

Research into the population genetics of  
*Eucalyptus tetrapleura* and related  
species (Glenugie Upgrade;  
16.00002544.0771).

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FINAL REPORT

*The Royal Botanic Gardens & Domain Trust*



*The Royal*  
**BOTANIC GARDEN**  
*Sydney*



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We acknowledge the Traditional Custodians of the land on which the plant species in this study are found on, and pay respects to Elders past and present. We acknowledge all collectors who conducted the field sampling.

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## Glossary

**Allele frequency:** is the relative frequency of a genetic variant in a population, expressed as a fraction or percentage. Specifically, it is the fraction of all individuals in the population that carry that allele.

**DArTseq:** this is a genotype-by-sequencing approach of **DArT PL**, where genome complexity reduction methods are applied to efficiently select low copy fractions of plant genomes to obtain high numbers of single nucleotide polymorphisms (**SNPs**). After selection of the low copy markers the fragments are sequenced on the Illumina platform. After a rigorous bioinformatic process a selection of highly reproducible single markers, along with information on the quality of each of these markers are provided to the end user.

**Discriminant analysis of principle component (DAPC):** a multivariate method designed to identify and describe clusters of genetically related individuals. Unlike **PCoA**, **DAPC** is less impacted by within-group variance and allows for group-specific assignment.

**Diversity Arrays Technology Pty Ltd (DArT PL):** is a Canberra, Australia, based company that specializes in delivering affordable products and services in genome profiling, genetic analysis and modern breeding, including development and provision of data storage and data mining technologies. DArT is a generic and cost-effective genotyping technology.

**F-statistics:** The measures  $F_{is}$ ,  $F_{st}$ , and  $F_{it}$  measure the expected degree of (usually) a reduction in heterozygosity when compared to Hardy-Weinberg expectation. In population genetics, *F*-statistics (also known as fixation indices) are related to the amounts of heterozygosity at various levels of population structure. They are derived from *F*, the inbreeding coefficient, and partition inbreeding to the individual component ( $F_{is}$ ), the bi-parental component ( $F_{it}$ ), and the population component ( $F_{st}$ ).

**Heterozygosity** refers to the number of loci where the two alleles an individual are different. Average heterozygosity is calculated across all loci within an individual, and within populations. The observed heterozygosity ( $H_o$ ) is defined as the percentage of loci heterozygous per individual or the number of individuals heterozygous per locus. Average heterozygosity at each locus and for each population can be estimated from allele frequencies at each locus. Individual population or species average heterozygosity is estimated by summing heterozygosities at each locus and averaging these values over all loci. The expected heterozygosity (also called gene diversity) is estimated from individual allele frequencies.

**Hybrid:** For the purpose of this report a hybrid is an individual who's **DArTseq** profile indicate that its genome consists of genetic material originating from two species. In this report hybrids were identified using several types of analyses including principal coordinate analysis, the snmf analysis

incorporated in LEA and NewHybrid. A hybrid does not belong to a specific taxonomic species although in some cases it is possible to identify either or both of the parental species.

**Principal Coordinate Analysis (PCoA):** this is analytical tool that can be used to visualise the genetic similarity/dissimilarity between individuals. The **SNP** data matrix is used to build the individual by individual dissimilarity matrix and from these eigenvectors giving the PC. In brief, **PCoA** is a multivariate technique that allows one to find and plot the major patterns within a multivariate data set (e.g., multiple loci and multiple samples). The mathematics is complex, but in essence **PCoA** is a process by which the major axes of variation are located within a multidimensional data set. Each successive axis explains proportionately less of the total variation, such that when there are distinct groups, the first 2 or 3 axes will typically reveal most of the separation among them.

**‘Pure’ population:** in this report the term pure population is used to refer to those populations where all sampled *E. tetrapleura* were identified by the genomic data as *E. tetrapleura* i.e. no hybrids were found. Note that the sampling at each site was not always exhaustive and that unsampled hybrids can still be present at these populations. ‘Pure’ populations can also include other ironbark species that were recognised as such in the field.

**Restore and Renew iforms App:** This is an application designed for data entry in the field when collecting leaf tissue samples and herbarium voucher material for the Restore and Renew project run at the Royal Botanic Gardens & Domain Trust. The application allows users to record spatial data (latitude and longitude) using the GPS chip on a mobile device, scan the barcode number (a unique number for each sample) used on the tissue collecting envelopes, therefore reducing the number of errors introduced into dataset by manual data entry. The data is uploaded to a server and from there accessed and incorporated into a relational database.

**Rstudio:** is an integrated development environment for the R. It includes a console, syntax-highlighting editor that supports direct code execution, as well as tools for plotting, history, debugging and workspace management. In all cases for this report the open source RStudio was run on a Linux desktop.

**Single Nucleotide Polymorphism (SNP; plural Single Nucleotide Polymorphisms, SNPs):** is a variation in a single nucleotide that occurs at a specific position in the genome, where each variation is present to some appreciable degree within a population.

## Executive summary

Genetic diversity among sampled individuals and populations was measured using high quality genome scans. The genome scans define between-population relatedness and connectivity, differentiate among species, and identify hybrids. The results show that *Eucalyptus tetrapleura* is a genetically differentiated species, and it actively hybridises with co-distributed ironbarks. Similar levels of genetic diversity exist within *E. tetrapleura* as in related taxa, and the species is represented by a single genetic provenance. The findings show that *E. tetrapleura* is most likely more narrowly distributed (only south of the Clarence River), and potentially rarer (due to miss-identifications) than previously suggested. Though many of the ironbarks north of the Clarence River resembled *E. tetrapleura* the genetic data indicated that these are all of hybrid origin. Morphological identification can be misleading and the use of genomic tools appears to be the safest method for identification. For long-term management and conservation, we suggest that a re-established population should include material sourced from multiple individuals and multiple sites. However, to increase the chances of excluding hybrids from re-established populations seed material should be preferentially sourced from 'pure' populations (i.e. those populations that do not appear to include hybrid individuals), and ideally tested using genomic scans.

# 1. INTRODUCTION

*Eucalyptus tetrapleura*, the square fruited Ironbark, is listed as vulnerable under the Environment Protection and Biodiversity Conservation Act 1999 (Commonwealth). The main threats to this species are all related to habitat loss and habitat degradation including processes such as clearing for agriculture, road construction and maintenance and suburban development.

The species is found in the Northern Rivers Natural Resource area of New South Wales and was thought to be restricted to a small area of approximately 100km x 40km around Grafton (one of the outcomes of this report indicates a much reduced area of approximately 40 x 40 km). Within this small area it occurs in patches of various sizes with a number of small disjunct populations on the periphery such as the most southern records of the species in Nymboi-Binderay National Park. Records from the Atlas of Living Australia (ALA) indicate the most northern distribution as being in the area just north of Whiporie, east of Camira Creek railway siding, with one record from Mount Neville Nature Reserve.

*Eucalyptus tetrapleura* was named by L.A.S. Johnson in 1962, based on a specimen from near Halfway Creek (between Corindi and Grafton). Its name is a reference to the buds and fruits that are distinctly 4-ribbed. Following the taxonomic systems of Brooker (2000) and (Nicolle 2015) the most closely related species is *Eucalyptus fusiformis*, a species that has a wider range (Gold Coast south to around Bulahdelah). *E. tetrapleura* and *E. fusiformis* differ in bud and fruit shape and in leaf size and colour as previously outlined in the literature review provided by the Royal Botanic Gardens, Sydney to RMS. Two other ironbark species, *E. fibrosa* and *E. siderophloia* co-occur with *E. tetrapleura*. All of these overlapping species of ironbarks have a wider distribution than *E. tetrapleura*.

Though prior to this study there were no formal investigations into hybridisation between *E. tetrapleura* and other species of eucalyptus, there were a few recorded hybrids (previously outlined in the literature review). There are several reports of, and investigations into, hybridisation between species of *Eucalyptus* including controlled hybrids and natural hybrids. Cryptic hybrids (where the morphology suggests that the individual belongs to one species but information such as genetic data indicate that the genome of the species contains genetic components from other species) can only be identified through genetic screening. Identification of hybrids and their origin relies heavily on sampling, as ideally both parents should be included in the sampling. Interspecific hybrids (offspring from two differentiated parental species) do not belong to either of the parent species (which are represented by pure, non-admixed lineages). Hybrids can alter the reproductive fitness of a species, and often they do not produce viable seed while still competing for pollen and other limited and valuable resources. Although hybridisation is a natural and relatively common process in the genus *Eucalyptus*, it tends to be increasingly common in circumstances where a small population is surrounded by individuals from a closely related but different species. This can result in genetic swamping where the preference for outcrossed pollen results in the preferential uptake of inter-specific pollen.

In order to understand the full extent of the range of *E. tetrapleura*, to quantify and qualify genetic diversity within its range and the presence of hybridisation, the Royal Botanic Gardens & Domain Trust was contracted by Roads and Maritime Services NSW (RMS) to undertake an extensive population genetic study of *E. tetrapleura* that included representatives of other local ironbark species.



## 2. OBJECTIVES:

The main objectives of the project (listed below as requested by RMS) aimed at assessing genetic diversity and genetic structure within the whole distribution of *Eucalyptus tetrapleura* (Square-Fruited Ironbark), and investigate the extent of hybridisation with other local ironbarks.

1. Determine the genetic distinctness of *E. tetrapleura* from other close ironbark relatives. To address this objective we will answer the questions:

- Is *E. tetrapleura* a genetically distinct lineage?
- Where can it be found?
- How can it be identified?

To achieve this, the Royal Botanic Gardens & Domain Trust (from here on RBG&DT) sampled across the full extent of *E. tetrapleura* known (and accessible) range, and included multiple collections of other local ironbark species. We analysed genome-wide (DArTseq) datasets to explore genetic structure and bi-parental assignment (i.e. hybrid detection).

2. Assess any potential differences between the populations of Square-Fruited Ironbark, the degree of genetic variation within populations and the degree to which it is geographically and ecologically determined. This objective was addressed by investigating classic population genetic parameters, such as allelic diversity, heterozygosity and measures of inbreeding ( $F$  statistics).
3. Assess levels of gene flow between populations (based on  $F_{st}$  values), levels of heterozygosity and rare alleles within each population and if relevant provision of provenance boundaries based on the genetic structure obtained from the genome-wide data. The analyses required to address objectives 1, 2 and 3 overlap.
4. Characterise the mating system of *E. tetrapleura* i.e. the degree to which it has a typical eucalypt mating system and the potential for inbreeding to impact on population viability. A preliminary exploration into the mating system of *E. tetrapleura* was obtained via the population genetic analyses required to address objective 2.

## 3. METHODS:

### 3.1 Sampling

The sampling strategy was aimed at addressing each of the objectives outlined above. *Eucalyptus tetrapleura* was sampled extensively at locations where more than three individuals were found or known to occur. Sites were selected using records obtained from the Atlas of Living Australia (ALA) and field observation data obtained from John O'Donnell. We used Google Earth Pro (available from <https://www.google.com.au/earth/download/gep/agree.html>) to visualize locations and plan field-work logistics.

Prior to collecting leaf material in the field, a Scientific License (SL101766) for collecting leaf material from a threatened species and collecting any flora within National Parks and Wildlife Estate was obtained from the Office of Environment and Heritage (OEH), NSW Government.

In order to investigate the genetic distinctness of *E. tetrapleura* from other ironbarks leaf material and voucher material were collected from all ironbarks that co-occur with *E. tetrapleura*. Co-occurring species were: *E. fibrosa*, *E. fusiformis* and *E. siderophloia*. In order to obtain comparative (control) material for the analyses, leaf material for each of the co-occurring species was also collected from sites where the species distributions did not overlap with that of *E. tetrapleura*. Table 2 and Figure 1 outline all collection sites and taxonomic identifications of material collected at each site.

At each site, approximately 2g of fresh leaf material was collected per individual for 2 to 16 individuals (Table 1). Fresh leaf material was placed in pre-barcoded envelopes. Using the Restore and Renew iforms App, the barcodes were scanned and for each collection other relevant information such as location data (latitude and longitude) and location information, site description, disturbance history, was recorded. At each site material suitable for herbarium vouchers was also collected. Where multiple species co-occurred voucher material was collected from each of the species recognizable in the field. In all cases where it was not possible to identify the plants in the field, additional voucher material was collected. Upon return from the field, the vouchers were inspected for taxonomic determination by RBG&DT staff. The leaf material collected for genetic analyses was kept cool until returning to Sydney where the material was placed in a -80C freezer prior to freeze drying.

## 3.2 DNA extraction and sequencing:

DNA was extracted from each sample using the Plant DNA Extraction Protocol for DArT as available from the Diversity Arrays Technology Pty Ltd (DArT PL) website (<http://www.diversityarrays.com/sites/default/files/resources>). All samples were sent to DArT PL, Canberra, for the DArT PL genotype by sequencing analysis (referred to as DArT seq analysis).

## 3.3 Data analysis:

A range of dedicated analyses was carried out using the single nucleotide polymorphism (SNP) data. The quality control, principal coordinate analysis (PCoA) and discriminant analysis of principal components (DAPC) and sparse non-negative matrix factorization (snmf) using the programs adagenet and Lea (see details in each section) were implemented in Rstudio using the commands and scripts prepared by Dr. Jason Bragg (RBG&DT) for the Restore and Renew project.

### 3.3a Quality screening and control of Single Nucleotide Polymorphism data:

Once RBG&DT received the single nucleotide polymorphism data (SNP) it was checked for quality. Samples and loci that did not pass standardized quality thresholds were removed from the data and not used in further analyses. Samples that did not pass the quality control (QC) are indicated in Table 2. To ensure that only the higher quality DArTseq markers were used for analyses all SNPs with a reproducibility (proportion of replicate assay pairs for which the marker score is consistent) of less than 98 % and which had more than 10 % missing data were excluded from the dataset (this left 17,637 SNPs remaining from 42,787 SNPs). To ensure results were not influenced by linkage one SNP was randomly selected from each of the clones that contained more than one SNP. Finally, samples that had greater than 40 % missing data were removed from the dataset. After the rigorous quality control 9,369 SNPs and 237 samples (table 1) were used in the subsequent data exploration and analysis.

### 3.3b Genetic relationships considering reticulate evolution

DArTseq analysis produced a dataset comprising a total of 42,787 SNPs. *Splitstree4*<sup>1</sup> was used to infer the genetic relationships among a set of samples as a network, based on information from multiple loci using the total number of SNPs obtained rather than the quality trimmed data set. The network can represent evolutionary histories with substantial reticulation, arising from incomplete lineage sorting and hybridisation. In this study, a relationship network of the total dataset was

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<sup>1</sup> Huson DH, Bryant D (2006). Application of phylogenetic networks in evolutionary studies. *Mol Biol Evol* 23: 254–267

generated in *Splitstree4* using the default settings of the software. The output from the *Splitstree* analysis was used as part of the data exploration process (Appendix 1; Figure A2).

### 3.3c Principal coordinate and discriminant analyses

We used the package *adegenet* 2.0.1<sup>2</sup> in R (version 3.3.0, R Core Development Team) to perform a principle coordinate analysis (PCoA) to better understand relationships between individuals and populations. In addition, a discriminant analysis of principle components (DAPC)<sup>3</sup>, was performed using *adegenet* to explore genetic structure within and between the study species. DAPC takes into account the multilocus genotype of each individual and identifies and describes clusters on the basis of genetic similarity without considering a model of evolution (Figure S1).

### 3.3d Genetic structure

Genetic structure analyses were performed using LEA (an R Package for Landscape and Ecological Association Studies)<sup>4</sup>. LEA includes statistical methods for estimating ancestry coefficients from large genotypic matrices and evaluating the number of ancestral populations. LEA implements the *snmf* function (sparse Non-Negative Matrix Factorization algorithms) and estimates individual admixture coefficients from the genotype matrix. Cross-entropy values were estimated for between 1 and 12 ancestral populations (K values or structure groups). The K value with the lowest cross-entropy value was selected for further analysis.

### 3.3e Extent of genetic introgression

Outputs from the PCoA and LEA analyses identified the presence of hybrids. A model-based Bayesian clustering analyses as implemented in the software *NewHybrids* v. 1.1<sup>5</sup> was thus used to further investigate the extent of introgression between species. This software assigns individuals to one of six genetic classes (species A, species B, F1 hybrid, F2 hybrid, backcross to species A, or backcross to species B). Due to the complex computation analysis implemented in *NewHybrids* only subsets of the data can be used in the analyses. The subsets reported on here were selected using the outputs from the LEA and PCoA analysis. The number of SNP markers was also reduced by removing markers with missing data and reproducibility less than 100%. From this reduced but high quality dataset 200 markers were randomly selected and the data imported into *NewHybrids*<sup>6</sup>.

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<sup>2</sup> Jombart T (2008). *Adegenet*: an R package for the multivariate analysis of genetic markers. *Bioinformatics* 24: 1403–1405

<sup>3</sup> Jombart T, Devillard S, Balloux F (2010). Discriminant analysis of principal components: a new method for the analysis of genetically structured populations. *BMC Genet* 11: 94.

<sup>4</sup> Frichot E, François O (2015) LEA: An R package for landscape and ecological association studies. *Methods in Ecology and Evolution* 6: 925–929.

<sup>5</sup> Anderson EC, Thompson EA (2002) A model-based method for identifying species hybrids using multilocus genetic data. *Genetics* 160: 1217–1229.

<sup>6</sup> Analyses were conducted using Jeffrey's priors for  $\pi$  and  $\vartheta$  and were computed for a burn-in period of 100,000 sweeps (to ensure convergence) and a run-time of 1,000,000 sweeps.

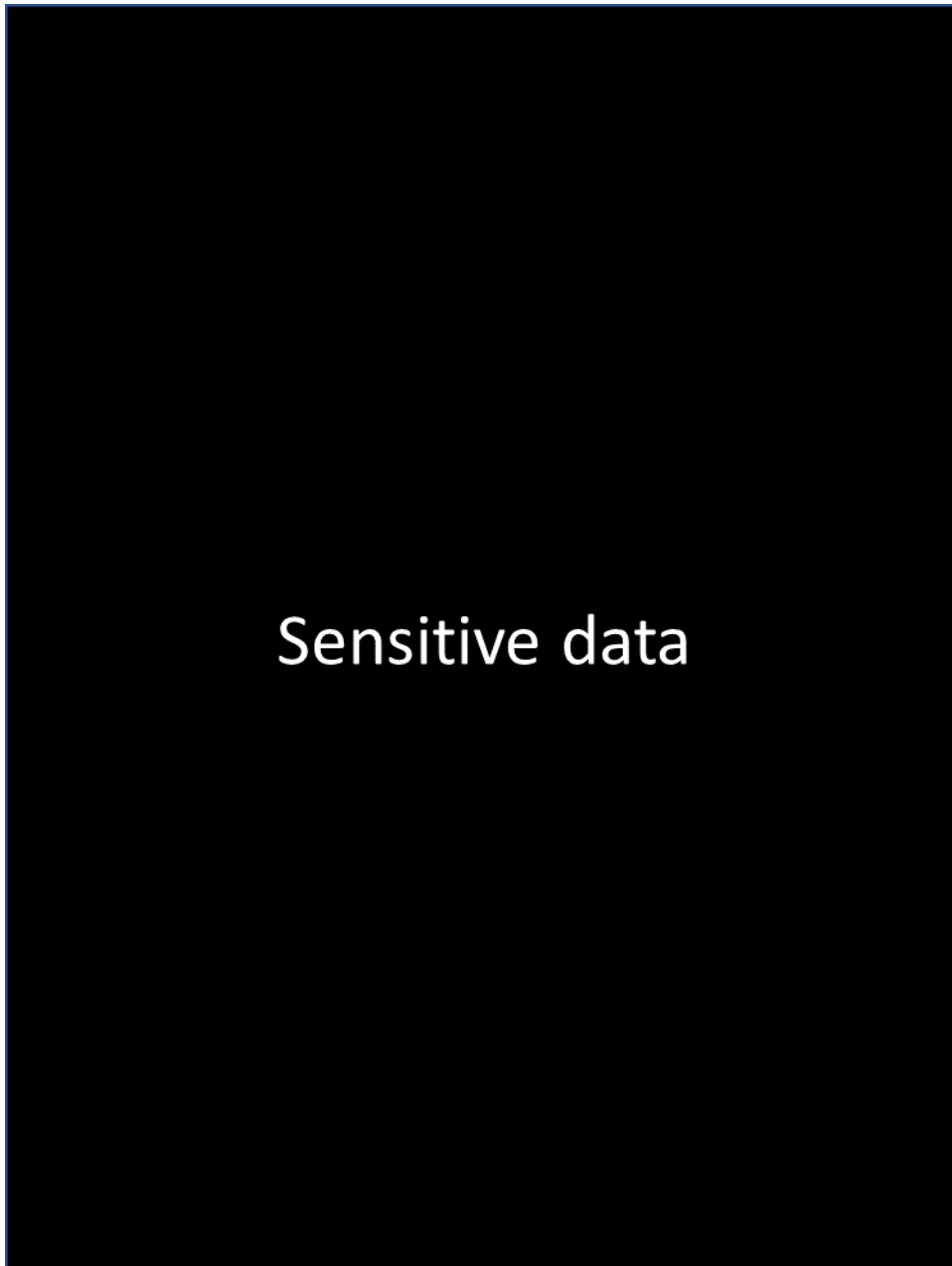
### 3.3f Population genetic diversity

A subset of the highest quality markers from the dataset was used for population genetic analyses, since the software used, could not process more than 8,000 loci. All markers with a reproducibility of less than 100 % were removed from the dataset. In addition, all markers with missing data at any locus were excluded. This left 4,179 markers for analyses. To ensure this subset was representative of the larger dataset, a PCoA was conducted based on Nei's unbiased genetic distances in the PCoA module of GenALex v6.501<sup>7</sup>.

We calculated genetic diversity parameters from the highest quality markers. The expected heterozygosity ( $H_e$ ), observed heterozygosity ( $H_o$ ), number of observed alleles ( $N_a$ ), genetic differentiation between populations ( $F_{ST}$ ) and degree of inbreeding ( $F_{IS}$ ) were determined using the Frequency Module of GenALex v6.501. Genetic diversity parameters were calculated for the groups found in the present study using the PCoA, DAPC and LEA analyses described above. Analyses were also conducted on groups identified by NewHybrids analysis.

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<sup>7</sup> Peakall R, Smouse PE (2006). GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Mol Ecol Notes 6: 288-295. Peakall R, Smouse PE (2012). GenALEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research – an update. Bioinformatics 28: 2537–2539.



**Figure 1:** The sites where collections were made for the *Eucalyptus tetrapleura* genetic study. The species identification on this map is based on field identifications (map also supplied as high quality PDF file).

## 4. RESULTS AND INTERPRETATIONS

### Summary

The high quality genome scans (DArTseq) were able to measure genetic diversity among sampled individuals and populations, define between-population relatedness and connectivity, differentiate among species, and identify hybrids as well as estimate levels of genomic individual introgression.

The main significant (and in some cases unexpected) findings are:

- *E. tetrapleura* is a genetically differentiated species,
- *E. tetrapleura* actively hybridises with co-distributed related species,
- Several populations are not pure, and the identification of hybrids (and species in some instances) based on morphology alone is complex and often equivocal;
- Similar levels of genetic diversity exist within *E. tetrapleura* as in related taxa;
- There is little genetic divergence between populations of *E. tetrapleura*;
- *E. fusiformis* is not supported as an independent lineage.

## 4.1 Field collections

Samples were collected at 28 sites covering the recorded distribution of the species. Due to unsuitable and potentially dangerous road conditions, sampling was not attempted at Mount Neville (only one observational record from NSW vegetation surveys exists for this location). A total of 245 samples were collected for DNA analyses. Apart from 12 samples collected in the Sydney area (6 x *E. fibrosa* samples and 6 x *E. siderophloia* samples, see inset in figure 1) all sampling occurred around Grafton in northern NSW (figure 1). Not all samples could be identified in the field and vouchers were thus scrutinised upon return. After careful identification of the vouchers using morphology the total number of *E. tetrapleura* samples were 170 (however see the discussion below on the genetic identity of the samples). A summary of field and voucher identification is provided in Table 2. A descriptive summary of each collection sites is provided in the supplementary section. Collection data has been submitted to BioNet, and voucher (a total of 47) and leaf tissue collections will be housed at the National Herbarium of NSW.

## 4.2 Single Nucleotide Polymorphisms: data quality

DNA was successfully extracted from all 242 samples and submitted to Diversity Arrays Technology Ltd Pty for genomic analyses. SNP data was received for 241 samples and four of these samples (of which three were *E. tetrapleura*) were omitted from analysis after the data quality control, thus leaving 237 samples with good quality SNP data. One hundred and sixty six putative *E. tetrapleura* (though see discussion following on the genetic identification of the samples) produced high quality genomic SNP data for our analyses and interpretations. Over 42,000 SNPs were identified across nearly 27,000 sequences (loci), and after quality control checks and verifications close to 10,000 SNPs were used (Table 1). Overall, quality controls indicated that data was of very high standard, and enabled us to maintain an extremely large number of polymorphic (variable) loci to complete our investigations.

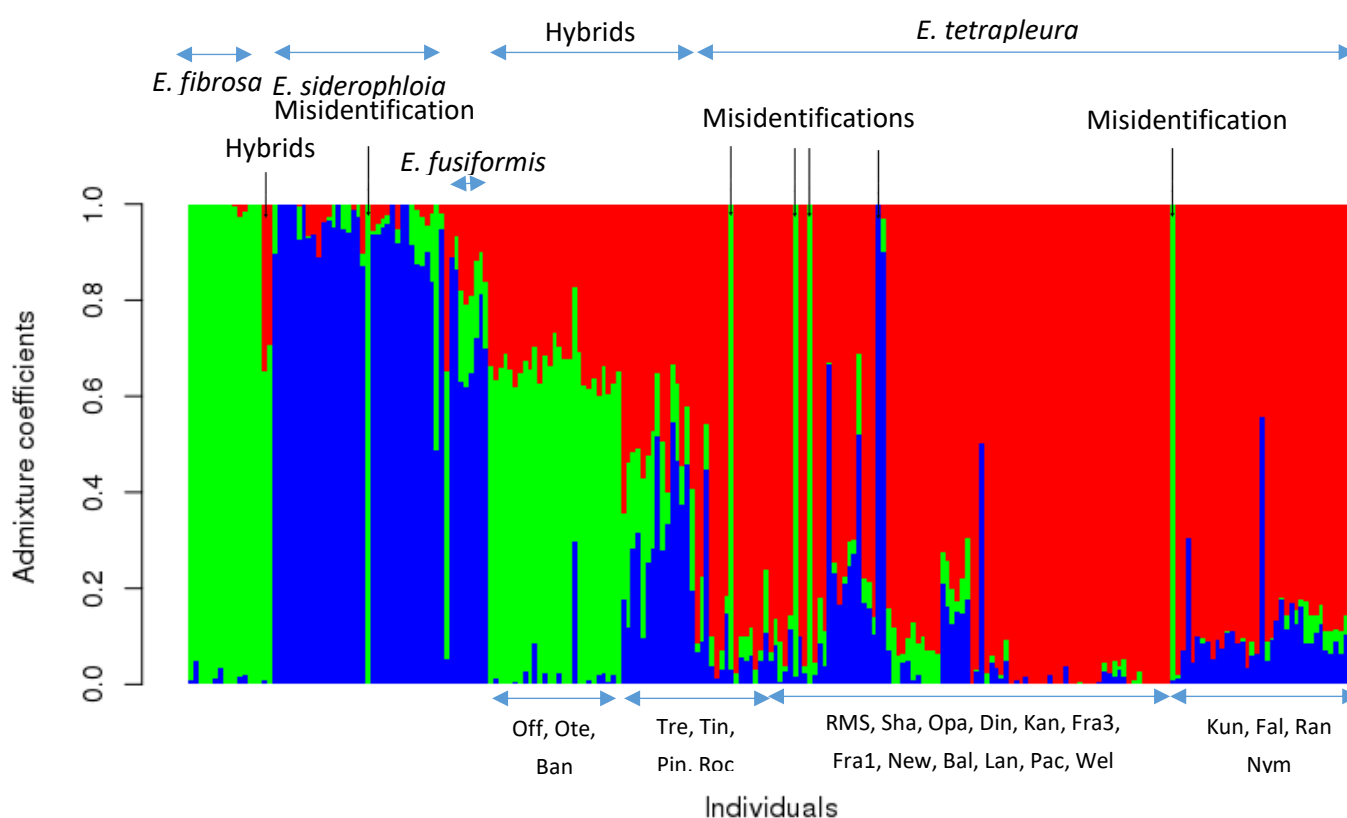




## 4.3 Genetic data results and interpretations

4.3a Species delimitation: is *E. tetrapleura* a genetically distinct lineage? And where can we find individuals belonging to this group?

The genomic signature obtained with nearly 10,000 (9,369) genome-wide markers enabled the recognition of individuals belonging to a genetically distinct *E. tetrapleura* group, as illustrated by the Principal Component Analysis (PCoA) output in Figure 2, and Bayesian clustering (LEA) output in Figure 3.



**Figure 3:** Bayesian assignment analysis as implemented by the software LEA based on genomic-wide data (from 9,369 markers derived from DArTseq) obtained from most of the distribution of *E. tetrapleura*, and from co-distributed ironbark relatives. Vertical strips represent single individuals grouped into populations as described at the bottom. This figure is aimed visualising broad patterns and not at enabling the identification of single individuals. The red colour is assigned to *E. tetrapleura* genomes (more than 0.80 identifies individuals belonging to this group), green is assigned to *E. fibrosa* genomes (more than 0.80 identifies individuals as belonging to this group), and blue is assigned to *E. siderophloia* / *E. fusiformis*. Individuals that have multiple colours are of admixed (hybrid) origin. Some of the taxonomic misidentifications are highlighted (additional information on the location of each individual and their genetic status is listed in Table 2).

Overall the two analytical approaches investigated (as visualised in Figs. 2 and 3) were in agreement in the separation of pure vs. admixed individuals and in identifying possible issues with identification based on morphology alone. Additional analytical methods DAPC (see Supplementary Information) and NewHybrid (see section 4c) further supported these findings.

Genomically *E. tetrapleura* is a clearly distinct species, however based on the whole-genome analyses, only 121 individuals were identified as *E. tetrapleura*, and only 7 sites originally sampled as *E. tetrapleura* sites included exclusively this species: Dinjerra road; Franklin's road 1; Newfoundland State Forest; Bald Knob road; Pacific Highway (north of Wells Crossing); Wells Crossing; Near Nymboi Bunderay National Park (see map in Fig. 4). However note that at Dinjerra Rd and Franklins rd other ironbark species were present but not sampled for this study. Interestingly 161 individuals were originally collected as *E. tetrapleura*, and 169 were given that putative ID back at the Herbarium. Of the 121 individuals identified as *E. tetrapleura* with the genetic data, some had originally been sampled as 'un-identified ironbark', or *E. siderophloia*. Conversely most individuals wrongly identified in the field as *E. tetrapleura* turned out to be either hybrids or *E. fibrosa*.

While it was expected that some hybrids would be present the findings suggest that the species might be more restricted than initially thought, and that surveys relying on morphological identification alone might produce a proportion of inaccurate identifications. As the map shows, *E. tetrapleura* plants have not been found north of the Clarence River, a recognised biogeographic barrier. It is important to remember that hybrid individuals are as closely related to both parental species and cannot be included in any numerical account of *E. tetrapleura*. Also the presence of hybrids at a site does not imply that both parental species were there in the past, as hybrid offspring can be produced by pollen dispersal (e.g. *E. siderophloia* maternal plants located north of the Clarence could be pollinated by *E. tetrapleura* pollen originating from paternal individuals located south of the Clarence). There is one additional observational record at Mount Neville that we could not sample but, based on the findings of this report, it is most likely that its original identification was incorrect.

4.3b Genetic distinctiveness and relationships with other square-fruited ironbarks: species delimitation and identification issues.

The results presented in the section above (4.3a) suggest that although differentiated, the local ironbark species are close relatives and this relationship is likely to facilitate the level of hybridisation that have been identified in this study (see next section, 4.3 c). The short branches at the base of the unrooted phylogenetic tree presented in the Appendix (Fig. A2) provide some support for this. However, only a thorough phylogenetic study that includes multiple individuals

from multiple sites and much wider taxon sampling can address the systematics and evolution of the ironbarks.

An unexpected finding (although not directly relevant to *E. tetrapleura*), was that none of the analyses resolved *E. siderophloia* and *E. fusiformis* as distinctly differentiated species, but more as a part of a single lineage continuum (despite the fact that both were also sampled from geographically wide representatives). Two of the populations (Tamban State Forest and Whiporie) were previously identified as *E. fusiformis* (in the database). However, observations in the field raised doubts about this identification on the basis of morphology and it was concluded they were *E. siderophloia*. Only the Bongil Bongil NP population had fruits ‘typical’ of *E. fusiformis*. However, while the PCoA suggests that this population is somewhat differentiated, it still fits within a broader genetic interpretation of *E. siderophloia*. In the future, dedicated fine-scale genomic and morphological studies will be needed to support the necessary taxonomic clarifications for these two species.

	<i>E. fibrosa</i>	<i>E. tetrapleura x fibrosa</i>	<i>E. siderophloia</i>	<i>E. tetrapleura x siderophloia</i>
<i>E. tetrapleura x fibrosa</i>	0.1524455			
<i>E. siderophloia</i>	0.2065591	0.14895104		
<i>E. tetrapleura x siderophloia</i>	0.1871294	0.06651775	0.07975758	
<i>E. tetrapleura</i>	0.2447085	0.11800971	0.17331334	0.05050259

**Table 1:** Pairwise  $F_{st}$  values among the five groups identified on the basis of the PCoA, DAPC and LEA analyses. The higher the number the more genetically differentiated these groups are. This confirms genomic exchange between the lineages and suggests that overall *E. tetrapleura* is more differentiated (as far as gene flow) from *E. fibrosa* than from *E. siderophloia*.

#### 4.3c Admixture among square-fruited ironbarks: hybrids their parental sources and distribution.

We found extensive evidence of hybridisation between *E. tetrapleura* and all other species investigated (Table 2). Hybrids were found at five *E. tetrapleura* sites south of the Clarence River and hybrids but no *E. tetrapleura* were found at any of the putative sites north of the Clarence (i.e. *E. tetrapleura* is not found north of the Clarence River).

Generally the genomic analyses suggest that hybridisation events between *E. tetrapleura* and *E. fibrosa* might be more constrained and involve less backcrossing back to parent, than hybridisation events between *E. tetrapleura* and *E. siderophloia*.

Nevertheless, pollen exchange is taking place (and has taken place in the past) between ironbark species when the species are in close proximity to each other, and when the opportunity arises. Hybridisation events are likely to be driven by a range of mechanisms such as slightly asynchronous

flowering events that lead to the dominance of one pollen type over another. Such a process could for example explain the presence of hybrids north of the Clarence, where no *E. tetrapleura* are currently found. However it is important to remember that seed fertilisation by foreign pollen does not necessarily imply the production of a hybrid individual, as many other factors impact on successful establishment (such as the need for environmental disturbance to generate un-occupied habitat for example).



Sensitive data

**Figure 4:** A map showing the locations where *E. tetrapleura* (red circle) and hybrids (grey) were identified using the full genome scan data obtained from DArTseq. Circles ½ red & ½ grey indicates pure and hybrid individuals identified at the site. High quality Georeferenced maps were supplied separately in PDF format.

**Table 2:** A summary of species and hybrid identification with the final genetic analyses based on the results from the PCoA, DAPC and LEA (Genetic ID). Species and hybrids are colour coded and location coded as in previous figures. Full name of each location as well as three sets of ID (as done in the field when collected; as verified on voucher at the NSW Herbarium; and as confirmed through genomic analyses); Genetic ID includes species names or hybrid, # hybrid that does not involve *E. tetrapleura*, all other hybrids showed introgression with *E. tetrapleura*; did not pass QC = samples for which data was received but did not pass the data quality control and lat/long information are included;

Sample	Code	Location	Field ID	Voucher ID	Genetic ID	Latitude	Longitude
NSW1019049	Fib_Opa	Old Pacific Highway	<i>E. fibrosa</i>	<i>E. fibrosa</i>	<i>E. fibrosa</i>	-29.820171	153.037799
NSW1013155	Fib_Opa	Old Pacific Highway	<i>E. fibrosa</i>	<i>E. fibrosa</i>	<i>E. fibrosa</i>	-29.820209	153.037753
NSW1020990	Fib_Opa	Old Pacific Highway	<i>E. fibrosa</i>	<i>E. fibrosa</i>	<i>E. fibrosa</i>	-29.820209	153.037753
NSW1020989	Fib_Fra2	Franklins Road 2	<i>E. fibrosa</i>	<i>E. fibrosa</i>	<i>E. fibrosa</i>	-29.849522	153.074872
NSW1018981	Fib_Fra2	Franklins Road 2	<i>E. fibrosa</i>	<i>E. fibrosa</i>	<i>E. fibrosa</i>	-29.849444	153.074952
NSW1018352	Fib_Fra2	Franklins Road 2	<i>E. fibrosa</i>	<i>E. fibrosa</i>	<i>E. fibrosa</i>	-29.849572	153.075224
NSW1013186	Fib_Fra2	Franklins Road 2	<i>E. fibrosa</i>	<i>E. fibrosa</i>	<i>E. fibrosa</i>	-29.849403	153.075169
NSW1018992	Fib_Fra2	Franklins Road 2	<i>E. fibrosa</i>	<i>E. fibrosa</i>	<i>E. fibrosa</i>	-29.849434	153.075346
NSW1018614	Fib_Fra2	Franklins Road 2	<i>E. fibrosa</i>	<i>E. fibrosa</i>	<i>E. fibrosa</i>	-29.849489	153.07503
NSW1019398	Fib_Cas	Castlereagh, Sydney	<i>E. fibrosa</i>	<i>E. fibrosa</i>	<i>E. fibrosa</i>	-33.684657	150.762737
NSW1019358	Fib_Cas	Castlereagh, Sydney	<i>E. fibrosa</i>	<i>E. fibrosa</i>	<i>E. fibrosa</i>	-33.684766	150.76273
NSW1019349	Fib_Cas	Castlereagh, Sydney	<i>E. fibrosa</i>	<i>E. fibrosa</i>	<i>E. fibrosa</i>	-33.685072	150.763
NSW1018720	Fib_Cas	Castlereagh, Sydney	<i>E. fibrosa</i>	<i>E. fibrosa</i>	<i>E. fibrosa</i>	-33.685072	150.763
NSW1019410	Fib_Cas	Castlereagh, Sydney	<i>E. fibrosa</i>	<i>E. fibrosa</i>	<i>E. fibrosa</i>	-33.685149	150.763255
NSW1013150	Fib_Cas	Castlereagh, Sydney	<i>E. fibrosa</i>	<i>E. fibrosa</i>	<i>E. fibrosa</i>	-33.685123	150.763356
NSW1018715	Fib_Sum	Summerland Way	<i>E. fibrosa</i>	<i>E. fibrosa</i>	Hybrid	-29.26217	152.984203
NSW1018716	Fib_Sum	Summerland Way	<i>E. fibrosa</i>	<i>E. fibrosa</i>	Hybrid	-29.262029	152.984205
NSW1019440	Fib_Sum	Summerland Way	<i>E. fibrosa</i>	<i>E. fibrosa</i>	Did not pass QC	-29.262607	152.984318
NSW1015802	Sid_Whi	Whiporie	<i>E. fusiformis</i>	<i>E. siderophloia</i>	<i>E. siderophloia</i>	-29.298722	152.964454
NSW1019375	Sid_Whi	Whiporie	<i>E. fusiformis</i>	<i>E. siderophloia</i>	<i>E. siderophloia</i>	-29.298737	152.964403
NSW1019376	Sid_Whi	Whiporie	<i>E. fusiformis</i>	<i>E. siderophloia</i>	<i>E. siderophloia</i>	-29.29873	152.964357
NSW1019377	Sid_Whi	Whiporie	<i>E. fusiformis</i>	<i>E. siderophloia</i>	<i>E. siderophloia</i>	-29.299035	152.964394
NSW1019378	Sid_Whi	Whiporie	<i>E. fusiformis</i>	<i>E. siderophloia</i>	<i>E. siderophloia</i>	-29.299015	152.964388
NSW1018520	Sid_Whi	Whiporie	<i>E. fusiformis</i>	<i>E. siderophloia</i>	<i>E. siderophloia</i>	-29.29888	152.964584
NSW1013090	Sid_Whi	Whiporie	<i>E. fusiformis</i>	<i>E. siderophloia</i>	<i>E. siderophloia</i>	-29.29888	152.964584
NSW1019364	Sid_Whi	Whiporie	<i>E. fusiformis</i>	<i>E. siderophloia</i>	<i>E. siderophloia</i>	-29.29888	152.964584
NSW1018727	Sid_Pin	Pine Brush State Forest	<i>E. siderophloia</i>	<i>E. siderophloia</i>	<i>E. siderophloia</i>	-29.658662	153.190017
NSW1018808	Sid_Pin	Pine Brush State Forest	<i>E. siderophloia</i>	<i>E. siderophloia</i>	<i>E. siderophloia</i>	-29.658736	153.189971
NSW1018728	Sid_Pin	Pine Brush State Forest	<i>E. siderophloia</i>	<i>E. siderophloia</i>	<i>E. siderophloia</i>	-29.658736	153.189971
NSW1019421	Sid_Pin	Pine Brush State Forest	<i>E. siderophloia</i>	<i>E. siderophloia</i>	<i>E. siderophloia</i>	-29.658728	153.189999
NSW1018729	Sid_Old	Old Genn Innes Road	<i>E. siderophloia</i>	<i>E. siderophloia</i>	<i>E. siderophloia</i>	-29.763514	152.755253

Sample	Code	Location	Field ID	Voucher ID	Genetic ID	Latitude	Longitude
NSW1018824	Sid_New	Newfoundland State Forest	<i>E. ironbark</i>	<i>E. siderophloia</i>	<i>E. siderophloia</i>	-29.867721	153.152677
NSW1018814	Sid_New	Newfoundland State Forest	<i>E. ironbark</i>	<i>E. siderophloia</i>	<i>E. siderophloia</i>	-29.867911	153.152699
NSW1018964	Sid_New	Newfoundland State Forest	<i>E. ironbark</i>	<i>E. siderophloia</i>	<i>E. siderophloia</i>	-29.867883	153.152707
NSW1018825	Sid_New	Newfoundland State Forest	<i>E. ironbark</i>	<i>E. siderophloia</i>	<i>E. siderophloia</i>	-29.867883	153.152707
NSW1018813	Sid_New	Newfoundland State Forest	<i>E. ironbark</i>	<i>E. siderophloia</i>	<i>E. siderophloia</i>	-29.867721	153.152677
NSW1013166	Sid_Kun	Kungala Road	<i>E. siderophloia</i>	<i>E. siderophloia</i>	<i>E. siderophloia</i>	-29.937628	153.033969
NSW1020935	Sid_Nym	Near Nymboi Binderay NP	<i>E. siderophloia</i>	<i>E. siderophloia</i>	<i>E. fibrosa</i>	-30.006651	152.778549
NSW1015804	Sid_Nym	Near Nymboi Binderay NP	<i>E. siderophloia</i>	<i>E. siderophloia</i>	<i>E. siderophloia</i>	-30.00214	152.77285
NSW1015803	Sid_Nym	Near Nymboi Binderay NP	<i>E. siderophloia</i>	<i>E. siderophloia</i>	<i>E. siderophloia</i>	-30.00214	152.77285
NSW1020949	Sid_Tam	Tamban State Forest	<i>E. fusiformis</i>	<i>E. siderophloia</i>	<i>E. siderophloia</i>	-30.8803	152.86986
NSW1020941	Sid_Tam	Tamban State Forest	<i>E. fusiformis</i>	<i>E. siderophloia</i>	<i>E. siderophloia</i>	-30.880401	152.870102
nsw1020942	Sid_Tam	Tamban State Forest	<i>E. fusiformis</i>	<i>E. siderophloia</i>	<i>E. siderophloia</i>	-30.8803	152.87006
NSW1020993	Sid_Tam	Tamban State Forest	<i>E. fusiformis</i>	<i>E. siderophloia</i>	<i>E. siderophloia</i>	-30.880205	152.869625
NSW1020992	Sid_Tam	Tamban State Forest	<i>E. fusiformis</i>	<i>E. siderophloia</i>	<i>E. siderophloia</i>	-30.880013	152.869405
NSW1015810	Sid_Tam	Tamban State Forest	<i>E. fusiformis</i>	<i>E. siderophloia</i>	<i>E. siderophloia</i>	-30.880126	152.869354
NSW1019037	Sid_Yar	Yarramundi, Sydney	<i>E. siderophloia</i>	<i>E. siderophloia</i>	<i>E. siderophloia</i>	-33.615892	150.650879
NSW1019454	Sid_Yar	Yarramundi, Sydney	<i>E. siderophloia</i>	<i>E. siderophloia</i>	<i>E. siderophloia</i>	-33.615863	150.650871
NSW1019406	Sid_Yar	Yarramundi, Sydney	<i>E. siderophloia</i>	<i>E. siderophloia</i>	<i>E. siderophloia</i>	-33.615731	150.650668
NSW1019409	Sid_Yar	Yarramundi, Sydney	<i>E. siderophloia</i>	<i>E. siderophloia</i>	#Hybrid	-33.615731	150.650668
NSW1019407	Sid_Yar	Yarramundi, Sydney	<i>E. siderophloia</i>	<i>E. siderophloia</i>	<i>E. siderophloia</i>	-33.615763	150.650491
NSW1019408	Sid_Yar	Yarramundi, Sydney	<i>E. siderophloia</i>	<i>E. siderophloia</i>	<i>E. siderophloia</i>	-33.615331	150.650712
NSW1015808	Tet_x_Sid_Ban	Banyabba Area	<i>E. tetrapleura x siderophloia</i>	<i>E. tetrapleura x siderophloia?</i>	<i>E. siderophloia</i>	-29.258405	153.016438
NSW1019374	Tet_x_Sid_Ban	Banyabba Area	<i>E. tetrapleura x siderophloia</i>	<i>E. tetrapleura x siderophloia?</i>	Hybrid	-29.328953	153.016438
NSW1019420	Tet_x_Sid_Pin	Pine Brush State Forest	<i>E. tetrapleura x siderophloia</i>	<i>E. tetrapleura x siderophloia?</i>	<i>E. siderophloia</i>	-29.658631	153.18971
NSW1015805	Sid_x_Cre_Old	Old Glenn Innes Road	<i>E. bright green ironbark</i>	<i>E. siderophloia x crebra</i>	<i>E. siderophloia</i>	-29.763101	152.753393
NSW1019385	Fus_Bon	Bongil Bongil National Park	<i>E. fusiformis</i>	<i>E. fusiformis</i>	<i>E. siderophloia</i>	-30.419693	153.034674
NSW1019382	Fus_Bon	Bongil Bongil National Park	<i>E. fusiformis</i>	<i>E. fusiformis</i>	<i>E. siderophloia</i>	-30.41734	153.037527
NSW1018719	Fus_Bon	Bongil Bongil National Park	<i>E. fusiformis</i>	<i>E. fusiformis</i>	<i>E. siderophloia</i>	-30.417221	153.037473
NSW1019381	Fus_Bon	Bongil Bongil National Park	<i>E. fusiformis</i>	<i>E. fusiformis</i>	<i>E. siderophloia</i>	-30.417238	153.037398
NSW1013216	Fus_Bon	Bongil Bongil National Park	<i>E. fusiformis</i>	<i>E. fusiformis</i>	<i>E. siderophloia</i>	-30.416262	153.039572
NSW1019455	Fus_Bon	Bongil Bongil National Park	<i>E. fusiformis</i>	<i>E. fusiformis</i>	<i>E. siderophloia</i>	-30.415997	153.039965
NSW1015801	Tet_Off	Off Old Tenterfield Road	<i>E. tetrapleura</i>	<i>E. terapleura</i>	Hybrid	-29.256113	152.975699
NSW1019427	Tet_Off	Off Old Tenterfield Road	<i>E. tetrapleura</i>	<i>E. terapleura</i>	Hybrid	-29.256127	152.975709
NSW1019426	Tet_Off	Off Old Tenterfield Road	<i>E. tetrapleura</i>	<i>E. terapleura</i>	Hybrid	-29.256018	152.975071
NSW1019437	Tet_Off	Off Old Tenterfield Road	<i>E. tetrapleura</i>	<i>E. terapleura</i>	Hybrid	-29.255981	152.974953
NSW1019425	Tet_Off	Off Old Tenterfield Road	<i>E. tetrapleura</i>	<i>E. terapleura</i>	Hybrid	-29.255983	152.974952
NSW1018802	Tet_Off	Off Old Tenterfield Road	<i>E. tetrapleura</i>	<i>E. terapleura</i>	Hybrid	-29.255983	152.974952
NSW1019399	Tet_Off	Off Old Tenterfield Road	<i>E. tetrapleura</i>	<i>E. terapleura</i>	Hybrid	-29.258405	152.975316
NSW1018846	Tet_Off	Off Old Tenterfield Road	<i>E. tetrapleura</i>	<i>E. terapleura</i>	Hybrid	-29.258376	152.97518

Sample	Code	Location	Field ID	Voucher ID	Genetic ID	Latitude	Longitude
NSW1018845	Tet_Off	Off Old Tenterfield Road	<i>E. tetrapleura</i>	<i>E. terapleura</i>	Hybrid	-29.257846	152.975862
NSW1018843	Tet_Off	Off Old Tenterfield Road	<i>E. tetrapleura</i>	<i>E. terapleura</i>	Hybrid	-29.258108	152.976079
NSW1018840	Tet_Off	Off Old Tenterfield Road	<i>E. tetrapleura</i>	<i>E. terapleura</i>	Hybrid	-29.257585	152.975986
NSW1018836	Tet_Off	Off Old Tenterfield Road	<i>E. tetrapleura</i>	<i>E. terapleura</i>	Hybrid	-29.257793	152.976184
NSW1018844	Tet_Off	Off Old Tenterfield Road	<i>E. tetrapleura</i>	<i>E. terapleura</i>	Hybrid	-29.256986	152.975707
NSW1018842	Tet_Off	Off Old Tenterfield Road	<i>E. tetrapleura</i>	<i>E. terapleura</i>	Hybrid	-29.257004	152.975724
NSW1018838	Tet_Off	Off Old Tenterfield Road	<i>E. tetrapleura</i>	<i>E. terapleura</i>	Hybrid	-29.256897	152.975974
NSW1019379	Tet_x_Fib_Ote	Old Tenterfield Road	<i>E. tetrapleura x fibrosa?</i>	<i>E. tetrapleura x fibrosa?</i>	Hybrid	-29.253231	152.970377
NSW1019452	Tet_x_Fib_Ote	Old Tenterfield Road	<i>E. tetrapleura x fibrosa?</i>	<i>E. tetrapleura x fibrosa?</i>	Hybrid	-29.253231	152.970377
NSW1015826	Tet_x_Fib_Ote	Old Tenterfield Road	<i>E. tetrapleura x fibrosa?</i>	<i>E. tetrapleura x fibrosa?</i>	Hybrid	-29.253231	152.970377
NSW1013125	Tet_x_Fib_Ote	Old Tenterfield Road	<i>E. tetrapleura x fibrosa?</i>	<i>E. tetrapleura x fibrosa?</i>	Hybrid	-29.253264	152.970416
NSW1018830	Tet_x_Fib_Ote	Old Tenterfield Road	<i>E. tetrapleura x fibrosa?</i>	<i>E. tetrapleura x fibrosa?</i>	Hybrid	-29.253253	152.970367
NSW1013120	Tet_x_Fib_Ote	Old Tenterfield Road	<i>E. tetrapleura x fibrosa?</i>	<i>E. tetrapleura x fibrosa?</i>	Hybrid	-29.253253	152.970367
NSW1013109	Tet_Ban	Banyabba	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	Hybrid	-29.331056	153.01603
NSW1018822	Tet_Ban	Banyabba	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	Hybrid	-29.330892	153.016076
NSW1019428	Tet_Ban	Banyabba	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	Hybrid	-29.330892	153.016076
NSW1019371	Tet_Ban	Banyabba	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	Hybrid	-29.32936	153.016094
NSW1019372	Tet_Ban	Banyabba	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	Hybrid	-29.328953	153.016438
NSW1019373	Tet_Ban	Banyabba	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	Hybrid	-29.328953	153.016438
NSW1019011	Tet_Tre	Trenayr Lane	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	Hybrid	-29.58433	152.971088
NSW1015829	Tet_Tre	Trenayr Lane	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	Hybrid	-29.592677	152.966656
NSW1015828	Tet_Tre	Trenayr Lane	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	Hybrid	-29.592651	152.966694
NSW1015823	Tet_Tre	Trenayr Lane	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	Did not pass QC	-29.591853	152.963645
NSW1018986	Tet_Tre	Trenayr Lane	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	Did not pass QC	-29.592519	152.965896
NSW1019069	Tet_Tre	Trenayr Lane	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	Hybrid	-29.592519	152.965896
NSW1020998	Tet_Tre	Trenayr Lane	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	Hybrid	-29.592627	152.966652
NSW1019070	Fib_Tre	Trenayr Lane	<i>E. ironbark</i>	<i>E. fibrosa?</i>	Hybrid	-29.592807	152.966752
NSW1018983	Fib_Tre	Trenayr Lane	<i>E. ironbark</i>	<i>E. fibrosa?</i>	Hybrid	-29.592706	152.966712
NSW1019009	Fib_Tre	Trenayr Lane	<i>E. ironbark</i>	<i>E. fibrosa?</i>	Hybrid	-29.592831	152.966688
NSW1019014	Fib_Tre	Trenayr Lane	<i>E. ironbark</i>	<i>E. fibrosa?</i>	Hybrid	-29.592831	152.966688
NSW1013105	Tet_Tin	Tindal Road	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	Hybrid	-29.633361	152.822084
NSW1013100	Tet_Tin	Tindal Road	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	Hybrid	-29.633361	152.822084
NSW1013095	Tet_Tin	Tindal Road	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	Hybrid	-29.633348	152.822091
NSW1018709	Tet_Tin	Tindal Road	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	Hybrid	-29.633256	152.822315
NSW1018708	Tet_Tin	Tindal Road	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	Hybrid	-29.635977	152.827444
NSW1018707	Tet_Tin	Tindal Road	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	Hybrid	-29.635977	152.827444
NSW1018975	Tet_Pin	Pine Brush State Forest	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.654486	153.180416
NSW1019370	Tet_Pin	Pine Brush State Forest	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.654461	153.180409
NSW1019369	Tet_Pin	Pine Brush State Forest	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	Hybrid	-29.654461	153.180409



Sample	Code	Location	Field ID	Voucher ID	Genetic ID	Latitude	Longitude
NSW1019368	Tet_Pin	Pine Brush State Forest	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.653422	153.181007
NSW1018827	Tet_Pin	Pine Brush State Forest	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.653422	153.181007
NSW1018851	Tet_Pin	Pine Brush State Forest	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.653422	153.181007
NSW1018826	Tet_Pin	Pine Brush State Forest	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.658555	153.190104
NSW1019429	Tet_Roc	Rockview Road, near Chambigne NP	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. fibrosa</i>	-29.780814	152.74571
NSW1019039	Tet_Roc	Rockview Road, near Chambigne NP	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.780814	152.74571
NSW1018839	Tet_Roc	Rockview Road, near Chambigne NP	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.78066	152.745852
NSW1019453	Tet_Roc	Rockview Road, near Chambigne NP	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	Did not pass QC	-29.780684	152.745896
NSW1018815	Tet_Roc	Rockview Road, near Chambigne NP	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.780684	152.745896
NSW1018816	Tet_Roc	Rockview Road, near Chambigne NP	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.780684	152.745896
NSW1018817	Tet_Roc	Rockview Road, near Chambigne NP	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.781311	152.74537
NSW1020937	Tet_RMS	RMS Offset Property	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.794024	153.082346
NSW1015814	Tet_RMS	RMS Offset Property	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.794175	153.082658
NSW1015813	Tet_RMS	RMS Offset Property	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.794249	153.082548
NSW1015815	Tet_RMS	RMS Offset Property	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.794263	153.082457
NSW1015816	Tet_RMS	RMS Offset Property	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.794398	153.081789
NSW1020987	Tet_RMS	RMS Offset Property	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.794485	153.082094
NSW1018353	Tet_RMS	RMS Offset Property	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.8035	153.079963
NSW1019345	Tet_RMS	RMS Offset Property	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. fibrosa</i>	-29.804005	153.079877
NSW1020986	Tet_RMS	RMS Offset Property	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.804659	153.079796
NSW1019015	Tet_RMS	RMS Offset Property	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.803804	153.079356
NSW1018987	Tet_RMS	RMS Offset Property	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. fibrosa</i>	-29.803808	153.079473
NSW1015821	Tet_RMS	RMS Offset Property	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.804104	153.079849
NSW1018956	Tet_Sha	Shannondale Road	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.811133	152.857878
NSW1015822	Tet_Sha	Shannondale Road	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.811133	152.857878
NSW1019044	Tet_Sha	Shannondale Road	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	Hybrid	-29.810936	152.857183
NSW1019048	Tet_Sha	Shannondale Road	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.81354	152.826838
NSW1018966	Tet_Sha	Shannondale Road	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.813198	152.826865
NSW1013149	Tet_Sha	Shannondale Road	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.813126	152.827298
NSW1018957	Tet_Sha	Shannondale Road	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.810618	152.828845
NSW1018958	Tet_Sha	Shannondale Road	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	Hybrid	-29.810438	152.82897
NSW1013129	Tet_Sha	Shannondale Road	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	Hybrid	-29.810403	152.828994
NSW1013114	Tet_Sha	Shannondale Road	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.810403	152.828994
NSW1018959	Tet_Sha	Shannondale Road	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.810403	152.828994
NSW1018960	Tet_Sha	Shannondale Road	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.811279	152.828705
NSW1013135	Tet_Sha	Shannondale Road	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. siderophloia</i>	-29.811325	152.828218
NSW1018961	Tet_Sha	Shannondale Road	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. siderophloia</i>	-29.811446	152.828162
NSW1019132	Tet_Sha	Shannondale Road	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.811305	152.82834
NSW1018984	Tet_Sha	Shannondale Road	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.811305	152.82834

Sample	Code	Location	Field ID	Voucher ID	Genetic ID	Latitude	Longitude
NSW1013144	Tet_Opa	Old Pacific Highway	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.820094	153.0372
NSW1018954	Tet_Opa	Old Pacific Highway	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.820256	153.036977
NSW1013154	Tet_Opa	Old Pacific Highway	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.820242	153.036969
NSW1019348	Tet_Din	Dinjerra Road	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.82624	153.026366
NSW1019367	Tet_Din	Dinjerra Road	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.826239	153.026367
NSW1018706	Tet_Din	Dinjerra Road	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.82624	153.026367
NSW1018705	Tet_Din	Dinjerra Road	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.826239	153.026367
NSW1019366	Tet_Din	Dinjerra Road	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.826239	153.026367
NSW1018837	Tet_Din	Dinjerra Road	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.826239	153.026367
NSW1015820	Tet_Kan	Kangaroo Creek Road	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.839335	152.889595
NSW1013104	Tet_Kan	Kangaroo Creek Road	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.839317	152.889519
NSW1013159	Tet_Kan	Kangaroo Creek Road	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.839317	152.889519
NSW1018994	Tet_Kan	Kangaroo Creek Road	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.839317	152.889519
NSW1013119	Tet_Kan	Kangaroo Creek Road	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.839317	152.889519
NSW1013134	Tet_Kan	Kangaroo Creek Road	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.839317	152.889519
NSW1020991	Tet_Fra3	Franklins Road 3	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.845425	153.094972
NSW1018962	Tet_Fra3	Franklins Road 3	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.845468	153.094949
NSW1013124	Tet_Fra3	Franklins Road 3	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	Hybrid	-29.845491	153.094962
NSW1019129	Tet_Fra3	Franklins Road 3	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.844638	153.095125
NSW1019123	Tet_Fra3	Franklins Road 3	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.844473	153.095168
NSW1019075	Tet_Fra3	Franklins Road 3	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.844482	153.09518
NSW1018995	Tet_Fra1	Franklins Road 1	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.852226	153.051814
NSW1019008	Tet_Fra1	Franklins Road 1	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.85245	153.051855
NSW1015885	Tet_Fra1	Franklins Road 1	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.852683	153.051886
NSW1018818	Tet_New	Newfoundland State Forest	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.866317	153.153878
NSW1018819	Tet_New	Newfoundland State Forest	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.866317	153.153878
NSW1018820	Tet_New	Newfoundland State Forest	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.866264	153.153904
NSW1018821	Tet_New	Newfoundland State Forest	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.866241	153.153971
NSW1018704	Tet_New	Newfoundland State Forest	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.866241	153.153971
NSW1018823	Tet_New	Newfoundland State Forest	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.866101	153.153822
NSW1013191	Tet_Bal	Bald Knob Road	<i>E. siderophloia</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.88084	153.067347
NSW1015853	Tet_Bal	Bald Knob Road	<i>E. siderophloia</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.880847	153.067228
NSW1019084	Tet_Bal	Bald Knob Road	<i>E. siderophloia</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.880815	153.066644
NSW1018951	Tet_Bal	Bald Knob Road	<i>E. siderophloia</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.881032	153.066639
NSW1019128	Tet_Bal	Bald Knob Road	<i>E. siderophloia</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.881032	153.066639
NSW1019068	Tet_Bal	Bald Knob Road	<i>E. siderophloia</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.880858	153.067752
NSW1019036	Tet_Bal	Bald Knob Road	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.880811	153.06745
NSW1019102	Tet_Bal	Bald Knob Road	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.880882	153.066674
NSW1020943	Tet_Bal	Bald Knob Road	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.88125	153.070562

Sample	Code	Location	Field ID	Voucher ID	Genetic ID	Latitude	Longitude
NSW1018950	Tet_Bal	Bald Knob Road	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.881243	153.070739
NSW1018834	Tet_Lan	Lanitza Road	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.883574	152.991888
NSW1018689	Tet_Lan	Lanitza Road	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.883563	152.991696
NSW1019434	Tet_Lan	Lanitza Road	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.883563	152.991696
NSW1013139	Tet_Lan	Lanitza Road	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.883605	152.991863
NSW1019446	Tet_Lan	Lanitza Road	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.883969	152.991864
NSW1018701	Tet_Lan	Lanitza Road	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.883503	152.992417
NSW1018841	Tet_Lan	Lanitza Road	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.883544	152.994592
NSW1019046	Tet_Pac	Pacific Highway, north of Wells Crs	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.886217	153.0591
NSW1018955	Tet_Pac	Pacific Highway, north of Wells Crs	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.886217	153.0591
NSW1015817	Tet_Pac	Pacific Highway, north of Wells Crs	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.886217	153.0591
NSW1020940	Tet_Pac	Pacific Highway, north of Wells Crs	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.886217	153.0591
NSW1020939	Tet_Pac	Pacific Highway, north of Wells Crs	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.886217	153.0591
NSW1020936	Tet_Pac	Pacific Highway, north of Wells Crs	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.886217	153.0591
NSW1020996	Tet_Wel	Wells Crossing	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.899149	153.063588
NSW1020988	Tet_Wel	Wells Crossing	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.899149	153.063588
NSW1015825	Tet_Wel	Wells Crossing	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.899149	153.063588
NSW1013211	Tet_Kun	Kungala Road	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. fibrosa</i>	-29.937766	153.034984
NSW1019053	Tet_Kun	Kungala Road	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.937742	153.034863
NSW1013171	Tet_Kun	Kungala Road	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	Hybrid	-29.937257	153.035064
NSW1020947	Tet_Kun	Kungala Road	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.931391	153.06316
NSW1013115	Tet_Kun	Kungala Road	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.93138	153.063131
NSW1013196	Tet_Fal	Falconers Lane	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.96635	153.136374
NSW1019010	Tet_Fal	Falconers Lane	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.966529	153.136168
NSW1013160	Tet_Fal	Falconers Lane	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.965757	153.136521
NSW1020945	Tet_Fal	Falconers Lane	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.965757	153.136521
NSW1020944	Tet_Fal	Falconers Lane	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.965725	153.13651
NSW1020997	Tet_Fal	Falconers Lane	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.965667	153.136531
NSW1020938	Tet_Fal	Falconers Lane	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.965667	153.136531
NSW1018963	Tet_Fal	Falconers Lane	<i>E. ironbark</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.966514	153.136096
NSW1013085	Tet_Fal	Falconers Lane	<i>E. ironbark</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.965838	153.136441
NSW1015819	Tet_Ran	Range Road South	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.98862	153.143711
NSW1013130	Tet_Ran	Range Road South	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.988672	153.143324
NSW1019131	Tet_Ran	Range Road South	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.988672	153.143324
NSW1013201	Tet_Ran	Range Road South	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.988326	153.143371
NSW1020946	Tet_Ran	Range Road South	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	Hybrid	-29.988323	153.143372
NSW1015827	Tet_Ran	Range Road South	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.988344	153.142726
NSW1019133	Tet_Ran	Range Road South	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-29.988292	153.142605
NSW1015806	Tet_Nym	Near Nymboi Binderay NP	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-30.006954	152.777134

Sample	Code	Location	Field ID	Voucher ID	Genetic ID	Latitude	Longitude
NSW1019052	Tet_Nym	Near Nymboi Binderay NP	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-30.006829	152.77717
NSW1013145	Tet_Nym	Near Nymboi Binderay NP	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-30.006829	152.77717
NSW1018967	Tet_Nym	Near Nymboi Binderay NP	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-30.006829	152.77717
NSW1015818	Tet_Nym	Near Nymboi Binderay NP	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-30.006829	152.77717
NSW1018952	Tet_Nym	Near Nymboi Binderay NP	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-30.006829	152.77717
NSW1019105	Tet_Nym	Near Nymboi Binderay NP	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-30.006765	152.778046
NSW1019106	Tet_Nym	Near Nymboi Binderay NP	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-30.006767	152.778055
NSW1018980	Tet_Nym	Near Nymboi Binderay NP	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-30.006607	152.778486
NSW1013176	Tet_Nym	Near Nymboi Binderay NP	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-30.006601	152.778286
NSW1019043	Tet_Nym	Near Nymboi Binderay NP	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-30.006601	152.778286
NSW1013181	Tet_Nym	Near Nymboi Binderay NP	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-30.006601	152.778286
NSW1015824	Tet_Nym	Near Nymboi Binderay NP	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-30.006719	152.778676
NSW1019051	Tet_Nym	Near Nymboi Binderay NP	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-30.006651	152.778549
NSW1013140	Tet_Nym	Near Nymboi Binderay NP	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-30.006651	152.778549
NSW1013110	Tet_Nym	Near Nymboi Binderay NP	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	<i>E. tetrapleura</i>	-30.006651	152.778549

**Table 3:** Heterozygosity, Fixation index and allele frequency across populations, based on 4179 markers (Reproducibility=0.98, No missing data).

Species	Pop	N	$N_E$ (SE)	$I$ (SE)	$H_o$ (SE)	$H_e$ (SE)	$uH_e$ (SE)	$F$	No. Private Alleles (SE)
<i>E. fibrosa</i>	Opa	3	1.333 (0.009)	0.298 (0.007)	0.239 (0.007)	0.199 (0.005)	0.239 (0.006)	-0.200 (0.011)	0.064 (0.006)
	Fra2	6	1.334 (0.008)	0.321 (0.006)	0.231 (0.006)	0.207 (0.004)	0.226 (0.005)	-0.103 (0.009)	0.139 (0.009)
	Cas	6	1.379 (0.009)	0.354 (0.006)	0.243 (0.006)	0.230 (0.004)	0.251 (0.005)	-0.056 (0.009)	0.194 (0.010)
<i>E. siderophloia</i> (including <i>E. fusiformis</i> )	Whi	8	1.270 (0.007)	0.262 (0.005)	0.184 (0.004)	0.168 (0.004)	0.179 (0.004)	-0.082 (0.006)	0.058 (0.005)
	Pin	5	1.254 (0.007)	0.234 (0.005)	0.185 (0.005)	0.154 (0.004)	0.171 (0.004)	-0.180 (0.007)	0.037 (0.004)
	New	5	1.263 (0.007)	0.251 (0.005)	0.180 (0.005)	0.163 (0.004)	0.181 (0.004)	-0.107 (0.007)	0.040 (0.004)
	Nym	2	1.232 (0.007)	0.195 (0.006)	0.178 (0.006)	0.133 (0.004)	0.178 (0.005)	-0.334 (0.010)	0.017 (0.003)
	Tam	6	1.270 (0.007)	0.258 (0.005)	0.187 (0.005)	0.167 (0.004)	0.182 (0.004)	-0.108 (0.007)	0.047 (0.004)
	Yar	5	1.262 (0.007)	0.243 (0.005)	0.182 (0.005)	0.159 (0.004)	0.177 (0.004)	-0.130 (0.008)	0.045 (0.004)
	Bon	6	1.251 (0.007)	0.238 (0.005)	0.169 (0.004)	0.154 (0.004)	0.169 (0.004)	-0.088 (0.007)	0.047 (0.004)
<i>E. tetrapleura</i>	Pin	6	1.193 (0.005)	0.188 (0.004)	0.133 (0.004)	0.120 (0.003)	0.131 (0.003)	-0.104 (0.005)	0.128 (0.006)
	Roc	5	1.190 (0.005)	0.181 (0.004)	0.130 (0.004)	0.118 (0.003)	0.131 (0.003)	-0.109 (0.006)	0.110 (0.005)
	RMS	10	1.196 (0.005)	0.196 (0.004)	0.127 (0.003)	0.124 (0.003)	0.130 (0.003)	-0.027 (0.005)	0.166 (0.006)
	Sha	11	1.205 (0.005)	0.213 (0.004)	0.137 (0.003)	0.132 (0.003)	0.138 (0.003)	-0.037 (0.004)	0.206 (0.007)
	Opa	3	1.186 (0.005)	0.170 (0.004)	0.137 (0.004)	0.113 (0.003)	0.135 (0.004)	-0.212 (0.007)	0.077 (0.005)
	Din	6	1.191 (0.005)	0.184 (0.004)	0.135 (0.004)	0.119 (0.003)	0.130 (0.003)	-0.121 (0.006)	0.122 (0.006)
	Kan	6	1.195 (0.005)	0.192 (0.004)	0.137 (0.004)	0.123 (0.003)	0.134 (0.003)	-0.106 (0.005)	0.142 (0.006)
	Fra3	5	1.186 (0.005)	0.177 (0.004)	0.132 (0.004)	0.115 (0.003)	0.127 (0.003)	-0.136 (0.006)	0.101 (0.005)
	Fra1	3	1.175 (0.005)	0.158 (0.004)	0.124 (0.004)	0.105 (0.003)	0.126 (0.004)	-0.181 (0.008)	0.067 (0.004)
	New	6	1.175 (0.005)	0.165 (0.004)	0.127 (0.004)	0.107 (0.003)	0.117 (0.003)	-0.163 (0.005)	0.092 (0.005)
	Bal	10	1.197 (0.005)	0.195 (0.004)	0.132 (0.003)	0.124 (0.003)	0.130 (0.003)	-0.062 (0.005)	0.160 (0.006)
	Lan	7	1.188 (0.005)	0.185 (0.004)	0.126 (0.003)	0.118 (0.003)	0.127 (0.003)	-0.069 (0.005)	0.124 (0.006)
	Pac	6	1.178 (0.005)	0.169 (0.004)	0.121 (0.004)	0.109 (0.003)	0.119 (0.003)	-0.098 (0.006)	0.094 (0.005)
	Wel	3	1.172 (0.005)	0.155 (0.004)	0.122 (0.004)	0.103 (0.003)	0.124 (0.004)	-0.190 (0.007)	0.067 (0.004)
	Kun	3	1.186 (0.005)	0.168 (0.004)	0.135 (0.004)	0.112 (0.003)	0.134 (0.004)	-0.208 (0.007)	0.071 (0.004)
	Fal	9	1.174 (0.005)	0.166 (0.004)	0.124 (0.004)	0.107 (0.003)	0.113 (0.003)	-0.125 (0.004)	0.111 (0.005)
Ran	6	1.189 (0.005)	0.179 (0.004)	0.130 (0.004)	0.116 (0.003)	0.126 (0.003)	-0.114 (0.006)	0.113 (0.006)	
Nym	16	1.205 (0.005)	0.208 (0.004)	0.139 (0.003)	0.130 (0.003)	0.134 (0.003)	-0.050 (0.004)	0.189 (0.007)	

$N$  = No. of Individuals;

$N_E$  = No. of Effective Alleles =  $1 / (\sum \pi_i^2)$

$I$  = Shannon's Information Index =  $-1 * \sum (\pi_i * \ln(\pi_i))$

$H_o$  (SE) = Observed Heterozygosity = No. of Hets /  $N$

$H_e$  = Expected Heterozygosity =  $1 - \sum \pi_i^2$

$uH_e$  = Unbiased Expected Heterozygosity =  $(2N / (2N-1)) * H_e$

$F$  = Fixation Index =  $(H_e - H_o) / H_e = 1 - (H_o / H_e)$

No. Private Alleles = No. of Alleles Unique to a Single Population

4.3d Genetic diversity, heterozygosity and connectivity of *E. tetrapleura*: understanding dynamics, and a preliminary investigation of the mating system of *Eucalyptus tetrapleura*

Although,  $F$  values (inbreeding measures),  $H_e$  and rare allele counts (measures of diversity) are marginally lower in *E. tetrapleura* than in the other two species (which are however much less extensively sampled), these values are not outside the expected range. There is no sign of inbreeding (in fact all populations register an excess of observed heterozygosity in respect to expectation) and diversity in *E. tetrapleura* is well within the range of the other two species. In fact even smaller populations do not appear to have a comparative loss of diversity when compared to larger ones. It is therefore unlikely that hybridisation is a result of lack of diversity or inbreeding, but it is more likely to be the results of chance, bi-directional events (all species are likely to receive foreign pollen, not *E. tetrapleura* alone) and the occasional overlap in flowering time.

This is supported by the fact that  $F_{st}$  measures (estimates of gene flow) between *E. tetrapleura* populations (0.037 to 0.106) are within the range of the measures recorded for other species (0.071 to 0.078 for *E. fibrosa*; and for 0.055 to 0.121 for *E. siderophloia*). There is no apparent relationship between the extent of gene flow and the geographic location of populations, suggesting that genetic exchange within *E. tetrapleura* appears to be unrestricted, and that this process contributes to the maintenance of genetic diversity across the entire distribution of the species.

## 5. CONCLUSIONS AND IMPLICATIONS

This project highlighted the following facts:

- *Eucalyptus tetrapleura* is geographically more narrowly distributed (only south of the Clarence River), and potentially rarer (due to miss-identifications) than previously suggested. Only hybrids were identified north of the Clarence.
- The additional surveys undertaken by RMS and RBG have confirmed a number of new *E. tetrapleura* locations in the core range of the species and outer edges south of the Clarence River. Additional *Eucalyptus tetrapleura* locations have been found at the RMS offset on the Woolli Road, east Franklins Road, east Bald Knob Road, Dinjera Road, south of Falconers Rd, Kungala road area, Lanitza area, Kangaroo Creek area and in the Nymboi-Binderay area. All these additional locations had a number of *Eucalyptus tetrapleura* individuals that were confirmed by the RBGDT genetic collections and testing. This is apparent in Figure 5 that provides a summary of all survey and collection sites of *Eucalyptus tetrapleura*.
- Although *E. tetrapleura* is a clearly genomically distinct species, morphological identification can be problematic. Within large populations individuals of other ironbark species and hybrids can be misidentified. The use of genomic tools appears to be the safest method for identification.
- It is likely that land clearing for agriculture and other environment degradation processes around the Grafton area have reduced habitat availability, decreased overall numbers, and potentially facilitated the establishment of hybrid individuals and hybrid populations.
- Although rare, this species behaves as one single meta-population with extensive gene flow among sites (i.e. no distinct provenances), and no sign of inbreeding or excessive loss of overall diversity (aside for the potential localised risk of genetic swamping from excessive hybridisation).
- Pollen exchange between closely related ironbarks in the area appears to be a natural process (as ascertained among many other eucalypt groups), but the potential establishment of hybrid individuals and even whole-hybrid populations is often linked to post-disturbance habitat disruption. Small groups of individuals that have become isolated by disturbance events are generally thought to be more susceptible to genetic swamping, as there is an increasing chance that more foreign pollen becomes locally available. The presence of cleared habitat (and hence limited competition from pure mature plants and/or offspring) can also increase the chances of hybrid offspring to establish.
- Although the necessary studies and experimentation needs to be carried out, like most other eucalypts *E. tetrapleura* appears to be a preferential outcrosser. Based on genetic evidence (heterozygosity, genetic diversity and gene flow), there are no reasons to expect reduced fertility of seeds across any of the major populations.

As a consequence, we recommend for *E. tetrapleura* conservation, and for the establishment of new populations that:

- Where possible, *E. tetrapleura* populations should be preserved, and disturbance levels minimised (to reduce the potential establishment of hybrids).
- New populations should preferentially be established away from other ironbarks when possible. Although some pollen exchange will occur regardless, isolation from other ironbarks will reduce its frequency.
- A re-established population can include material sourced from as many individuals and as many sites as possible, as they are all interconnected and can all contribute to its overall diversity.
- If the material sourced for re-establishing populations is seed-based, then extreme caution has to be taken to ensure that no hybrid material is introduced. The collection of seed material from pure populations only would reduce the likely incidence of hybrid propagules, but would not guarantee their exclusion (as pollen appears to travel far). Morphological differences among seedlings might help differentiate between species and hybrids, but this remains to be verified experimentally. At this stage secure identification can only be obtained via genomic analyses.





Sensitive data

**Figure 5:** A summary of survey and collection sites for studies of *Eucalyptus tetrapleura* from four different sources as indicated in the legend. This map was also supplied to RMS in high quality georeferenced PDF format.

## List of Figures and Tables

**Figure 1:** The sites where collections were made for the *Eucalyptus tetrapleura* genetic study. The species identification on this map is based on field identifications (map also supplied as high quality PDF file).

**Figure 2:** PCoA of genome wide SNP data (from 9,369 markers derived from DArTseq) obtained from the whole distribution of *E. tetrapleura*, and co-distributed ironbark relatives. This figure is not aimed at enabling the identification of single individuals. The stippled lines serve as a guide to highlight which individuals cluster together: red circle highlights the cluster of *E. tetrapleura*, the black circles a range of hybrid individuals (see section 4c for additional information and interpretation), the green circle highlights *E. fibrosa*, and the blue circle pure *E. siderophloia* / *E. fusiformis* (see section 4.3c for additional information and interpretation). Information on the location of each individual and their genetic status is listed in Table 1 and Figure 1.

**Figure 3:** Bayesian assignment analysis as implemented by the software LEA based on genomic-wide data (from 9,369 markers derived from DArTseq) obtained from most of the distribution of *E. tetrapleura*, and from co-distributed ironbark relatives. Vertical strips represent single individuals grouped into populations as described at the bottom. This figure is not aimed at enabling the identification of single individuals. The red colour is assigned to *E. tetrapleura* genomes (more than 0.80 identifies individuals belonging to this group), green is assigned to *E. fibrosa* genomes (more than 0.80 identifies individuals as belonging to this group), and blue is assigned to *E. siderophloia* / *E. fusiformis*. Individuals that have multiple colours are of admixed (hybrid) origin. Some of the taxonomic misidentifications are highlighted (additional information on the location of each individual and their genetic status is listed in Table 2).

**Figure 4:** A map showing the locations where *E. tetrapleura* (red circle) and hybrids (grey) were identified using the full genome scan data obtained from DArTseq. Circles ½ red & ½ grey indicates pure and hybrid individuals identified at the site. High quality Georeferenced maps are supplied separately in PDF format.

**Figure 5:** A summary of survey and collection sites for studies of *Eucalyptus tetrapleura* from four different sources as indicated in the legend.

**Table 1:** Pairwise  $F_{st}$  values among the five groups identified on the basis of the PCoA, DAPC and LEA analyses. The higher the number the more genetically differentiated these groups are. This confirms genomic exchange between the lineages and suggests that overall *E. tetrapleura* is more differentiated (as far as gene flow) from *E. fibrosa* than from *E. siderophloia*.

**Table 2:** A summary of species and hybrid identification based on the genetic analyses using PCoA, DAPC and LEA (Genetic ID). Species and hybrids are colour coded and location coded as in previous figures. Full name of each location as well as three sets of ID (as done in the field when collected; as verified on voucher at the NSW Herbarium; and as confirmed through genomic analyses); and lat/long information are included; did not pass QC = samples for which data was received but did not pass the data quality control.

**Table 3:** Heterozygosity, Fixation index and allele frequency across populations, based on 4,179 markers (Reproducibility=0.98, No missing data).

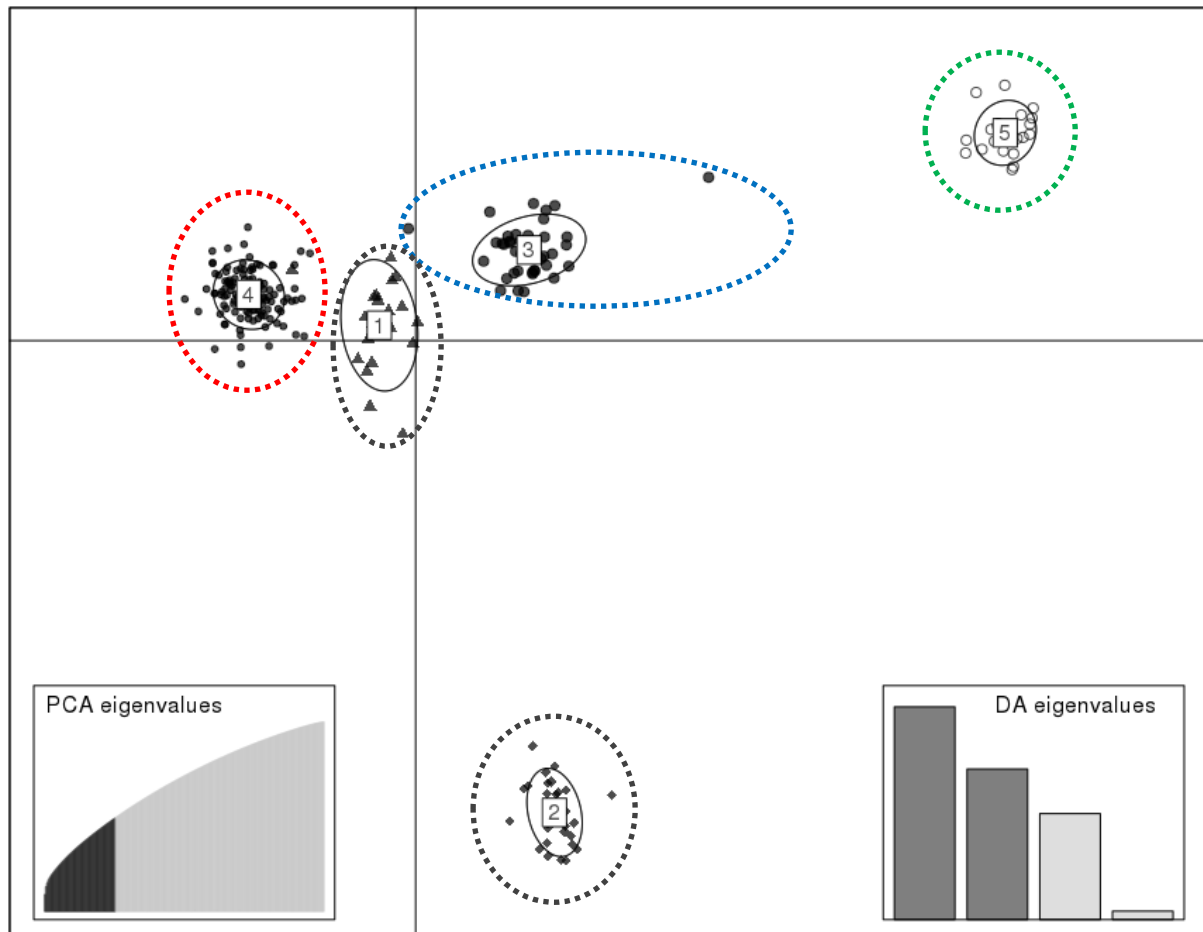
## List of supplementary files

Appendix 1 Additional results

Appendix 2 A descriptive summary for each sampled population of ironbark for the 'Research into the population genetics of *Eucalyptus tetrapleura* and related species' report.

## Appendix 1

### A1 Additional results:



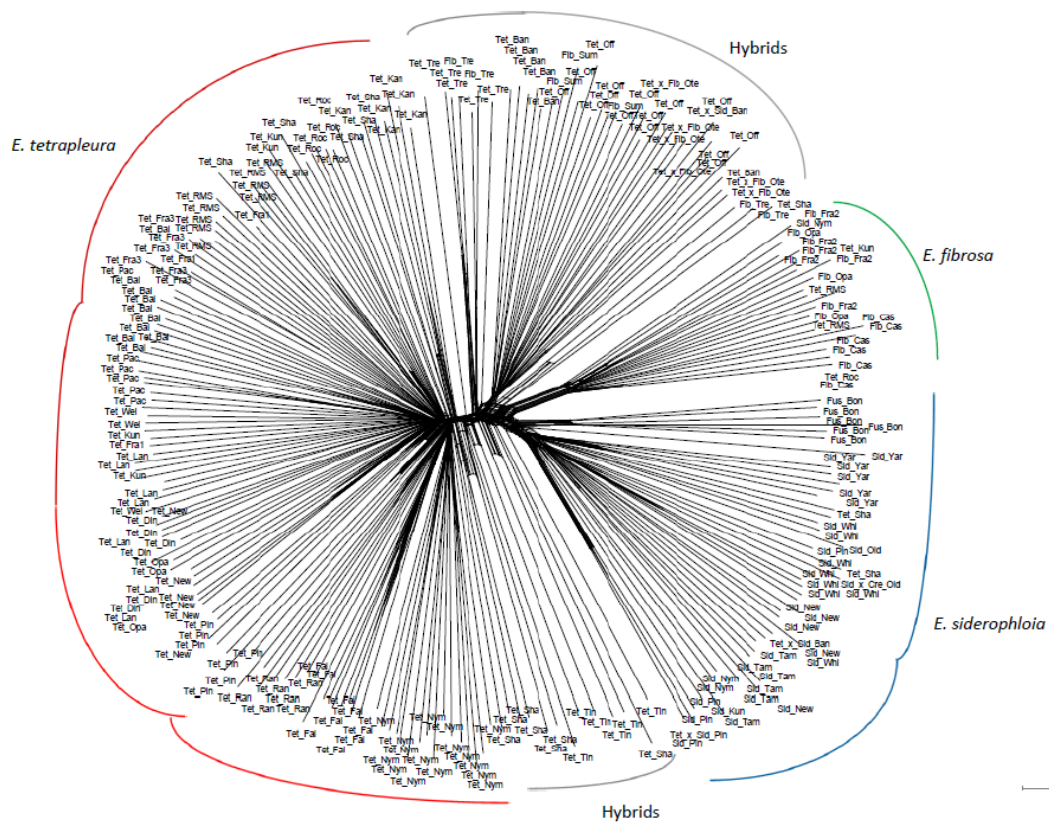
**Figure A1:** Discriminant analysis of principal components (DAPC) results of genomic-wide data obtained from the whole putative distribution of *E. tetrapleura*, and from co-distributed ironbark relatives. Group 1 = Hybrids: *E. tetrapleura* x *E. siderophloia*. All samples from Tre and Tin. Also 5 other samples collected as *E. tetrapleura* (Pin: NSW1019369; Sha: NSW1018958 and NSW1013129; Fra3: NSW1013124; Ran: NSW1020946).

Group 2 = Hybrids: *E. tetrapleura* x *E. fibrosa*. Includes all samples from Off, Ote and Ban (collected as *E. tetrapleura*), 2 samples collected as *E. fibrosa* (Sum: NSW1018715 and NSW1018716) and one sample collected as a putative *E. tetrapleura* x *E. siderophloia* (Ban: NSW1019374).

Group 3= *E. siderophloia* and *E. fusiformis*. Also in this group are 3 samples collected as *E. tetrapleura* (Sha: NSW1019044, NSW1013135 and NSW1018961).

Group 4 = *E. tetrapleura*.

Group 5 = *E. fibrosa*. Also in this group are 4 samples collected as *E. tetrapleura* (Roc: NSW 1019429; RMS: NSW1019345 and NSW1018987; Kun: NSW1013211) and 1 collected as *E. siderophloia* (Nym: NSW1020935).



**Figure A2:** *Splitstree4* analysis of all DArTseq markers. Four samples were excluded from the dataset so that it would be consistent with the PCoA. Codes for species names (as determined from voucher and field data) and current codes for site. The figure illustrates the presence of several hybrid types and the relationship between *E. fusiformis* and *E. siderophloia*. This figure is simply to illustrate the results from the analysis and the reader is not required to identify each individual in the analysis.

## Appendix 2

A descriptive summary for each sampled population of ironbarks for the 'Research into the population genetics of *Eucalyptus tetrapleura* and related species' report.

The following summary provide information on the collection sites, field observations at each site and a summary of the findings based on all the genetic analyses performed on the DArTseq data as in Table 2 ( PCoA, DAPC, LEA and NewHybrids). The majority of the analyses suggested that there are 10 populations of *E. tetrapleura* where all the individuals sampled as *E. tetrapleura* were in fact genetically identified to be this species (these are listed in Table 2). The species names under the site names summarises the results of the genetic data. All populations where all the sampled *E. tetrapleura* individuals were found to be this species are indicated with *E. tetrapleura* in red font. An asterisk after *E. tetrapleura*\* indicates that there were other ironbark species present but these were not sampled. The location of the sites can be found on the maps provided in Figure 1 and Figure 4 as well as in Table 2.

### ***Eucalyptus tetrapleura* populations**

#### **Northern populations**

##### **Off Old Tenterfield Rd, Old Tenterfield Rd and Summerland Way (Ote, Off, Sum)**

#### **hybrids**

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RBG&DT sampled 3 populations in this area (which were c. 50 km from Grafton and were very isolated from other *E. tetrapleura* populations). ALA records suggested that *E. tetrapleura* occurs in this area however as outlined below RBG&DT could not verify the presence of *E. tetrapleura* in the area.

The individuals from Summerland Way looked morphologically like *E. fibrosa* (2 individuals are represented in the genetic data). There were a small number of individuals at this site (<5) and the area was heavily disturbed (it was beside the road).

The individuals from the Old Tenterfield Rd looked like hybrids between *E. tetrapleura* and *E. fibrosa* (more like *E. fibrosa* on basis of morphology, with elongated caps on buds and ribs on bottom of fruits). We sampled 6 individuals here, there were probably fewer than 15 individuals.

Individuals from the unnamed track off the Old Tenterfield Rd looked more like *E. tetrapleura* with elongated caps (due to admixture from *E. fibrosa*). We saw more than 20 individuals here. Areas of this site had been cleared, so was disturbed.

Our analyses (PCoA, DAPC and LEA) suggest that these 3 populations (23 individuals) were hybrids between *E. tetrapleura* and *E. fibrosa* (either F2 or backcrossing to *E. fibrosa*). The NewHybrids analysis suggests that these were a mixture of *E. fibrosa* and hybrids (perhaps due to backcrossing with *E. fibrosa*).

### **Banyabba Area (Ban)**

#### ***E. siderophloia*, hybrids**

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This site is c. 40 km north of Grafton. RBG&DT sampled a population adjacent to the railway at Banyabba (we sampled from 6 individuals, there were probably no more than 10 present). This population also looked to have admixture from another species that we assumed was *E. siderophloia* (2 individuals resembling this species were also collected at this site). Our genetic analyses (PCoA, DAPC and LEA) suggested that the admixture detected in the individuals sampled as *E. tetrapleura* was actually from *E. fibrosa* and that these 6 individuals were genetically in the same group as the Off Old Tenterfield Rd-Old Tenterfield Rd-Summerland Way group). The NewHybrids analysis suggested that the 6 *E. tetrapleura* sampled at Banyabba were in fact a mixture of *E. fibrosa* and hybrids (possibly due to backcrossing with *E. fibrosa*).

All our genetic analyses (PCoA, DAPC, LEA and NewHybrids) have also shed light on what we sampled as *E. siderophloia* hybrids: 1 individual is *E. siderophloia*, and the other is genetically a hybrid between *E. tetrapleura* and *E. fibrosa*.

Though many individuals resembled *E. tetrapleura* somewhat in morphology the genetic data indicated that none of the samples that RBG&DT collected were from this species.

### **Trenayr Lane (Tre)**

#### **hybrids**

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This site was just to the north of Grafton (in the Junction Hill area, 10 km out of Grafton) and morphologically the ironbarks looked to have admixture from *E. fibrosa* (9 individuals are represented in the genetic analysis). The PCoA placed some individuals between *E. tetrapleura* and *E. siderophloia*. Other hybrid individuals were ambiguously placed between *E. tetrapleura*, *E. siderophloia* and *E. fibrosa*. The DAPC suggested that these are all hybrids between *E. tetrapleura* and *E. siderophloia*, while the LEA plots show admixture from *E. tetrapleura*, *E. siderophloia* and *E. fibrosa* (although most of the admixture were from *E. tetrapleura* and *E. siderophloia*). The NewHybrids analysis also supported that these were all hybrids.

This site included remnant trees that looked like *E. tetrapleura*, with a few seedlings next to farmland. There was some remnant bushland across the road from where RBG&DT sampled in the

road corridor (much of the bush was in private property where more ironbark trees could be seen but could not determine the species and did not collect).

### **Tindal Rd (Tin)**

#### **hybrids**

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This site was located 10 km to the north-west of Grafton. This population (6 individuals) was sampled as *E. tetrapleura* (on basis of morphology). There were fewer than 10 individuals at this site. The *E. tetrapleura* trees were all mature remnant trees next to farmland. Our genetic analyses (PCoA, DAPC and LEA) suggested that all individuals collected were hybrids (probably between *E. tetrapleura* and *E. siderophloia*). The NewHybrids analysis suggested that 5 individuals here are hybrids with one sample of *E. tetrapleura* (but note that even this sample had more than 10% genomic signals from other species suggesting it could have its origin from a hybrid backcrossing with *E. tetrapleura* as indicated by the other analysis).

### **Pine Brush State Forest (Pin)**

#### ***E. siderophloia*, *E. tetrapleura* and hybrids**

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This site is 30 km north-east of Grafton. Here, RBG&DT sampled 7 individuals of *E. tetrapleura* (based on morphology) and 4 individuals of *E. siderophloia* (also based on morphology). This population occurred in an area that had been recently logged. RBG&DT staff saw fewer than 10 individuals of *E. tetrapleura* here.

The genetic analyses (PCoA, DAPC and LEA) suggested that most of the samples identified as *E. tetrapleura* were *E. tetrapleura* (with one sample being a hybrid between *E. tetrapleura* and *E. siderophloia*). The NewHybrids analysis suggested that 3 individuals were hybrids and 3 were pure *E. tetrapleura*.

All the individuals sampled as *E. siderophloia* were identified with the genetic analyses as *E. siderophloia*.



## Southern-eastern populations: along Pacific Highway (close to/at where the RMS upgrade is occurring), Lanitza Rd and Newfoundland State Forest

### RMS Offset (RMS)

#### *E. fibrosa* and *E. tetrapleura*

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RBG&DT staff sampled 6 individuals from the lower (closer to the forest) and 6 individuals from the upper (closer to the road) areas of this property. This site is used for agricultural purposes and areas have been left for *E. tetrapleura* to revegetate and rehabilitate. The upper site consisted of younger *E. tetrapleura* trees (mostly less than 5 m tall), which we identified from fruits and buds to be *E. tetrapleura*. All of the individuals collected here were *E. tetrapleura* in all the genetic analyses (PCoA, DAPC, LEA and NewHybrids).

The lower site consisted of tall trees (>20 m), most of which we were able to confirm on the basis of morphology to be *E. tetrapleura* (i.e. fruits and buds were obtained from the trees); the remaining individuals were identified on the basis of fruits on the ground below the tree. Our genetic analyses (PCoA, DAPC and LEA) indicated that 4 individuals were *E. tetrapleura* and 2 individuals were *E. fibrosa*. Our NewHybrids and LEA analysis suggested that there were 3 *E. tetrapleura*, 1 hybrid and 2 *E. fibrosa*.

### Old Pacific Highway (Opa)

#### *E. fibrosa* and *E. tetrapleura*

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At this site along the Old Pacific Highway, RBG&DT found *E. tetrapleura* and *E. fibrosa* trees (identified on the basis of morphology). The fruits and buds suggested typical *E. tetrapleura* fruits and typical *E. fibrosa* fruits. Collections were made from both ironbark species. On the basis of morphology only 4 individuals could be identified as *E. tetrapleura* at this site (one of which could not be sampled due to its height). The PCoA, DAPC and LEA suggested that there were 3 *E. tetrapleura* and 3 *E. fibrosa*. Our NewHybrids analysis indicated that there were 2 *E. tetrapleura*, 1 hybrid and 3 *E. fibrosa*.

### Dinjerra Rd (Din)

#### *E. tetrapleura*\*

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This site was in a suburban area with remnant bushland next to houses. The site is close to and upland from a creek. A population of *E. tetrapleura* was identified here from which we sampled 6 individuals. These were the only 6 individuals we could confirm to be *E. tetrapleura*. \*Other ironbark

species were present but not sampled. We sampled only *E. tetrapleura* individuals that were morphologically typical of the species (on the basis of fruits). All our genetic analyses (PCoA, DAPC, LEA and NewHybrids) suggest these samples are *E. tetrapleura*.

### **Sampling along Franklins road:**

We sampled three sites along Franklins Road. This is a long stretch of road off the Pacific Highway, with extensive bushland and with many ironbark species present.

#### **Franklins Rd 1 (Fra 1)**

##### ***E. tetrapleura*\***

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At a site at the Pacific Highway itself (Fra 1) a small number of individuals of *E. tetrapleura* (we have 3 samples in our genetic analyses). We saw other ironbark species present, but did not sample from them. The area next to this had been cleared for the RMS road upgrade and so the site had experienced recent habitat disturbance. All of our genetic analyses (PCoA, DAPC, LEA and NewHybrids) suggest that the individuals sampled were *E. tetrapleura*.

#### **Franklins Rd 2 (Fra2)**

##### ***E. fibrosa***

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We found a population of *E. fibrosa* at Franklins Rd 2 (Fra 2) from which we sampled 6 individuals (all had fruits and buds typical of *E. fibrosa*). In all our genetic analyses (PCoA, DAPC, LEA and NewHybrids), these have been confirmed to be *E. fibrosa*.

#### **Franklins Rd 3 (Fra 3)**

##### ***E. tetrapleura* and hybrids**

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A third site further along the road (Fra 3) RBG&DT sampled 6 individuals of what could be identified as *E. tetrapleura* (fruits and buds were typical of the species). This was a more extensive population and there were probably more than 10 individuals. The PCoA, DAPC and LEA results suggest that 5 individuals are pure *E. tetrapleura*, while the DAPC indicates that 1 individual is a hybrid between *E. tetrapleura* and *E. siderophloia*. In our NewHybrids analysis, 3 individuals are assigned as pure *E. tetrapleura* and 3 are assigned as hybrids.

## Newfoundland State Forest (New)

### *E. siderophloia* and *E. tetrapleura*

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This site was well within the State Forest and away from the Pacific Highway (and was thus relatively undisturbed and in good condition). This area was more elevated than the sites around the Pacific Highway and was on a ridge. We found *E. tetrapleura* and *E. siderophloia* occurring here (identified on basis of morphology) and sampled both. We sampled 6 individuals of *E. tetrapleura*, which were found to be this species in all our genetic analyses (PCoA, DAPC, LEA and NewHybrids). Our genetic analyses suggest that all samples of the other ironbark species were *E. siderophloia* (5 individuals). The *E. tetrapleura* population was larger than others we had seen (at least 15 individuals, maybe 20).

## Bald Knob Road (Bal)

### *E. tetrapleura*

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This site was off the Pacific Highway and was less disturbed than some of the other sites along the Pacific Highway. RBG&DT sampled from trees that could be confirmed to be *E. tetrapleura* on the basis of morphology (the fruits were typical of the species). RBG&DT also sampled from younger trees with no fruits, across the road. The identity of these young trees were initially unknown but were later identified as *E. tetrapleura*. In total 10 individuals were sampled (this was a larger population). All the genetic analyses (PCoA, DAPC, LEA and NewHybrids) suggested that all individuals sampled from this site were *E. tetrapleura*.

## Pacific Highway (Pac)

### *E. tetrapleura*\*

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This site was close to but slightly north of Wells Crossing (where the type specimen of *E. tetrapleura* was collected). The population of trees was next to a construction site used for the upgrade (where there were RMS work stations and RBG&DT staff needed supervision from RMS to enter). RBG&DT found only 6 individuals at this site (all of which we sampled) that had fruits typical of *E. tetrapleura*. Other ironbark species were observed nearby, although we did not sample them. All our genetic analyses (PCoA, DAPC, LEA and NewHybrids) suggest the individuals sampled from this site were *E. tetrapleura*.

## Wells Crossing (Wel)

### *E. tetrapleura*\*

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This site was very close to where the type specimen for *E. tetrapleura* was found. We only found 3 individuals of *E. tetrapleura* here (which are represented in our genetic study). There were other ironbark species present. This site was very close to the Pacific Highway and was fenced off from the road (RBG&DT also needed supervision to enter this site). All the genetic analyses (PCoA, DAPC, LEA and NewHybrids) suggested that these individuals were *E. tetrapleura*, and according to our LEA plots there were very little admixture from other ironbark species.

## Lanitza Rd (Lan)

### *E. tetrapleura*\*

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This site was to the west of the Pacific Highway and was surrounded by farms. Many individuals were identified as *E. tetrapleura* in the road corridor (the fruits found were morphologically similar to those found at and near the Wells Crossing site). We sampled from 7 individuals. Other ironbark species were present, but we did not sample them. Most of the genetic analyses (PCoA, DAPC and LEA) suggested that the individuals sampled were *E. tetrapleura*. In the NewHybrids analysis, 5 individuals were assigned as *E. tetrapleura*, and 2 individuals as hybrids (with high levels of backcrossing with *E. tetrapleura*). While there were many properties, the site looked to be in relatively good condition and the road corridor extended a long way (i.e. we had to drive to get to our last *E. tetrapleura* samples).

## Kungala Rd (Kun)

### *E. fibrosa*, *E. siderophloia* and *E. tetrapleura* and hybrids

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We found 3 ironbark species along Kungala Rd (*E. tetrapleura*, *E. siderophloia* and *E. fibrosa*). There were many properties and we saw mostly adult trees in road corridors and very few seedlings. We sampled 5 individuals of what we identified as *E. tetrapleura* (on the basis of morphology). All our genetic analyses (PCoA, DAPC, LEA and NewHybrids) suggest that 1 of these individuals was *E. fibrosa*. The PCoA, DAPC and LEA indicate that the other 4 individuals were *E. tetrapleura*. However, the NewHybrids analysis suggests that only 2 are *E. tetrapleura* and the other 2 are hybrids (although one of these hybrids had high levels of backcrossing with *E. tetrapleura*).

We also sampled an individual which was identified on the basis of morphology to be *E. siderophloia*. All our genetic analyses (PCoA, DAPC, LEA and NewHybrids) suggest that this is *E. siderophloia*.

## Falconers Lane (Fal)

### *E. tetrapleura*\*

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This was a site where RMS were actively working on the upgrade and RBG&DT needed supervision to enter. Nine individuals (both tall trees and younger trees) in a very narrow road corridor were sampled. Many of these were identified as *E. tetrapleura* on the basis of morphology but the fruit morphology suggested some level of admixture from another species, possibly *E. fusiformis* and/or *E. siderophloia*. Although there were other ironbark species present, these were not sampled. All the genetic analyses (PCoA, DAPC, LEA and NewHybrids) suggested that these trees are *E. tetrapleura*. The LEA plots suggest that there is a small amount of admixture from *E. siderophloia*, which is relatively higher than that found at Wells Crossing, Pacific Highway, Bald Knob Rd and Newfoundland.

## Range Rd South (Ran)

### *E. tetrapleura*\* and hybrids

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This was another site where RMS was directly working on the upgrade and supervision was needed to enter. The individuals of *E. tetrapleura* were large old remnant trees with no understorey. RBG&DT sampled 7 individuals of *E. tetrapleura*. Other ironbark species were present across the road from the site. The morphology was similar to the Falconers Lane site where the fruits looked as though there could be admixture from another species. All the genetic analyses (PCoA, DAPC, LEA and NewHybrids) suggested 6 of the individuals are *E. tetrapleura* and 1 is a hybrid between *E. tetrapleura* and *E. siderophloia*. The LEA plots suggested that there was admixture from *E. siderophloia* and that this admixture was relatively higher than at Wells Crossing, Pacific Highway, Bald Knob Rd and Newfoundland.

## South-western populations

### Shannondale Rd (Sha)

### *E. siderophloia*, *E. tetrapleura* and hybrids

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This site was in a central position to the distribution of *E. tetrapleura*. The site was close to Shannondale Dam and was in relatively good condition. There were many trees and other ironbark species observed at this site. We sampled from 16 trees, most of which were identified as *E. tetrapleura* on the basis of fruits in trees (viewed through binoculars) and some were identified from fruits on the ground. The genetic analyses (PCoA, DAPC and LEA) suggest that 11 individuals were *E. tetrapleura*, while 2 were hybrids of *E. tetrapleura* and *E. siderophloia*. In the DAPC, 3 were found to be *E. siderophloia*. However, the NewHybrids analysis suggests that only 3 individuals are *E.*

*tetrapleura*, 2 are *E. siderophloia* and the remainder are hybrids. The LEA plots suggest that there is admixture from both *E. siderophloia* and *E. fibrosa* in all of these individuals, even the ones that are identified in the DAPC and PCoA as *E. tetrapleura*. The admixture in the LEA plots is much higher compared to the sites at Wells Crossing, Pacific Highway, Lanitza Rd, Bald Knob Rd and Newfoundland State Forest.

### **Kangaroo Creek Rd (Kan)**

#### ***E. tetrapleura***

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This population was close to Shannondale Rd. It was in a suburban area and the trees were along a road corridor. There was no understorey and we only saw mature trees. RBG&DT sampled from 6 trees, all identified as *E. tetrapleura* on the basis of fruits. Most of our genetic analyses (PCoA, DAPC and LEA) suggested these are all *E. tetrapleura*, although the LEA plots indicates that there is a small amount of admixture from *E. siderophloia* and *E. fibrosa* in all of the individuals. The NewHybrids analysis has assigned only 3 as pure *E. tetrapleura* and the remainder as hybrids.

### **Rockview Rd (Roc)**

#### ***E. fibrosa* and *E. tetrapleura***

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This site had farmland on both sides and was on a roadside corridor. There had been grazing and clearing in the understorey. RBG&DT saw many ironbark species, but could only identify a few *E. tetrapleura* trees on the basis of morphology (using binoculars to spot fruits and/or finding fruits on the ground under trees). RBG&DT took samples from 7 trees but 1 sample did not pass QC. The genetic analyses (PCoA, DAPC and LEA) suggest 1 sample is *E. fibrosa*. In the DAPC and PCoA, the remaining 5 were found to be *E. tetrapleura*, although the LEA plots suggest that there is a small amount of admixture in these individuals from *E. fibrosa* and *E. siderophloia*. The NewHybrids analysis suggests that 4 individuals are pure *E. tetrapleura* and 2 are hybrids.

### **Nymboi-Binderay National Park (Nym)**

#### ***E. fibrosa*, *E. siderophloia* and *E. tetrapleura***

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This site is isolated and was the southernmost sampled population of *E. tetrapleura* in this study. It is between the National Park and farmland. It was relatively undisturbed and in good condition. We found fruits of *E. tetrapleura* and sampled 15 individuals here. Our genetic analyses (PCoA, DAPC and LEA) suggest these are *E. tetrapleura*. The LEA plots suggest there is admixture from both *E. siderophloia* and *E. fibrosa*. The NewHybrids analysis assigns 14 to be *E. tetrapleura* and 1 as a hybrid with *E. fibrosa*.

## **Other *Eucalyptus fibrosa* populations**

### **Castlereagh Nature Reserve**

This site was in the Sydney region. It had fruits and buds typical of *E. fibrosa*. All our genetic analyses showed that it grouped with the *E. fibrosa* populations from around Grafton.

## **Other *Eucalyptus siderophloia* populations**

### **Yarramundi**

This population was found near a creek off the Grose River. We found fruits on the ground under the trees that were consistent with *E. siderophloia*. The genetic analyses grouped 5 individuals with the *E. siderophloia* populations from around Grafton. However, 1 individual was in an ambiguous position in the PCoA (between *E. siderophloia* and *E. fibrosa*) and the NewHybrids analysis suggested this to be a hybrid. In the DAPC, this individual grouped with *E. siderophloia*.

### **Whiporie**

This site was located near the northern *E. tetrapleura* (hybrid) sites, not far from Banyabba. It was listed in the NSW database as *E. fusiformis*. RBG&DT found remnant trees close to a creek. There were no fruits, although one tree had buds. Upon returning to the lab, the vouchers looked more like *E. siderophloia*. All the genetic analyses (PCoA, DAPC, LEA and NewHybrids) suggest that they are *E. siderophloia*.

### **Tamban**

This site is located south of Grafton and was listed in the Atlas of Living Australia as a site where *E. fusiformis* was found. It was upland and looked relatively dry. We found ironbark trees here with no fruits or buds. It did not look like typical *E. fusiformis*, although identifications can be very difficult without fertile material. Most of the genetic analyses (PCoA, DAPC and LEA) suggested that this population was *E. siderophloia*. The NewHybrids analysis suggested that all individuals are *E. siderophloia*, with the exception of 1 sample (which is a hybrid with *E. siderophloia* as one parent).

## ***Eucalyptus fusiformis* populations**

### **Bongil Bongil National Park**

This site was located in a gully on the edge of a rainforest. It was the wettest site visited, with tall ironbark trees spaced widely apart. We found fruits on the ground under the trees consistent with *E.*

*fusiformis*. The Splitstree analysis places this population on a branch at the base of a branch of the *E. siderophloia* populations. The PCoA places it next to (and very close to) *E. siderophloia*, while the DAPC suggests that it is not genetically differentiated from *E. siderophloia*. The LEA plots show that it grouped with *E. siderophloia*, but with some admixture from *E. tetrapleura*. The NewHybrids analysis assigned all individuals from this population to *E. siderophloia*.