13	23	0.51	0.59	0.10	5.12	3
AA 9	20	0.55	0.61	0.08	4.96	0
AA 10	21	0.56	0.61	0.06	4.91	0
AA 11	13	0.57	0.61	0.06	4.75	2
AA 12	23	0.54	0.60	0.04	4.57	0
MEAN	21	0.56	0.60	0.06	4.88	0.60

Abbreviations: number of individuals (n); observed heterozygosity (H_O); expected heterozygosity (H_E); inbreeding co-efficient (F_{IS}); allelic richness (*AR*); observed number of private alleles (*PA)*

Table 3 Pairwise population F_{ST} values for all sites for twelve microsatellite loci in *Acacia adinophylla*

POP	*AA4*	*AA5*	*AA6*	*AA7*	*AA1*	*AA2*	*AA3*	*AA8*	*AA 13*	*AA 9*	*AA 10*	*AA11*	*AA12*
AA4	0.000												
AA5	0.027	0.000											
AA6	0.043	0.021	0.000										
AA7	0.022	0.011	0.012	0.000									
AA1	0.038	0.011	0.040	0.022	0.000								
AA2	0.040	0.023	0.041	0.019	0.002	0.000							
AA3	0.032	0.011	0.022	0.003	0.011	0.019	0.000						
AA8	0.067	0.039	0.061	0.046	0.011	0.030	0.036	0.000					
AA13	0.038	0.026	0.054	0.035	0.007	0.002	0.018	0.024	0.000				
AA9	0.073	0.033	0.066	0.060	0.014	0.022	0.037	0.045	0.021	0.000			
AA10	0.018	0.010	0.013	0.000	0.016	0.011	0.005	0.026	0.016	0.035	0.000		
AA11	0.032	0.038	0.022	0.019	0.041	0.034	0.015	0.069	0.040	0.067	0.016	0.000	
AA12	0.058	0.039	0.067	0.039	0.024	0.029	0.033	0.040	0.029	0.025	0.030	0.064	0.000

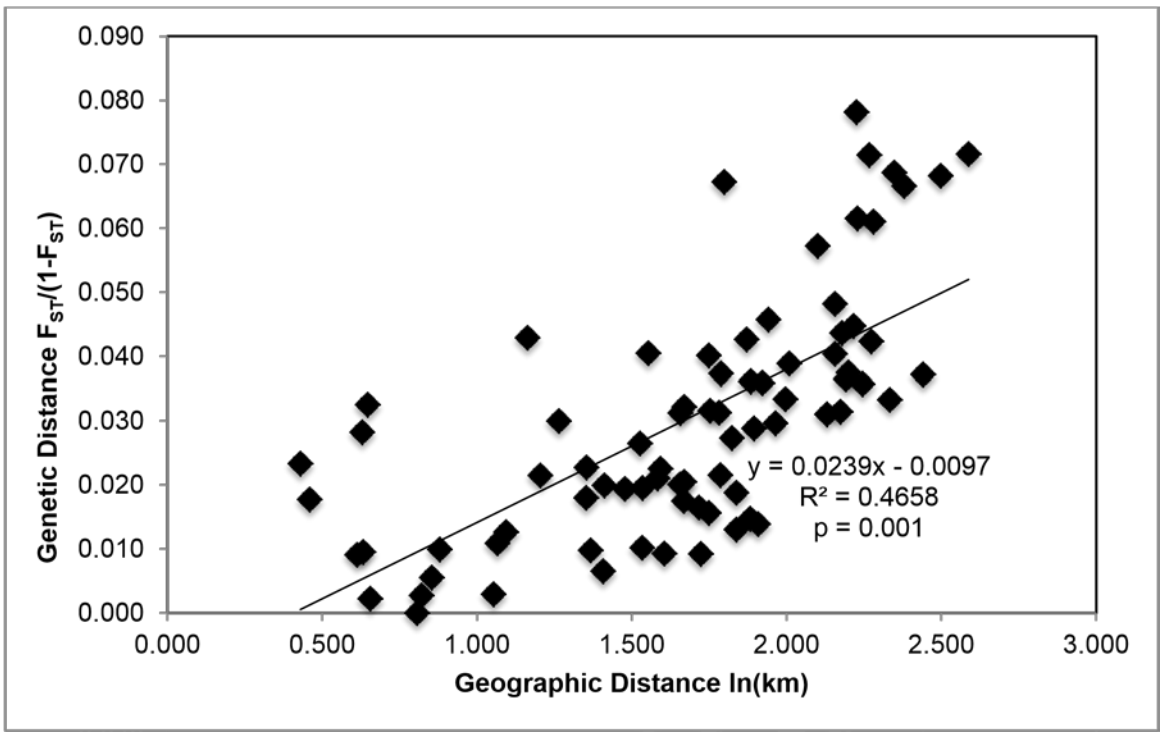


Figure 6 Mantel testing of isolation by distance for correlations between geographic distance and pairwise genetic distance from twelve microsatellite loci for all pairs of sites of *Acacia adinophylla*

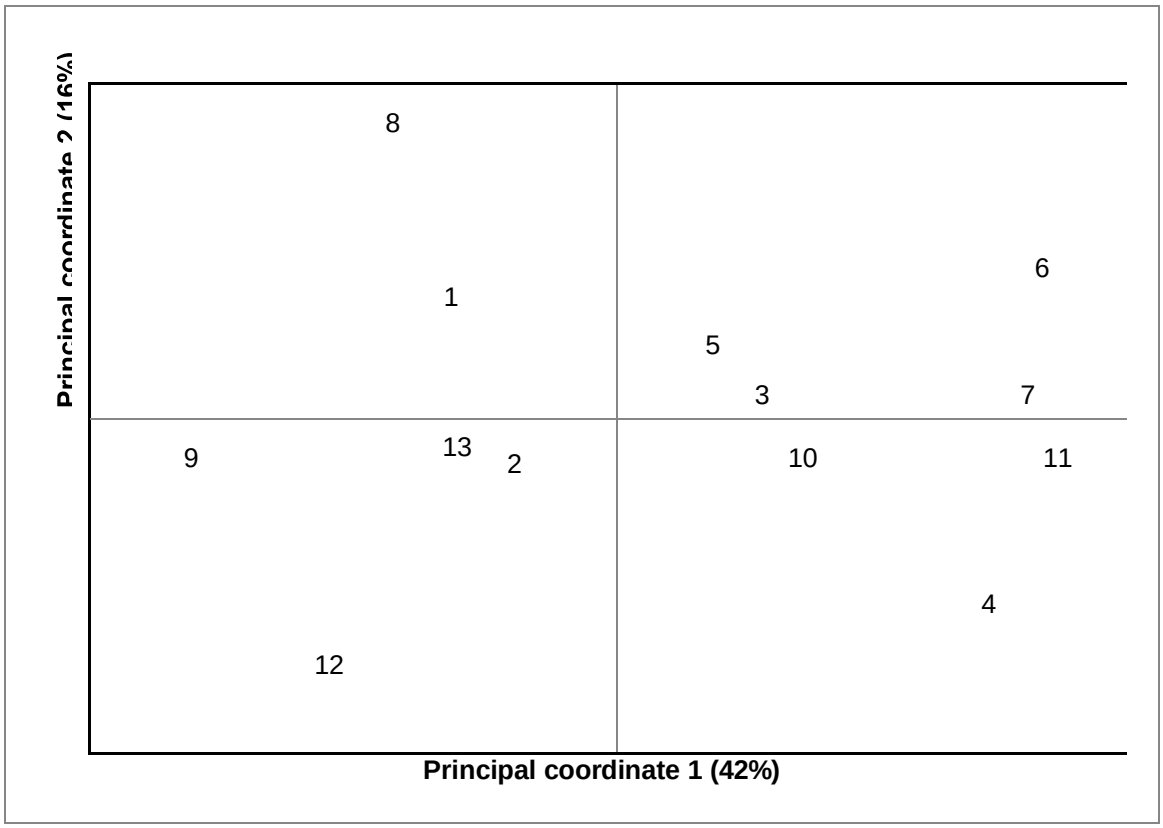
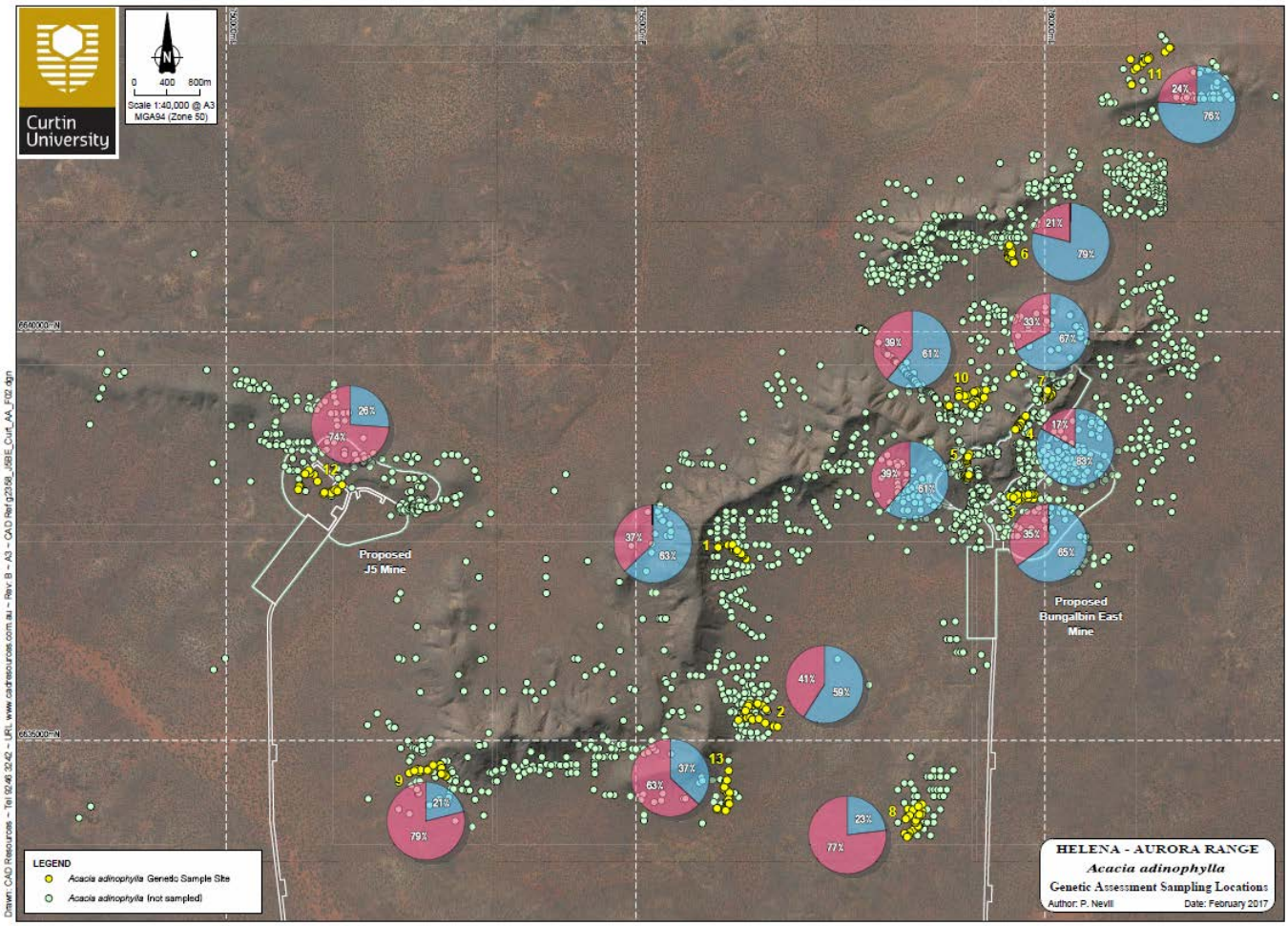


Figure 7 Principal coordinates analysis for all populations of *Acacia adinophylla* based on F_{ST}. These two dimensions account for 58% of the total variation



F**igure 8** Proportion of membership values from STRUCTURE analysis for each sampled site of *Acacia adinophylla*. Colours in pie charts indicate the mean proportion of assignment to one of two clusters (red, blue) for all individuals within a site, where optimal number of clusters was defined by the Evanno et al (2005) method as K = 2. Yellow dots indicate the location of 300 plants sampled for genotyping. Green dots indicate the location of an un-sampled plant or aggregations of un-sampled plants. Numbers indicate site locations and the proposed disturbance area is outlined including the 20 m buffer zone

Acacia adinophylla *- Genetic assessment of impact*

The genetic impact of proposed mining on *A. adinophylla* was assessed on the basis of repeating analyses of genetic variation after removal of genotyped samples located within the disturbance area or buffer zone (AA3, AA 4, AA 5, AA 7 and AA 12), and comparing to the complete dataset (Table 4). The removal of impacted samples from these five sites resulted in the loss of two alleles - i.e. 96 of 98 alleles (98%) were recovered from within the remaining samples. One of these alleles was extremely rare and found only at the AA 4 site (frequency 0.05). All other parameters of genetic variation (mean H_O, H_E, AR) were unaffected. Similarly, overall measures of genetic differentiation were not affected by removal of impacted samples (F_{ST} = 0.030 and 0.028; D_{EST} = 0.050 and 0.047, for full and reduced dataset respectively) (Table 4) as the most genetically distinct sites (AA 8 and AA 9) were located in the southern part of the species range,

outside of disturbance areas. Consequently, while up to ~12% of all plants may be removed by mine development, ~2% of alleles detected at 12 microsatellite markers will be lost, and other genetic parameters remain unaffected.

Table 4 Genetic diversity and differentiation parameters for *Acacia adinophylla* from all sites, and from all except samples within the disturbance area and buffer zone

	A	H_O	H_E	AR	PA	F_{ST}	D_{EST}
ALL SITES	98	0.55 (0.02)	0.60 (0.02)	4.86	-	0.030 (0.006)	0.050 (0.015)
ALL SITES (-impacted)	96	0.55 (0.02)	0.62 (0.02)	4.90	1 (88%)	0.027 (0.015)	0.047 (0.015)

Abbreviations: total number of alleles across all loci (*A*); observed heterozygosity (H_O); expected heterozygosity (H_E); allelic richness (*AR*); observed number of private alleles lost (*PA*) with % remaining in brackets; and genetic differentiation indices F_{ST} and D_{EST} are shown. Standard errors in parentheses

Lepidosperma bungalbin *- Genetic variation and its spatial structure*

We did not find any identical multilocus genotypes and there was no evidence of linked loci after Bonferroni corrections. All loci were polymorphic in all populations and there was no consistent departure from Hardy-Weinberg equilibrium for any locus across all sites (results not shown). From MICROCHECKER, the average frequency of null alleles was < 6% across the whole data set.

The 12 microsatellites used in this study were all highly polymorphic with 9–29 alleles per locus (an average of 17 alleles per locus; Table 5). Observed heterozygosity (H_O) per population varied from 0.63 to 0.82 (overall mean = 0.73) and was lower than expected heterozygosity (H_E = 0.71 – 0.82; overall mean = 0.78; Table 6). Inbreeding was generally low except in populations LB 1, LB 3 and LB 4 (F_{IS} = 0 – 0.019; overall mean = 0.06) (Table 6). Allelic richness was similar among populations (*AR* = 6.78 – 9.08; overall mean = 8.11) and private alleles were found in very low frequencies at 9 of 13 sites (Table 6). No evidence of genetic bottlenecks was detected in any population ($p > 0.05$ and no mode shifts detected).

Pairwise F_{ST} comparisons ranged an order of magnitude from 0.005 (sites LB 7 and LB 5) to 0.11 (sites LB 1 and LB 2) but were generally low (Table 7) with a global F_{ST} of 0.068 ± 0.006. A similar pattern was observed with D_{EST} (pairwise data not shown; overall D_{EST} = 0.187 ± 0.045). A Mantel test showed that genetic distance was significantly correlated with geographic distance among all pairs of sites (Figure 9). Genetic differentiation exhibited a significant ($p<0.01$) positive association with geographic distance (IBD), habitat (CH), topographic complexity (CTC) and plant density (CD), irrespective of genetic differentiation metric used as the response variable (F_{ST} or D_{EST}). Multiple matrix regression showed that all variables exhibited high levels of multicollinearity so the most parsimonious models with a single variable were found from backward selection. This resulted in the choice of CH as the most explanatory variable for *L. bungalbin* (R^2 = 0.10 F_{ST}; 0.15 D_{EST}).The PCoA clearly segregated central, south

western and north eastern populations (Figure 10). As in the PCoA, the STRUCTURE analysis and the method of Evanno et al. (2005) identified K = 3 as the optimal number of clusters in *L. bungalbin* (Figure 11). These three genetic groups were similar to those found in the PCoA. However, there was some suggestion that K = 4 was the best of the number of genetic groups, with LB 1 and LB 10 each forming a distinct cluster.

Table 5 The 12 nuclear microsatellite loci used to study *Lepidosperma bungalbin* and their genetic diversity parameters

Name	*Size range (bp)*	A	H_O	H_E	F_{ST}
lbcur21	196-214	9	0.62	0.68	0.10
lbcur 41	236-284	21	0.53	0.54	0.10
lbcur 57	287-316	14	0.78	0.79	0.09
lbcur16	164-236	29	0.82	0.86	0.07
lbcur6	144-192	22	0.80	0.82	0.08
lbcur50	360-435	19	0.74	0.80	0.08
lbcur27	209-237	13	0.81	0.80	0.10
lbcur33	216-248	18	0.69	0.82	0.09
lbcur53	282-326	19	0.79	0.83	0.08
lbcur40	274-294	12	0.68	0.79	0.06
lbcur14	172-210	20	0.76	0.82	0.09
lbcur38	236-256	11	0.70	0.78	0.08
MEAN		17	0.73	0.78	0.08

Abbreviations: locus name; allele size range in base pairs; number of alleles observed per locus (*A*); observed heterozygosity (H_O); expected heterozygosity (H_E); and genetic differentiation indice F_{ST} per locus are shown

Table 6 Genetic diversity parameters for populations of *Lepidosperma bungalbin*

Population	n	H_O	H_E	F_{IS}	AR	PA
LB 3	23	0.70	0.80	0.13	8.75	7
LB 1	25	0.63	0.76	0.16	7.49	4
LB 5	21	0.76	0.78	0.01	8.18	1
LB 10	18	0.71	0.72	0	7.21	0
LB 2	21	0.70	0.71	0.01	6.78	1
LB 4	21	0.64	0.77	0.19	8.03	1
LB 6	23	0.75	0.83	0.09	9.04	5
LB 7	20	0.71	0.77	0.07	7.98	4
LB 8	21	0.82	0.80	0	8.31	0
LB 9	20	0.78	0.82	0.05	9.08	2
LB 11	23	0.80	0.80	0.01	8.31	2
MEAN	22	0.73	0.78	0.06	8.11	2.45

Abbreviations: number of individuals (n); observed heterozygosity (H_O); expected heterozygosity (H_E); inbreeding coefficient (F_{IS}); allelic richness (*AR*); observed number of private alleles

Table 7 Pairwise population F_{ST} values for all sites for 12 microsatellite loci in *Lepidosperma bungalbin*

POP	*LB 3*	*LB 1*	*LB 5*	*LB 10*	*LB 2*	*LB 4*	*LB 6*	*LB 7*	*LB 8*	*LB 9*	*LB 11*
LB 3	0.000										
LB 1	0.077	0.000									
LB 5	0.053	0.092	0.000								
LB 10	0.084	0.086	0.078	0.000							
LB 2	0.080	0.111	0.038	0.080	0.000						
LB 4	0.044	0.082	0.057	0.080	0.089	0.000					
LB 6	0.044	0.086	0.052	0.072	0.067	0.054	0.000				
LB 7	0.050	0.102	0.005	0.099	0.037	0.076	0.058	0.000			
LB 8	0.060	0.081	0.067	0.096	0.056	0.069	0.041	0.064	0.000		
LB 9	0.051	0.089	0.060	0.092	0.074	0.058	0.025	0.071	0.055	0.000	
LB 11	0.056	0.096	0.058	0.109	0.088	0.069	0.033	0.059	0.070	0.039	0.000

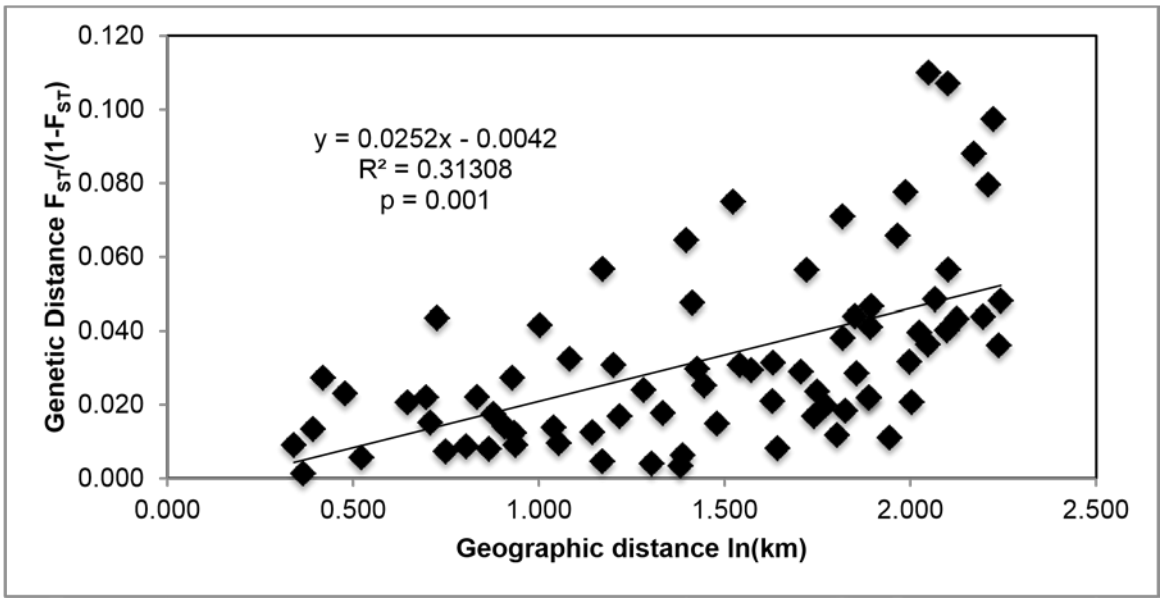


Figure 9 Mantel testing of isolation by distance for correlations between geographic distance and pairwise genetic distance from eleven microsatellite loci for all pairs of sites of *Lepidosperma bungalbin*

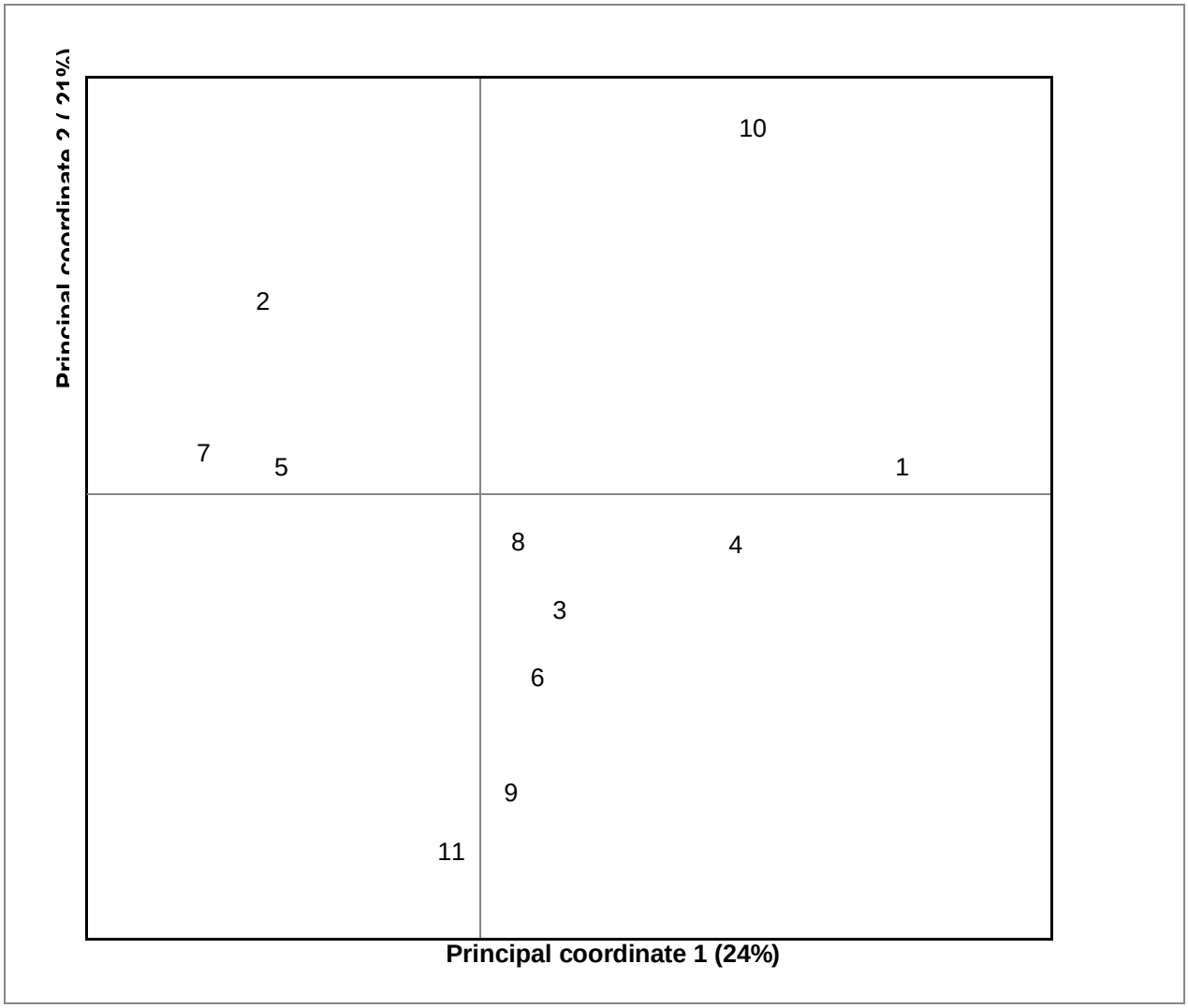


Figure 10 Principal coordinates analysis for all populations of *Lepidosperma bungalbin* based on F_{ST}. These two dimensions account for 45% of the total variation

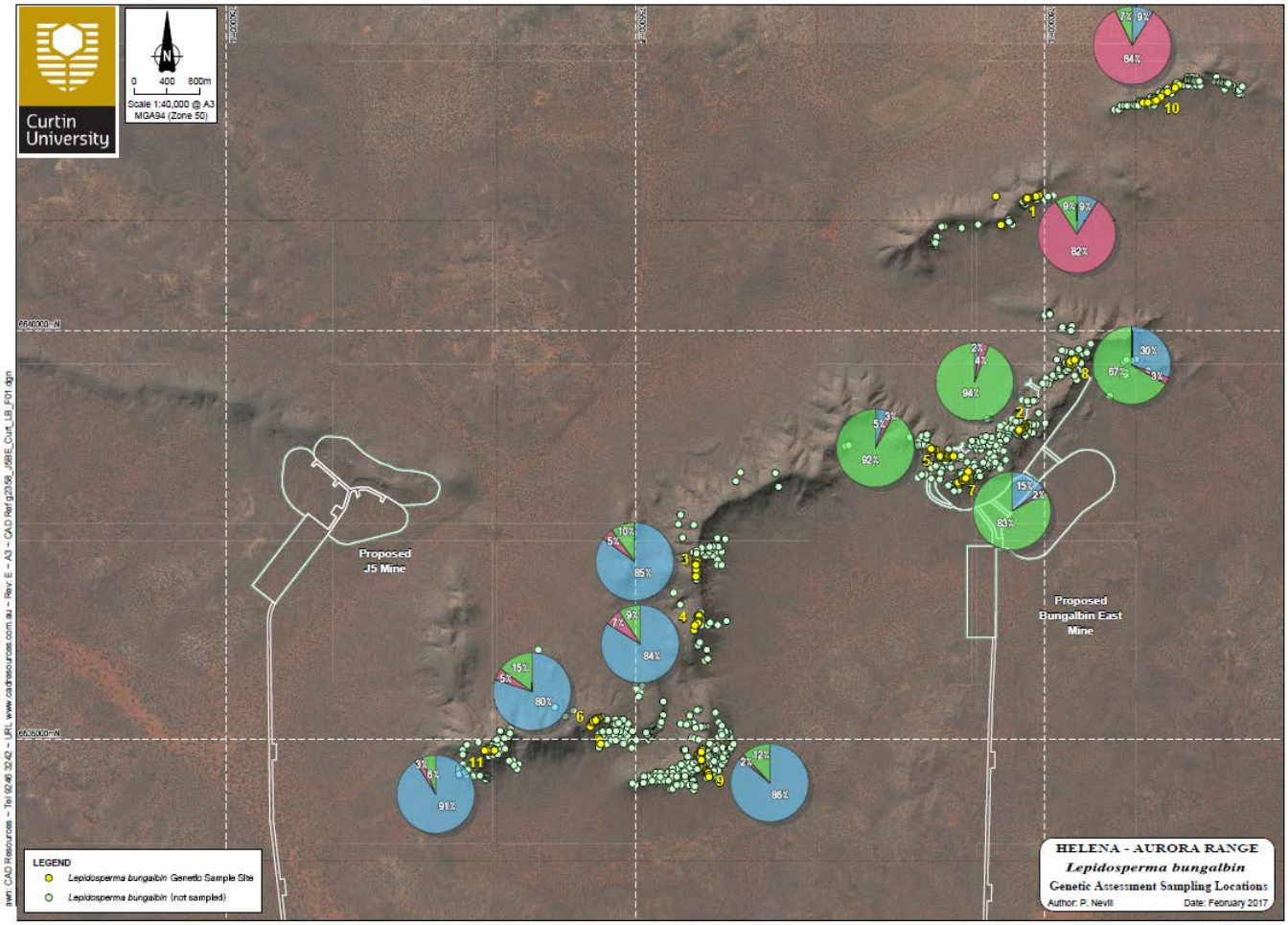


Figure 11 Proportion of membership values from STRUCTURE analysis for each sampled site of *Lepidosperma bungalbin*. Colours in pie charts indicate the mean proportion of assignment to one of two clusters (red, blue and green) for all individuals within a site, where optimal number of clusters was defined by the Evanno et al (2005) method as $K = 3$. Yellow dots indicate the location of 260 plants sampled for genotyping. Green dots indicate the location of an un-sampled plant or aggregations of un-sampled plants. Numbers indicate site locations and the proposed disturbance area is outlined including the 20 m buffer zone

Lepidosperma bungalbin *- Genetic assessment of impact*

The genetic impact of proposed mining on *L. bungalbin* was assessed on the basis of repeating analyses of genetic variation after removal of impacted samples from three sites located within (or partially), the proposed Bungalbin East mine (LB 2, LB 7 and LB 8), and comparing to the complete dataset (Table 8). The removal of impacted samples from these three sites resulted in the loss of 2 alleles (2 private) - i.e. 205 of 207 alleles (99%) were recovered from within the unaffected samples. These two alleles were extremely rare and found only in the LB 2 and LB 8 sites (lost allele frequencies all < 0.05). All other parameters of genetic variation (mean H_O, H_E, AR) were unaffected. Similarly, overall measures of genetic differentiation were not affected by removal of sites (F_{ST} = 0.068 and 0.067; D_{EST} = 0.187 and 0.190, for full and reduced dataset respectively) (Table 8) with the most genetically distinct sites (LB 1 and LB 10) located in the northern part of the species range, outside of disturbance areas. Therefore, while up to ~8% of

all plants may be removed by mine development, ~1% of alleles detected at 12 microsatellite markers will be lost, and other genetic parameters remain unaffected. However, most of the plants in the "green" genetic cluster will be removed with the proposal.

Table 8 Genetic diversity and differentiation parameters for *Lepidosperma bungalbin* from all sites, and from all except samples within the disturbance area and buffer zone

	A	H_O	H_E	AR	PA	F_{ST}	D_{EST}
ALL SITES	207	0.73 (0.01)	0.78 (0.01)	8.11	-	0.68 (0.010)	0.187 (0.045)
ALL SITES (-impacted)	205	0.72 (0.01)	0.78 (0.01)	8.26	2 (93%)	0.67 (0.007)	0.197 (0.045)

Abbreviations: total number of alleles across all loci (*A*); observed heterozygosity (H_O); expected heterozygosity (H_E); allelic richness (*AR*); observed number of private alleles lost (*PA*) with % remaining in brackets; and genetic differentiation indices F_{ST} and D_{EST} are shown. Standard errors in parentheses

Tetratheca aphylla subsp. aphylla *- Genetic variation and its spatial structure*

We did not find any identical multilocus genotypes and there was no evidence of linked loci after Bonferroni corrections. All loci were polymorphic in all populations and there was no consistent departure from Hardy-Weinberg equilibrium for any locus across all sites (results not shown). From MICROCHECKER the average frequency of null alleles was < 4% across the whole data set The 9 microsatellites used in this study were polymorphic with 4–28 alleles per locus (an average of 13 alleles per locus; Table 9). Observed heterozygosity (H_O) per population varied from 0.62 to 0.73 (overall mean = 0.66), which was slightly lower than expected heterozygosity (H_E = 0.64 – 0.72; overall mean = 0.68; Table 9). All populations deviated from Hardy-Weinberg proportions, however, the inbreeding statistic F_{IS} was generally low to moderate (F_{IS} = -0.07 – 0.012; overall mean = 0.03) (Table 10). Allelic richness was similar among populations (AR = 5.60 – 7.30; overall mean = 6.70) and private alleles were found in very low frequencies at 6 of 13 sites (Table 10). Evidence of genetic bottlenecks ($p<0.05$) was detected in 4 populations (TA 4, TA 7, TA 11 and TA 12) and there was no geographic pattern to their distribution.

Pairwise F_{ST} comparisons ranged an order of magnitude from 0.002 (sites TA 2 and TA 10) to 0.104 (sites TA 1 and TA 7) and were generally low (Table 11) with a global F_{ST} of only 0.031 ± 0.005. A similar pattern was observed with D_{EST} (pairwise data not shown; overall D_{EST} = 0.078 ± 0.027). Using Mantel tests we detected a significant association between pairwise genetic distance and geographic distance among all pairs of sites (Figure 12) and the ordination of the genetic data largely reflects the association (Figure 13). Using a landscape genetics approach genetic differentiation exhibited a significant ($p<0.01$) positive association with geographic distance (IBD), habitat (CH), topographic complexity (CTC) and plant density (CD), irrespective of genetic differentiation metric used as the response variable (F_{ST} or D_{EST}). Multiple matrix regression showed that all variables exhibited high levels of multicollinearity so the most parsimonious models with a single variable were found from backward selection. This resulted in the choice of CH as the most explanatory variable for *T. aphylla* subsp. *aphylla* (R^2 = 0.25 F_{ST}; 0.30 D_{EST}). STRUCTURE analysis and the method of Evanno et al. (2005) identified K = 2 as the optimal number of clusters in *T. aphylla* subsp. *aphylla* (Figure 14). We found an east/west cline

for proportions of membership in the two genetic clusters with more mixed proportions of membership for centrally located sites.

Table 9 The nine nuclear microsatellite loci used to study *Tetratheca aphylla* subsp*. aphylla* and their genetic diversity parameters

Name	*Size range (bp)*	A	H_O	H_E	F_{ST}
Te17bgt	162-234	28	0.78	0.87	0.06
c131	153-180	8	0.77	0.72	0.07
b11	174-184	6	0.59	0.65	0.07
c3	214-244	7	0.55	0.54	0.05
a106	158-172	7	0.70	0.72	0.04
te03bgt	444-489	10	0.54	0.61	0.07
b1	145-200	24	0.87	0.90	0.04
te15bgt	333-377	20	0.83	0.81	0.04
te09bgt	153-159	4	0.33	0.35	0.03
MEAN		12.7	0.66	0.68	0.05

Abbreviations: locus name; allele size range in base pairs; number of alleles observed per locus (*A*); observed heterozygosity (H_O); expected heterozygosity (H_E); and genetic differentiation indice F_{ST} per locus are shown

Table 10 Genetic diversity parameters for populations of *Tetratheca aphylla* subsp*. aphylla*

Population	n	H_O	H_E	F_{IS}	AR	PA
TA1	23	0.65	0.68	0.06	6.73	2
TA2	23	0.66	0.69	0.03	7.28	4
TA3	23	0.60	0.64	0.08	6.53	3
TA4	21	0.66	0.71	0.05	6.72	2
TA5	21	0.73	0.69	-0.05	7.26	0
TA6	19	0.64	0.69	0.10	6.72	1
TA7	23	0.65	0.66	0.02	5.60	0
TA8	24	0.63	0.69	0.08	7.30	0
TA9	26	0.67	0.67	-0.02	6.16	0
TA10	24	0.62	0.70	0.12	6.74	0
TA11	22	0.71	0.70	-0.04	6.63	0
TA12	23	0.67	0.72	0.07	6.73	2
TA13	23	0.70	0.66	-0.07	6.73	0
MEAN	22.6	0.66	0.68	0.03	6.70	1.07

Abbreviations: number of individuals (n); observed heterozygosity (H_O); expected heterozygosity (H_E); inbreeding coefficient (F_{IS}); allelic richness (*AR*); observed number of private alleles (*PA*)

Table 11 Pairwise population F_{ST} values for all sites for nine microsatellite loci in *Tetratheca aphylla* subsp*. aphylla*

POP	*TA1*	*TA2*	*TA3*	*TA4*	*TA5*	*TA6*	*TA7*	*TA8*	*TA9*	*TA10*	*TA11*	*TA12*	*TA13*
TA1	0.000												
TA2	0.011	0.000											
TA3	0.027	0.015	0.000										
TA4	0.032	0.009	0.021	0.000									
TA5	0.005	0.012	0.016	0.024	0.000								
TA6	0.011	0.002	0.013	0.006	0.008	0.000							
TA7	0.104	0.075	0.099	0.046	0.077	0.071	0.000						
TA8	0.033	0.024	0.055	0.022	0.030	0.007	0.064	0.000					
TA9	0.047	0.041	0.064	0.031	0.044	0.030	0.056	0.010	0.000				
TA10	0.009	0.002	0.023	0.018	0.014	0.005	0.085	0.020	0.037	0.000			
TA11	0.027	0.011	0.044	0.037	0.031	0.031	0.090	0.043	0.057	0.015	0.000		
TA12	0.048	0.034	0.042	0.015	0.039	0.023	0.041	0.019	0.022	0.044	0.046	0.000	
TA13	0.021	0.022	0.028	0.017	0.024	0.008	0.073	0.009	0.018	0.012	0.032	0.024	0.000

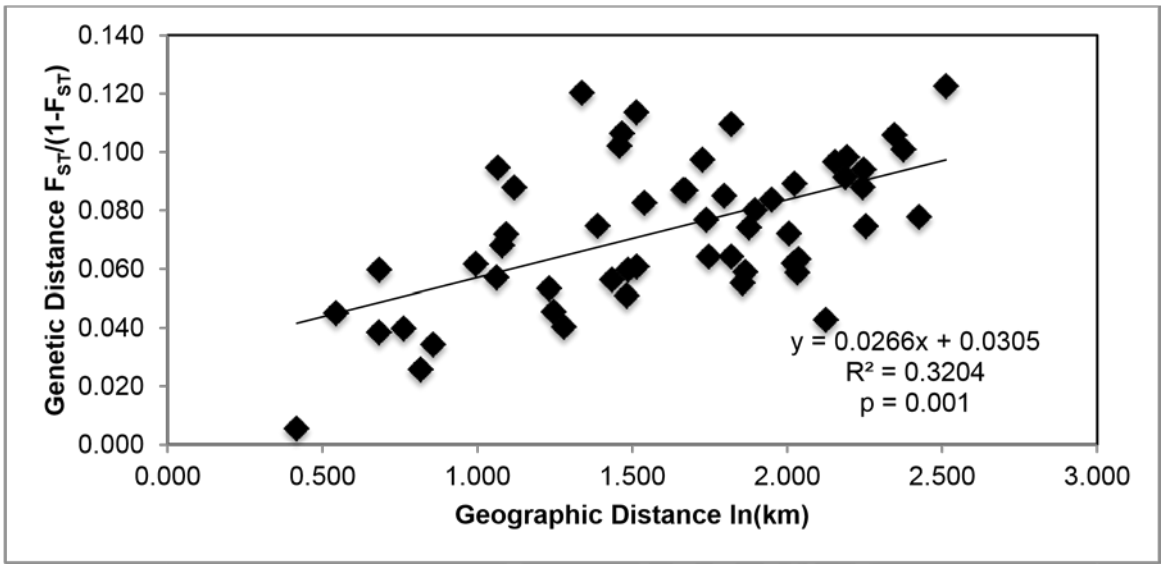


Figure 12 Mantel testing of isolation by distance for correlations between geographic distance and pairwise genetic distance from twelve microsatellite loci for all pairs of sites of *Tetratheca aphylla* subsp*. aphylla*

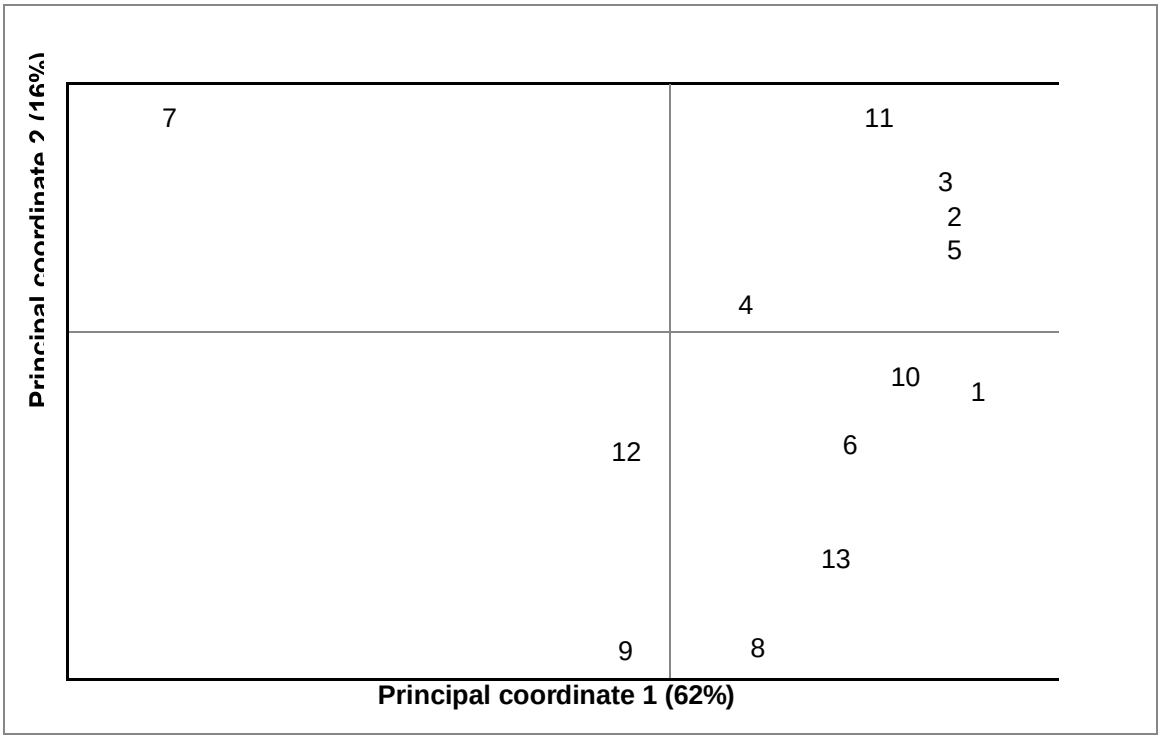


Figure 13 Principal coordinates analysis for all populations of *Tetratheca aphylla* subsp. *aphylla* based on F_{ST}. These two dimensions account for 78% of the total variation

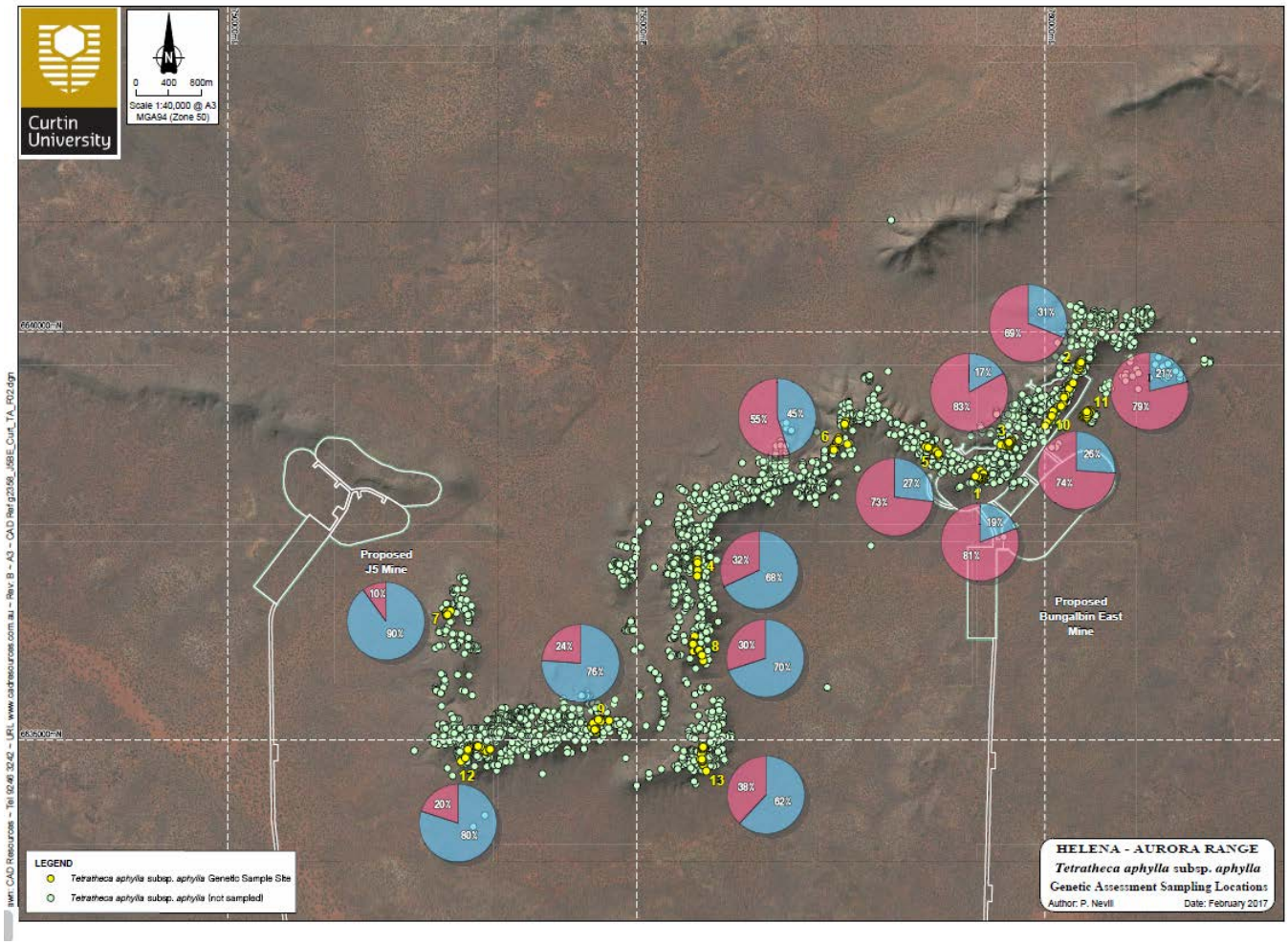


Figure 14 Proportion of membership values from STRUCTURE analysis for each sampled site of *Tetratheca aphylla* subsp*. aphylla*. Colours in pie charts indicate the mean proportion of assignment to one of two clusters (red, blue) for all individuals within a site, where optimal number of clusters was defined by the Evanno et al (2005) method as K = 2. Yellow dots indicate the location of 313 plants sampled for genotyping. Green dots indicate the location of an un-sampled plant or aggregations of un-sampled plants. Numbers indicate site locations and the proposed disturbance area is outlined including the 20 m buffer zone

Tetratheca aphylla subsp. aphylla *- Genetic assessment of impact*

The genetic impact of proposed mining on *Tetratheca aphylla* subsp. *aphylla* was assessed on the basis of repeating analyses of genetic variation after removal of samples located within the proposed Bungalbin East mine and buffer zone (impacted sites TA 1, TA 2, TA 3 and TA 10), and comparing to the complete dataset (Table 12). The removal of impacted samples resulted in the loss of 9 alleles (7 private) - i.e. 105 of 114 alleles (92%) were recovered from genotyped samples collected outside the mine/buffer zone. Seven of these alleles were extremely rare and found only in either the TA 1, TA 2 or TA 3 sites (lost allele frequencies all < 0.05). All other parameters of genetic variation (mean H_O, H_E, AR) were unaffected. Similarly, overall measures of genetic differentiation were not affected by removal of sites (F_{ST} = 0.031 and 0.031; D_{EST} =

0.078 and 0.076, for full and reduced dataset respectively) (Table 12) and the most genetically distinct sites (TA 7 and TA 9) were located in the south-western part of the species range, outside of disturbance areas. Consequently, while up to 20% of all plants may be removed by mine development, ~8% of alleles detected at 9 microsatellite markers will be lost, and other genetic parameters remain unaffected.

Table 12 Genetic diversity and differentiation parameters for *Tetratheca aphylla subsp. aphylla* from all sites, and from all except samples within the disturbance area and buffer zone at sites

	A	H_O	H_E	AR	PA	F_{ST}	D_{EST}
ALL SITES	114	0.66 (0.02)	0.68 (0.02)	6.7	-	0.031 (0.005)	0.078 (0.027)
ALL SITES (-impacted)	105	0.67 (0.02)	0.69 (0.02)	6.8	7 (50%)	0.031 (0.005)	0.076 (0.024)

Abbreviations: total number of alleles across all loci (*A*); observed heterozygosity (H_O); expected heterozygosity (H_E); allelic richness (*AR*); observed number of private alleles lost (*PA*) with % remaining in brackets; and genetic differentiation indices F_{ST} and D_{EST} are shown. Standard errors in parentheses

Discussion of key findings and outcomes

The proposed mines in the Helena-Aurora Range will require the removal of 12%, 8% and 20% of *A. adinophylla*, *L. bungalbin* and *T. aphylla* subsp. *aphylla* plants, respectively. Sites within the proposed disturbance area, including a 20 m buffer zone, and from across the entire range of each species were sampled for genotyping at microsatellite loci and our assessment of genetic impact is focused on the effect of removal of plants, on species genetic variation. The amount of genetic diversity and its distribution within populations are fundamental parameters in conservation biology as high levels of genetic variation are expected to increase the potential of populations to respond to selection and to maintain the health of individuals.

Similar genetic studies to ours on other short range BIF endemic flora provide context to our assessment of the genetic effects of proposed mine developments in the Helena-Aurora Range. For example, the genetic impact of mining at Windarling on *T. paynterae* subsp. *paynterae* to the approved W5 ridge (population size reduced by 2%) and to the conditionally approved “Area B” (population size reduced by 18%) was assessed using microsatellite markers (Butcher et al. 2009). In this study, 104 alleles were identified at 11 loci for 270 individuals across the range of *T. paynterae*. Removal of “Area B” would reduce the number of alleles by 6% and removal of W5 would reduce the number of alleles by 5%, and genetic differentiation by 31%. Removal of both areas would reduce allelic diversity by 10.6% and genetic differentiation by 28%, however, the impact on private alleles was not reported.

Another similar study was conducted on *T. erubescens*, which is restricted to the Koolyanobbing Range. Proposed mine development would require the removal of 22% of *T. erubescens* plants and any impact of mine development on range-wide spatial genetic structuring was assessed using 11 microsatellite loci (Krauss and Anthony 2014). In this study,

59 alleles were detected and removal of impacted plants would reduce the number of alleles to 58 (1 low frequency private allele lost; ~2% reduction overall) and genetic differentiation by 6%.

In contrast, the proposed mine disturbance area at the Helena-Aurora Range would reduce the number of alleles of *A. adinophylla*, *L. bungalbin* and *T. aphylla* subsp. *aphylla* by 2%, 1% and 8%, respectively and genetic differentiation would remain unchanged. The effect on private alleles was greater with 12%, 7% and 50% of largely very low frequency private alleles of *A. adinophylla*, *L. bungalbin* and *T. aphylla* subsp. *aphylla* lost with removal of plants in the disturbance area. However, all other measures of genetic diversity were unchanged.

Assuming that variation and structuring at microsatellite loci genotyped is representative of genetic variation across the entire distribution of the study species, the assessable genetic impact on *A. adinophylla* and *L. bungalbin* of proposed mining in the Helena-Aurora Range will be relatively low and similar to that of detectable genetic impacts of proposed mining on *T. erubescens* but less than those of approved mining on *T. paynterae* subsp. *paynterae.* Whilst the genetic diversity and differentiation parameters assessed in *L. bungalbin* will remain largely unaffected by proposed mining, the majority of plants in the distinct 'green' genetic cluster will be either removed or indirectly impacted. However, ~3,000 individuals will remain outside the impact area and 20 m buffer zone, immediately next to this division (site LB 5) of a larger continuous plant grouping.

The assessable genetic impact of the removal of *T. aphylla* subsp. *aphylla plants* from the disturbance area was greater than that of proposed mining on *T. erubescens* but similar to that of *T. paynterae* subsp. *paynterae* (loss of private alleles not reported). However, the loss of 8% of alleles and, in particular, 50% of private alleles in *T. aphylla* subsp. *aphylla*, represents a significant amount of the species genetic diversity and may impact on its ability for future adaptation and persistence although this is difficult to quantify.

Longer term and indirect impacts

The longer-term genetic consequences of increasing the geographic isolation of remaining plants (e.g. *L. bungalbin* sites LB 1 and LB 10; *T. aphylla* subsp. *aphylla* site TA 11) are unclear as habitat fragmentation can have both negative and positive impacts on plants (Young et al. 1996). Similarly unclear are the longer-term genetic consequences of reducing the number of individuals both overall and in particular genetic groups (i.e. 'green' cluster in *L. bungalbin*). If the impact of local mining on dispersal vectors of *A. adinophylla*, *L. bungalbin* and *T. aphylla* subsp. *aphylla* is minimal beyond the 20 m buffer zone, any genetic effects on increasingly fragmented sites or sites with reduced individuals may be limited because isolation and small genetic neighbourhoods appear to be a feature of many, short range, BIF endemics (e.g. Butcher et al. 2011). However, an understanding of pollinators, direct estimates of seed/pollen dispersal, and reproductive success across the range of the species' is required to properly understand the impacts of this proposal on genetic processes and structure in *A. adinophylla*, *L. bungalbin* and *T. aphylla* subsp. *aphylla*.

Management implications

Findings from this study also have management implications for minimizing any genetic impacts arising from the loss of plants from the disturbance area and buffer zone, should mining proceed. The persistence of small, disjunct populations of rare species may depend on gene flow with other populations to limit the loss of genetic variation through drift. The development of strategies that maintain connectivity should identify landscape feature or demographic characteristics that facilitate dispersal. In our study, we found different levels of population structure in the three study species and different variables that best explained genetic connectivity. Maintaining suitable habitat between populations appears important in the maintenance of gene flow, particularly for *L. bungalbin* and *T. aphylla* subsp. *aphylla,* and should be a priority in mine and restoration planning, if at all possible. Also, the pattern of isolation by distance found in all of the study species and the genetic similarity of geographically proximate populations suggests that pollen and seed dispersal are likely to be restricted and are key factors, along with the natural range disjunctions, in the shaping of spatial genetic structure in *A. adinophylla*, *L. bungalbin* and *T. aphylla* subsp. *aphylla*. Any genetic impacts of proposed mining could be minimized *in situ b*y maintaining plants from the distinct geographic clusters and particularly geographically adjacent sites that are genetically clustered but where one is to be removed as part of mine development. For example, sites AA 3 and AA 5 are genetically very similar, with the allele frequencies of both sites largely captured by the plants to be retained within site AA 3, which is partly outside the disturbance area. Similarly, the careful targeting and inclusion of sites in any *ex situ* seed collections needs to be guided by the genetic data. An example of this is to collect from the most north easterly or south westerly populations of each of the species, which are typically the most genetically distinct and, which, with the exception of AA 12, will not be impacted by proposed mining.

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References

Arnaud-Haond S, Belkhir K (2007) GENCLONE: a computer program to analyse genotypic data, test for clonality and describe spatial clonal organization. Molecular Ecology Notes 7, 15–17
Balkenhol N, Waits LP, Dezzani RJ (2009) Statistical approaches in landscape genetics: an evaluation of methods for linking landscape and genetic data. Ecography 32, 818-830

Barrett RL (2007) New species of *Lepidosperma* (Cyperaceae) associated with banded ironstone in southern Western Australia. *Nuytsia* 17, 37–60

Barrett MD, Wallace MJ, Anthony JM (2012) Characterization and cross application of novel microsatellite markers for a rare sedge, *Lepidosperma gibsonii* (Cyperaceae). American Journal of Botany 99, e14-e16

Beard JS (1981). Swan, Vegetation Survey of Western Australia. University of Western Australia Press, Nedlands

Binks RM, Gardner MG, Millar MA, Byrne M (2014) Characterization and cross-amplification of novel microsatellite markers for two Australian sedges, *Lepidosperma* sp. Mt Caudan and L. sp. Parker Range (Cyperaceae). Conservation Genetics Resources 6**,** 333–336

Binks RM, Millar MA, Byrne M (2015) Not all rare species are the same: contrasting patterns of genetic diversity and population structure in two narrow-range endemic sedges. Biological Journal of the Linnaean Society 114, 873–886

Butcher R (2007). New taxa of 'leafless' Tetratheca (Elaeocarpaceae, formerly Tremandraceae) from Western Australia. Australian Systematic Botany 20, 139-160

Butcher PA, Krauss SL (2009) Development of microsatellites from the rare ironstone endemic, *Tetratheca paynterae ssp. paynterae* and cross_species amplification. Molecular Ecology Resources 9, 386-389

Butcher PA, McNee SA, Krauss SL (2009) Genetic impacts of habitat loss on the rare ironstone endemic *Tetratheca paynterae* subsp. *paynterae*. Conservation Genetics 10, 1735- 1746

Butcher PA, Bradbury D, Krauss SL (2011) Limited pollen-mediated dispersal and partial self-incompatibility in the rare ironstone endemic *Tetratheca paynterae* subsp. *paynterae* increase the risks associated with habitat loss. Conservation Genetics 12, 1603-1618

Carlson JE et al. (1991) Segregation of random amplified DNA markers in F1 progeny of conifers. Theoretical and Applied Genetics 83, 194–200

Cornuet J, Luikar G (1997) Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. Genetics 144, 2001-2014

Crawford NG (2010) SMOGD: software for the measurement of genetic diversity. Molecular

Ecology Notes 10, 556–557

Davidson D, Morton S (1984) Dispersal adaptations of some Acacia species in the Australian arid zone. Ecology 65, 1038–1051

Di Virgilio G (2015). Topographic determinants of habitat suitability for rare ironstone plants in semi-arid Western Australia. Unpublished report prepared for Mineral Resources Limited. Perth.

Di Rienzo A, Peterson AC, Garza JC, Valdes AM, Slatkin M (1994) Mutational processes of simple sequence repeat loci in human populations. Proceeding of the National Academy of Sciences of the USA, 3166-3170

Earl DA, vonHoldt BM (2012) STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conservation Genetics Resources. Conservation Genetic Resources 4, 359-361

Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Molecular Ecology 14, 2611-2620

Falush D, Stephens , Pritchard K (2003) Inference of population structure: extensions to linked loci and correlated allele frequencies. Genetics 164, 1567-1587

Gibson N, Coates DJ, Thiele KR (2007) Taxonomic research and the conservation status of flora in the Yilgarn banded iron formation ranges. Nuytsia 17, 1-12

Gibson N (2010) Recommended Interim Protocol for Flora Surveys of Banded Ironstone Formations (BIF) of the Yilgarn Craton. Department of Conservation and Environment, Perth

Gibson N, Meissner R, Markey AS, Thompson WA (2011) Patterns of plant diversity in ironstone ranges in arid south western Australia. Journal of Arid Environments 77, 25-31

Goslee SC, Urban DL (2007) The ecodist Package for Dissimilarity-based Analysis of Ecological Data. Journal of Statistical Software 22. doi:10.18637/jss.v022.i07

Goudet J (2002) FSTAT version 2.9.3.2. Department of Ecology and Evolution, Lausanne University, Lausanne, Switzerland

Jakobsson M, Rosenberg N (2007) CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. Bioinformatics 23, 1801-1806

Jost L (2008) *G*ST and its relatives do not measure differentiation. Molecular Ecology 17, 4015–4026

Kalinowski ST (2005) HP-Rare: a computer program for performing rarefaction on measures of allelic diversity. Molecular Ecology Notes 5,187-189

Krauss SL, Anthony J (2014) Population Genetic Variation and its Spatial Structure in *Tetratheca erubescens* (Elaeocarpaceae)*:* Final Report. Submitted to Cliffs Asia Pacific Iron Ore Pty Ltd. Report # 67 from the Genetics Laboratory

Legendre P, Fortin MJ (2010) Comparison of the Mantel test and alternative approaches for detecting complex multivariate relationships in the spatial analysis of genetic data. Molecular Ecology Resources 10, 831-844

Lichstein JW (2006) Multiple regression on distance matrices: a multivariate spatial analysis tool. Plant Ecology 188, 117–131.

Maslin B (1999) *Acacia adinophylla* Maslin. Nuytsia 12, 318-320

McPherson H et al. (2008) Isolation and characterization of polymorphic microsatellite loci from *Tetratheca ericifolia* (Elaeocarpaceae). Molecular Ecology Resources 8, 867-869

McRae BH, Shah VB, Mohapatra TK (2013) Circuitscape 4 User Guide. The Nature Conservancy. http://www.circuitscape.org.

Meglécz E et al. (2014) QDD version 3.1: a user‐friendly computer program for microsatellite selection and primer design revisited: experimental validation of variables determining genotyping success rate. Molecular Ecology Resources 14, 1302-1313

Meirmans PG, Van Tienderen PH (2004) GENOTYPE and GENODIVE: two programs for the analysis of genetic diversity of asexual organisms. Molecular Ecology Notes 4, 792-794

Millar MA, Byrne M, Coates DJ (2010) The maintenance of disparate levels of clonality, genetic diversity and genetic differentiation in disjunct subspecies of the rare *Banksia ionthocarpa*. Molecular Ecology 19, 4217-4227

Millar MA, Coates DJ, Byrne M (2013) Genetic connectivity and diversity in inselberg populations of *Acacia woodmaniorum*, a rare endemic of the Yilgarn Craton banded iron formations. Heredity 111, 437-444

Millar MA, Coates DJ, Byrne M (2015) Extensive long-distance pollen dispersal and highly outcrossed mating in historically small and disjunct populations of *Acacia woodmaniorum* (Fabaceae), a rare banded iron formation endemic. Annals of Botany 114, 961-971

Mims MC et al. (2016) Genetic Differentiation, Isolation-by-Distance, and Metapopulation Dynamics of the Arizona Treefrog (*Hyla wrightorum*) in an Isolated Portion of Its Range. PLoS One 11, e0160655.

Nevill PG, Anthony JM, Krauss SL (2010) Isolation and characterization of microsatellite markers for the banded ironstone endemic *Acacia karina* (Leguminosae: Mimosaceae) and cross-species

amplification with *A. stanleyi* and *A. jibberdingensis*. Conservation Genetics Resources 2, 321-323

Nevill PG et al. (2013) Microsatellite primers identified by 454 sequencing in the floodplain tree species *Eucalyptus victrix* (Myrtaceae). Applications in Plant Sciences 1, doi: 10.3732/apps.1200402

Noguerales V, Cordero PJ, Ortego J (2016) Hierarchical genetic structure shaped by topography in a narrow-endemic montane grasshopper. BMC Evolutionary Biology 16. doi:10.1186/s12862-016-0663-7

Nowakowski AJ et al. (2015) Mechanistic insights into landscape genetic structure of two tropical amphibians using field-derived resistance surfaces. Molecular Ecology 24, 580–595.

Peakall R and Smouse PE (2006) GenAlEx 6: genetic analysis in excel. Population genetic software for teaching and research. Molecular Ecology Notes 6, 288-295

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. Genetics 155, 945-959

Rice WR (1989) Analyzing tables of statistical tests. Evolution 43, 223–225

Rozen S and Skaletsky H (1999) Primer3 on the WWW for general users and for biologist programmers. Bioinformatics methods and protocols, pp.365-386

Van Oosterhout C, Hutchinson WF, Wills DP, Shipley P (2004). MICRO_CHECKER: software for identifying and correcting genotyping errors in microsatellite data. Molecular Ecology Notes 4, 535–538

Wagner HH, Fortin MJ (2012) A conceptual framework for the spatial analysis of landscape genetic data. Conservation Genetics 14, 253–261

Weir BS, Cockerham CC (1984) Estimating F-statistics for the analysis of population structure. Evolution 38, 1358– 1370

Willoughby JR et al. (2015) The impacts of inbreeding, drift and selection on genetic diversity in captive breeding populations. Molecular Ecology 24, 98–110

Young A, Boyle T, Brown T (1996) the population genetic consequences of habitat fragmentation for plants. Trends in Ecology and Evolution 11, 413-418