BOTANIC GARDENS OF SYDNEY

Research Centre for Ecosystem Resilience

Conservation genomics of *Eucalyptus camaldulensis* in the Hunter in support of genetically informed seed sourcing

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Conservation genomics of *Eucalyptus camaldulensis* in the Hunter in support of genetically informed seed sourcing

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Executive summary

Eucalyptus camaldulensis is the most widespread eucalypt species in Australia and is an iconic part of many landscapes, however land use changes and clearing has had major negative impacts on many populations of the species. This includes the Hunter catchment that is listed as an endangered population and is the target of several conservation and restoration projects within the Hunter region. This study uses high throughput genomic data to assess the genetic relationships of the Hunter Catchment *E. camaldulensis* population to the species wider distribution, assess levels of genetic diversity and health of remnant stands in the region, and develop genetically informed seed sourcing strategies to maximise the success and self-sustainability of restored populations of the species in the region. It is shown that *E. camaldulensis* frequently hybridises with other Red Gum species where they co-occur both within and outside the Hunter catchment, and that all stands in the region are *E. camaldulensis* subsp. *camaldulensis*, with close genetic relationships to populations in the Darling River catchment, especially those in the Namoi catchment. Based upon the frequency of hybrids and levels of genetic diversity, it is recommended seeds are sourced from the largest stands of *E. camaldulensis*, and preferentially from stands along major waterways, rather than smaller tributaries, to minimise the collection of hybrid seed and maximise captured genetic diversity. Seedlings from seed collections by the Broke Bulga Landcare group are assessed and optimised supplementation plans are developed for both the Landcare managed restoration site and a local government managed remnant stand in Muswellbrook.

Acknowledgements

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 the collecting efforts of all collectors, including Katie Elsley (NSW DCCEEW), members of Broke Bulga Landcare, including Mark Lyons and Bruce Cowan and others not mentioned. We also thank Andrew Marsh and other staff from Trees in Newcastle for help with seed collection and for maintaining separate maternal lines in their nursery. Funding for outgroup Red Gum sampling and genotyping was provided by Transport for New South Wales. We also thank any landowners who allowed collecting for this study to take place.

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BOTANIC GARDENS OF SYDNEY

2025

Guidelines for sourcing genetically diverse climate-ready seed for restoration of River Red Gum within the Hunter catchment

Introduction

These guidelines summarise how to optimise the genetic diversity and climate-readiness of seed used in restoration of River Red Gum (*Eucalyptus camaldulensis*) within the Hunter River catchment.

They are based on a study by the Research Centre for Ecosystem Resilience (ReCER) (Fahey et al. 2024). Seed sourcing guidance for other areas of NSW can be found via the Restore and Renew webtool at restore-and-renew.org.au

Why is genetic diversity important?

Genetic information can help ensure seed are sourced from appropriate locations and are sufficiently genetically diverse. Seed sourced from inappropriate populations, or with too little genetic diversity, may result in restored populations with low reproductive fitness and an inability to adapt to environmental change. In other words, lots of trees may go in the ground, but they may fail to reproduce, or they may be less likely to cope with diseases or changing climatic conditions.

Read more about why avoiding low genetic diversity and inbreeding is especially important for eucalypts

Key findings

- All Hunter populations are *Eucalyptus camaldulensis* subsp. *camaldulensis* and belong to the same genetic neighbourhood as the Murray-Darling Basin populations, with close genetic relationships to populations in the Darling River catchment to the north, especially those in the Namoi, Castlereagh, Gwydir and Macintyre catchments.

- Hybridisation with other Red Gum species is common across NSW, including in the Hunter where hybridisation occurs with *E. blakelyi, E. amplifolia,* and *E. tereticornis*.

- Hybridisation and inbreeding are more apparent in small and isolated populations at the upper reaches of tributaries, and in planted populations with low genetic diversity.

- *Eucalyptus camaldulensis* along Wollombi Brook have genetic material originating from *E. amplifolia* in their genomes, making them more genetically divergent than would otherwise be expected. This process, termed introgression, occurred following a historical hybridisation where the hybrid individuals mated back with *E. camaldulensis* in subsequent generations. Given these introgressed individuals are mature and the hybridisation event happened many generations ago, it can be assumed this was a natural process.

- Future climate projections reveal the Hunter catchment will experience average annual climatic conditions within the range currently experienced in the region, due to the strong east-west rainfall gradient. This means seed sourced from within the Hunter catchment should result in populations adapted to future climate conditions.

- Current climate conditions in the closely related Namoi, Gwydir and Macintyre catchments, also match the future climate of the Hunter Catchment.

General collection guidelines

The following guidance is recommended for restoration of *Eucalyptus camaldulensis* within the Hunter River catchment. Given *Eucalyptus camaldulensis* is listed as an endangered population within the Hunter, seed collection and propagation require a Scientific Licence under the *Biodiversity Conservation Act (2016*).

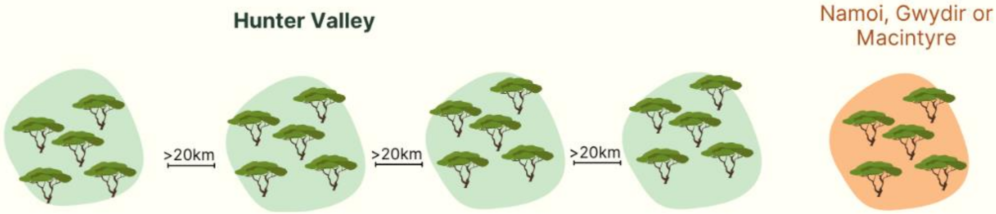
- Source seed from multiple trees across multiple sites.
- Collect from large natural stands along major waterways including Hunter River, Goulburn River and Dart Brook, rather than smaller tributaries.
- Avoid collecting from isolated trees, planted trees and from trees growing near other red gum species, including *E. amplifolia, E. blakelyi* and *E. tereticornis*.
- Undertake quality control checks of seed and seedling morphology to help identify hybrids before planting.
- Do not use seed from Wollombi Brook catchment elsewhere. Seed from elsewhere can be used within Wollombi Brook catchment.
- Supplement Hunter Valley sourced seed with climate-ready material from Namoi, Castlereagh, Gwydir and Macintyre catchments.
- Maintain maternal lines in seed collection and during propagation (van der Merwe et al. 2023).
- Mix maternal lines and provenances at planting to maximise outcrossing.

Optimising collections to maximise genetic diversity

Collecting seed from five unrelated maternal trees from four different stands located at least 20km apart should capture >90% of genetic diversity present in the Hunter catchment (see figure below).

To potentially increase climate resilience and further increase diversity, collect seed from an additional five unrelated maternal trees from the Namoi, Gwydir or Macintyre catchments (all five trees can be located within the same patch).

Maternal lines used should vary between restoration plantings, to avoid homogenizing and reducing the diversity of E. camaldulensis families present in the Hunter Catchment. For example, restoration plantings could use seed from the same four Hunter catchment stands but would ideally use seed from different mother trees.



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Fahey P, Hogbin P, van der Merwe M, Rossetto M (2024) Conservation genomics of *Eucalyptus camaldulensis* in the Hunter Catchment in support of genetically informed seed sourcing. Research Centre for Ecosystem Resilience. A report to the NSW Saving our Species Program, Department of Climate Change, Energy, the Environment and Water.

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More information

For a copy of the full report (Fahey et al. 2024), or advice on accessing genetically diverse *Eucalyptus camaldulensis* seed, please contact the NSW Saving our Species Program via Katie.Elsley@environment.nsw.gov.au

Key terms

Adaptability/Adaptive potential: The ability of a population to survive and reproduce under changing environmental conditions.

Allele: Alternate versions of DNA sequence at a given location within the genome.

Allelic diversity: The number of alleles present within a population of a species. One measure of genetic diversity (see below).

Climate-ready: Provenances that come from areas where current climatic conditions match those predicted to occur in a target area at a future point under a particular model of climatic change.

Ecological restoration: Assisting ecosystems that have been disturbed or cleared to restore ecological function.

Gene flow: The movement of genetic material such as differing alleles (see above) between individuals or a group of individuals by processes such as dispersal of seed and pollen.

Genetic bottleneck: A sharp reduction in genetic diversity caused by a reduction in population size or by sampling of seed from too few individuals.

Genetic distinctiveness: How different the genetic diversity of a population is compared to the genetic diversity in other populations of the same species.

Genetic diversity: The totality of genetic variation present in a population and a determinant of adaptability and adaptive potential.

Genetic lineage: a set of individuals or populations connected by a continuous line of descent from a shared ancestor.

Genetic neighbourhood: An area within the distribution of a species where there are no restrictions to gene flow other than the distance between individuals, such that alleles can be shared between all individuals within the area given sufficient time.

Genetic structure: The amount and distribution of genetic variation within and between populations across the landscape.

Genetic swamping: Loss of rare alleles as a result of repeated crossing with individuals from a different genetic neighbourhood of the same species or a different species via hybridisation.

Heterozygosity: The average proportion of DNA sites in the genome where the two copies (or alleles) are different across a sample of individuals. Heterozygous refers to having two different copies of DNA at a particular location within the genome, as opposed to homozygous (see below) where the two copies at a location are the same.

Homozygous: Having two identical copies of DNA at a particular location within the genome, as opposed to heterozygous where the two copies at a location are different. See above.

Inbreeding: The mating of individuals that are genetically closely related within a population leading to the production of progeny.

Inbreeding depression: a decrease in fitness of progeny from closely related parents due to an increase in homozygosity with the genome.

Isolation by distance: The term used to describe the change of shared genetic material across geographic space due to dispersal ability limiting the mating between individuals. Importantly this often does not lead to discrete population structure and local adaptation if populations are continuous across the landscape.

Maternal line: All offspring from the same mother plant.

Maintenance of maternal lines: The practice of keeping seed or other material collected from a particular mother plant identifiable and separate from that of other mother plants through the process of collection through to propagation and planting. Upon planting, maternal lines should be traceable to individuals, but should be interplanted to maximise interbreeding and mixing of genetic diversity in progeny.

Mother plant: An individual plant from which seed has been collected.

Outbreeding: The mating of individuals that are not genetically closely related leading to the production of progeny.

Outbreeding depression: A reduction of fitness of offspring resulting from the interbreeding of parents that are highly genetically distinct.

Population: A group of individual plants growing in the same place at the same time and interbreeding freely.

Private alleles: Alleles observed only in a single population, thus being private to that population.

Progeny: Offspring of a plant, typically seed.

Provenance: A source area for propagules (seed, cuttings etc.) used in restoration activities.

Seed Production Area: Areas where native plants of known provenance are planted to harvest seed for restoration or other purposes.

Site: a specific locality where genetic samples or seeds have been collected.

Sustainability/Self-sustaining: The ability of a planting to survive and reproduce in the long-term with minimal further active investment.

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1. Introduction

1.1 Background

Eucalyptus camaldulensis, the River Red Gum, is a widespread and iconic tree species native to all mainland states and territories of Australia. The species is a riparian zone specialist that primarily grows along waterways in the woodland and arid regions and is tolerant to a wide range of climatic and environmental conditions (McDonald et al. 2009). While the large distribution of the species means it is not at significant threat of extinction, the population in the Hunter River Catchment has been heavily impacted by land clearing and land use changes, and now numbers ~1000 mature individuals (Hughes 2021). Combined with the unique nature of the population being the only population along a river system that flows out to the east coast in NSW, this has led to the Hunter catchment *E. camaldulensis* population being listed as an endangered population under the Biodiversity Conservation Act 2016 (NSW) (Hughes 2021).

There is significant community and governmental interest in restoring *E. camaldulensis* vegetation along the Hunter and its tributaries, but the genetic health and uniqueness of the Hunter stands is unknown. Understanding these genetic factors is key to ensuring the restoration of the species in the region is successful through the establishment of self-sustaining populations. Another outstanding question is around which subspecies of *E. camaldulensis* grow in the Hunter, as it has been suggested both *E. camaldulensis* subsp. *camaldulensis*, which is widespread in the Murray-Darling Basin, and *E. camaldulensis* subsp. *acuta*, which is primarily distributed in Queensland with a small area of occurrence between Baradine and Moree in central north NSW (McDonald et al. 2009, Brooker et al. 2015), naturally occur in the Hunter Catchment. The third subspecies native to NSW, E. camaldulensis subsp. arida is only known from intermittent waterways in the far west of the state (McDonald et al. 2009, Brooker et al. 2015) and therefore is not relevant to any restoration activities in the Hunter.

It is important to identify which subspecies are present in the Hunter and their relative abundance so that this can be reflected in restoration plantings. Given the small number of remaining individuals of *Eucalyptus camaldulensis* in the Hunter it is also important to understand their relationship to the wider distribution of the species to determine if genetic rescue or the introduction of climate change ready provenances can be undertaken without impacting the uniqueness of the population. Furthermore, as is the case for many *Eucalyptus* species (Larcombe et al. 2015), there is a high likelihood that E. camaldulensis hybridises with other Red Gum species, so determining how widespread and common hybridisation is can inform how to mitigate its impacts on restoration plantings.

In addition to understanding the genetic relationships, understanding the genetic health of remnant populations in the Hunter is important to determine seed sourcing strategies and if genetic rescue is needed. If these small stands are experiencing high levels of kinship and inbreeding, and corresponding losses of genetic diversity, it may be inappropriate to source seed for restoration plantings from them. This is due to the remarkable negative impacts on individual survival and fitness (i.e. inbreeding depression) exhibited by Eucalyptus species when self-fertilisation or breeding of close relatives occurs (Potts and Wiltshire 1997, Costa e Silva et al. 2011, Breed et al. 2014).

1.2 Study objectives

To facilitate the recovery of *Eucalyptus camaldulensis* in the Hunter Valley via restoration plantings, genomic data was used to address the following:

1. Investigate the extent of hybridisation of *E. camaldulensis* with other Red Gum species across NSW and the Hunter, and how this may impact seed sourcing strategies.
2. Determine which subspecies of *Eucalyptus camaldulensis* are present in the Hunter Valley.
3. Assess the genetic distinctiveness of the Hunter Valley *E. camaldulensis* stands in comparison to other populations in NSW to allow for informed movement of seed in and out of the region.
4. Assess the genetic health of remnant stands of *E. camaldulensis* in the Hunter to determine population trajectories and suitability as seed sources for restoration plantings.
5. Test pilot seed collections from Wollombi Brook to determine the relationship between stand genetic health and quality of seed collected.
6. Guide the design of an optimised restoration seed collection strategy for the Hunter region.

Understanding population dynamics and patterns of genetic relatedness within and outside the Hunter will guide effective conservation and restoration efforts of this iconic tree species. By addressing the above-mentioned objectives, the report aims to provide a solid basis for the endangered populations' long-term management and conservation.

2. Methods

2.1 Sampling

The sampling of *Eucalyptus camaldulensis* at sites across NSW was conducted by several collectors (refer to the acknowledgements) (Figure 1, Table 1). In addition, ReCER had genetic data for several other Red Gum taxa that are native to NSW and the Hunter, and this data was used as outgroup sampling to test for hybridisation between *E. camaldulensis* and other closely related and co-occurring species.

2.2 DNA extraction and sequencing

Samples were sent to Diversity Arrays Technology (DArT) Pty Ltd in Canberra for DNA extraction and genotype-by-sequencing analysis, referred to as DArTseq analysis, following their documented in-house procedure. DNA was extracted from each sample using the Plant DNA Extraction Protocol for DArT.

2.3 Data analysis

All data analysis was performed using R v4.2 unless otherwise specified (R Development Core Team 2016)

2.3.1 Quality filtering

To ensure the use of high-quality DArTseq markers only, we applied quality checks to single nucleotide polymorphism (SNP) data using filtering scripts implemented by the in-house RRtools v1.0 package (as described by Rossetto *et al.* (2019)). Loci with a reproducibility of less than 96% or missing data exceeding 20% across all samples were excluded from the dataset. Additionally, one SNP per sequencing locus was randomly chosen for inclusion in the dataset for use in analyses as is standard practice in population genetics. Finally, samples with data missing for over 50% loci were excluded from the dataset. Two datasets were then created for separate analyses, one containing all outgroup samples in addition to *E. camaldulensis* and one containing only samples identified as *E. camaldulensis* using genetic data. All putative hybrid samples were excluded from the second dataset.

2.3.2 Analyses with outgroups

Principle components analysis (PCA) (using the glPca function from the *adegenet* R package (Jombart and Ahmed 2011)), admixture analysis (using the stand-alone program *STRUCTURE* v2.3 (Pritchard *et al.* 2000)) and phylogenetic network calculation (using the stand-alone program *SplitsTree* v4.17.2 (Huson and Bryant 2006)) were applied to the dataset containing outgroups to confirm samples were correctly identified and test for hybridisation with other Red Gum species. These analyses allow for the relationships between individual samples to be investigated, and therefore any samples that do not cluster with the other *E. camaldulensis* samples can be identified and, depending on whether they cluster with samples of another species or in an intermediate position, be assigned as either misidentifications or hybrid samples.

2.3.3 Analyses without outgroups

Further PCA and phylogenetic networks were generated for the dataset containing only the *E. camaldulensis* samples. This allowed for testing of the genetic distinctiveness and geographic extent of the three subspecies that occur in NSW. Further to this population divergence of individual sampling locations was also investigated using pairwise fixation index (F_{st}) values (calculated using the ReCER developed *RRtools* R package), which measure the level of genetic differentiation between two populations and can be used to look for gene flow between populations. This last measure was mainly used to test how much and how recent geneflow between the Hunter and Murray-Darling Basin may have occurred, as well as how much gene flow occurs between stands within the Hunter Catchment. As this is calculated from the genetic data from mature individuals, it measures gene flow patterns at the time when the seed, which became the mature trees, was produced rather than current processes. Given many remnant trees likely pre-date widespread clearing of the population, these values serve as way to investigate natural process that may have been interrupted by recent human activity in the catchment.

2.3.4 Population diversity analyses

For all sampled sites with at least five *E. camaldulensis* subsp. *camaldulensis* samples in the final dataset, population diversity measures were calculated using the *diveRsity* package (Keenan *et al.* 2013) (Table 2). This allows for comparisons of the genetic diversity of different populations across the subspecies distribution, and comparisons of how the diversity of the Hunter Catchment stands compares to diversity across the subspecies as a whole. Genetic diversity is highly dependent on species biology and evolutionary history, and therefore objective measures of what is a healthy level are hard to determine, however by comparing the genetic diversity of the Hunter stands to others outside the region, we can assess whether they are comparatively genetically unhealthy compared to what would be expected. To test for close relationships between individual trees in the Hunter stands, which may indicate that there is limited gene-flow occurring between stands, kinship values were calculated using the PLINK method in R. While a concern for other species with small numbers of isolated individuals, clonality is rare in *Eucalyptus* species with a tree growth habit such as *E. camaldulensis*, however this would be observed in the kinship values if it was occurring.

2.3.5 Assessment of Broke Bulga Landcare seed collections

Seed were collected from 10 mother trees at four sites along Wollombi Brook (Table 3) by the Broke Bulga Landcare group for use in their planting activities. This seed was germinated by Trees in Newcastle at their nursery facility. Leaf samples were collected from the mother trees and from the seedlings before they were planted at the Landcare restoration sites. Maternal lines (i.e., seeds and resultant seedlings coming from a single mother tree) were tracked through this whole process to allow for informed management of the restoration site. This process was used as a pilot for the practical implementations of genetically informed restoration of River Red Gums in the Hunter, with analysis of genotyped seedlings helping to inform the development of guidelines in this report.

Of particular interest was the level of hybrid seed, genetic diversity of the seedlings and the level of relatedness between them, termed kinship. To examine levels of kinship and diversity, PLINK kinship values were calculated using the *RRtools* R package along with standard genetic diversity parameters per methods used for the sampled adult populations.

2.3.6 Optimisation of seed sourcing strategies

One of the objectives of this project was to determine what collection effort is needed to ensure a target level of genetic diversity is represented in seed collections and restored populations of *E. camaldulensis* in the Hunter catchment. The genetic diversity goal ReCER applies in such scenarios is capturing 90% of common alleles (minor allele frequency > 0.03) in the dataset across a defined geographic region where seed is to be sourced. The level of sampling needed to achieve this is defined as the number of sites from which seed from 5 mother trees needs to be collected to guarantee this threshold is met when resampling of the genetic data is undertaken. In the case of the Hunter catchment, two geographic regions are defined across which this methodology was applied:

1. The remnant stands in the Hunter catchment.
2. The Hunter, Namoi, Gwydir and Macintyre catchments.

In addition to this generalised goal of determining minimum sampling efforts, we utilised the *optsitemix* analysis pipeline (described by Dimon *et al.* (2024)), which is an optimisation analysis to find the most complementary set of genotyped sites given a target number of sites to be collected from. This pipeline was used to determine the set of stands in the Hunter catchment which maximise the genetic diversity captured in seed collections. Furthermore, an additional *optsitemix* analysis was employed to determine the most complementary set of seed sources in the Hunter for the Muswellbrook stand, a target of upcoming restoration activities. In this case the genotyped individuals from this site are forced to be part of the optimised solution, thereby identifying a set of stands that maximise genetic diversity in the resultant supplemented population. This process was also repeated for the Broke restoration site forcing the seedlings already planted by the Broke Bulga Landcare group into the optimised selection.

2.4 Identification of climate-ready provenances

To determine where climate change ready seed can be sourced to supplement existing *E. camaldulensis* stands in the Hunter to help confer resilience in the face of climate change, a regional climate matching approach was taken. Firstly, all know stands of *E. camaldulensis* in the Hunter Valley were located from survey records and 10 km buffers were created around each in QGIS. These buffered areas were then merged to create a single region that represents the climatic envelope *E. camaldulensis* occupies in the Hunter. This region was saved as a shapefile and used in all further analyses undertaken in *R*. The mean annual rainfall and temperature ranges for this region were identified using the *CHELSA* v2.1 (Karger *et al.* 2017, Dirk Nikolaus *et al.* 2021) 1981-2010 climate data. Areas with both mean annual rainfall and temperature currently within this range across eastern Australia were then identified to represent areas which match the climate *E. camaldulensis* stands in the Hunter grow under.

All five models of future climate for the years 2071-2100 available through *CHELSA* v2.1 (Karger *et al.* 2017, Dirk Nikolaus *et al.* 2021) were used to identify climate change ready provenances. The minimum and maximum predicted future mean annual rainfall and temperature value across all five projections within the *E. camaldulensis* Hunter occupancy region was identified. Areas where the current climate conditions were within the range between these minimum and maximum projected values for both rainfall and temperature were then identified and a map of future climate matched areas for the Hunter produced.

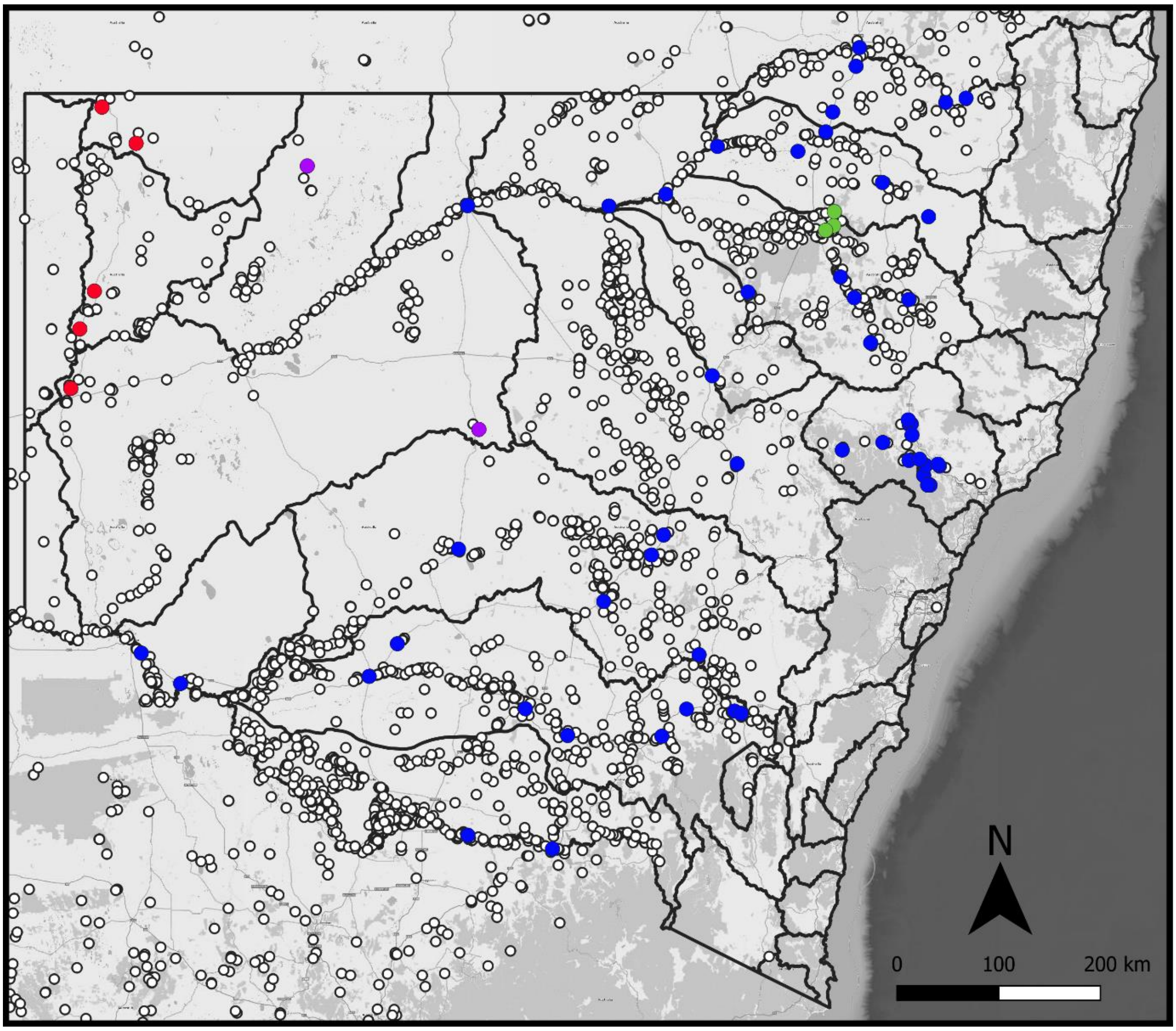

Figure 1: Map showing the sample collection locations of *E. camaldulensis* from across New South Wales genotyped in this study. Points are coloured to correspond to the subspecies they were identified as using genetic data: blue points are *E. camaldulensis* subsp. *camaldulensis*, green points are subsp. acuta, red points are subsp. *arida*, and purple points are populations genetically intermediate between subsp. camaldulensis and subsp. *arida*. Not shown is a single sampling site of *E. camaldulensis* subsp. *arida* from Oonartra Creek in South Australia from which samples were also genotyped. Also plotted as white points are the records of E. camaldulensis occurrences downloaded from the Atlas of Living Australia (2024) and the boundaries of the major river catchments in NSW.

Table 1: Summary of stands of E. *camaldulensis* sampled and genotyped in the Hunter Catchment. Stands are number to correspond to Figure 2, and the number of samples genotyped from each stand is listed along with the genetic identity of the genotyped individuals.

Stand number	Stand number	Number of samples genotyped
Wollombi Brook at Broke	1	1
Wollombi Brook north-west of Broke	2	3 – 1 *Eucalyptus camaldulensis subsp. camaldulensis*, 2 *Eucalyptus camaldulensis* x *amplifolia* hybrids
Planted Stand on Wollombi Brook west of Broke	3	5 – 4 planted *Eucalyptus camaldulensis subsp. camaldulensis*, 1 remnant *Eucalyptus camaldulensis* x *amplifolia* hybrid
Wollombi Brook at Bulga	4	10 – all *Eucalyptus camaldulensis* subsp. *camaldulensis* with *E. amplifolia* introgression.
Hunter River at Clydesdale Reserve, Singleton	5	6 – all *Eucalyptus camaldulensis subsp. camaldulensis*
Hunter River at Col Fisher Park, Singleton	6	6– all *Eucalyptus camaldulensis subsp. camaldulensis*
Hunter Valley Operations, Wollombi Brook Site	7	6– all *Eucalyptus camaldulensis subsp. camaldulensis*
Hunter Valley Operations, Billabong Site	8	6– all *Eucalyptus camaldulensis subsp. camaldulensis*
Apple Tree Creek at Apple Tree Flat	9	7 – 3 *Eucalyptus camaldulensis subsp. camaldulensis*, 4 *Eucalyptus camaldulensis subsp. camaldulensis* x *E. blakelyi-tereticornis* intergrade hybrids
Muscle Creek at Muswellbrook	10	5 – all *Eucalyptus camaldulensis subsp. camaldulensis*
Hunter River at Aberdeen	11	5 – all *Eucalyptus camaldulensis subsp. camaldulensis*
Natural stand, Dart Brook west of Aberdeen	12	6 – 5 *Eucalyptus camaldulensis*, 1 *Eucalyptus camaldulensis* x *E. blakelyi* hybrid
Planted stand, Dart Brook west of Aberdeen	13	6– 5 *Eucalyptus camaldulensis*, 1 *Eucalyptus camaldulensis* x *E. blakelyi* hybrid
Dart Brook on property Yaragin	14	18 – 6 mature remnant trees, 6 individuals recruited following flooding in 1945, 6 individuals recruited following flooding in 2007. All *Eucalyptus camaldulensis subsp. camaldulensis.*
Goulburn River at Sandy Hollow	15	6– all *Eucalyptus camaldulensis subsp. camaldulensis*
Bylong River at Bylong	16	6 – 4 *Eucalyptus camaldulensis*, 2 *Eucalyptus camaldulensis* x *E. blakelyi* hybrids

Table 2: Genetic diversity statistics for all Hunter Catchment stands with at least 5 Eucalyptus camaldulensis subsp. camaldulensis individuals successfully genotyped. For sites with more than 5 samples, 5 samples were randomly chosen for inclusion when calculating parameters to eliminate biases due to sampling effort. Cell colouration is proportional to its genetic diversity, with greener colours being more diverse and red colours being less diverse.

Stand number	Allelic Richness	Observed Heterozygosity	Expected Heterozygosity
4	1.51	0.19	0.219
5	1.518	0.196	0.22
6	1.519	0.195	0.223
7	1.55	0.204	0.232
8	1.583	0.206	0.244
10	1.516	0.196	0.221
11	1.563	0.196	0.242
12	1.579	0.202	0.246
13 – planted stand	1.49	0.18	0.214
14 – 1945 recruits	1.579	0.199	0.246
14 – 2007 recruits	1.566	0.193	0.242
14 – remnant individuals	1.571	0.197	0.242
15	1.511	0.2	0.219

Table 3: Table 3: Genetic diversity statistics for all maternal lines collected by Broke Bulga Landcare and used in their restoration planting. Note sample numbers have not been standardised, however the higher level of sampling in this dataset makes this unnecessary. Cell colouration is proportional to its genetic diversity, with greener colours being more diverse and red colours being less diverse. Note the average kinship between seedlings from maternal lines at stands 1, 2 and 3 were all much higher than observed for seedlings from maternal lines collected from the larger stand 4.

Stand number	Mother tree	Number of seedlings genotyped	Inbred seedlings	Hybrid seedlings	Allelic Richness	Observed Heterozygosity	Expected Heterozygosity	Average Kinship
1	NSW1151629	20	14	0	1.269	0.142	0.149	0.236
2	NSW1151630	19	6	1	1.315	0.16	0.168	0.198
3	NSW1151619	16	10	0	1.308	0.164	0.168	0.210
4	NSW1151675	19	8	0	1.395	0.188	0.198	0.127
4	NSW1151656	20	9	0	1.393	0.177	0.203	0.092
4	NSW1151646	20	3	0	1.447	0.204	0.225	0.059
4	NSW1151653	20	0	0	1.484	0.219	0.239	0.040
4	NSW1151673	20	2	1?	1.48	0.212	0.24	0.037
4	NSW1151690	10	1	0	1.437	0.205	0.219	0.052
4	NSW1151618	20	2	0	1.47	0.202	0.236	0.031

Table 4: Summary of seed collecting effort needed to reach threshold of 90% of common alleles represented in collections when collecting 5 E. camaldulensis subspecies camaldulensis maternal lines form each site. Optimised site combinations are only provided for stands in Hunter Catchment, as the maternal lines sourced from outside the catchment used for different plantings should be varied to avoid homogenizing the families present in stands across the Hunter Catchment.

Scenario	Number of sites from which seed is required	Total number of maternal lines	Optimised site combination
Within Hunter Valley	4 sites	20 maternal lines	N/A
Supplementation with provenances from Namoi, Gwydir and Macintyre catchments	5 sites- 4 within Hunter Catchment, 1 from outside Hunter Catchment	25 maternal lines	N/A
Supplementation with 50% climate change ready provenances from Namoi, Gwydir and Macintyre catchments	8 sites- 4 within Hunter Catchment, 4 from outside Hunter Catchment	40 maternal lines	N/A
Supplementation of Musswelbrook stand	4 sites- 3 within Hunter Catchment, 1 from outside Hunter Catchment	20 maternal lines	Stand 6 – Col Fisher Park Singleton, stand 11 – Hunter River at Aberdeen, stand 12 – Dart Brook west of Aberdeen, plus site from Macintyre River catchment
Supplementation of Broke Bulga Landcare planting	4 sites- 3 within Hunter Catchment, 1 from outside Hunter Catchment	20 maternal lines	Stand 6 – Col Fisher Park Singleton, stand 11 – Hunter River at Aberdeen, stand 12 – Dart Brook west of Aberdeen, plus site from Macintyre River catchment

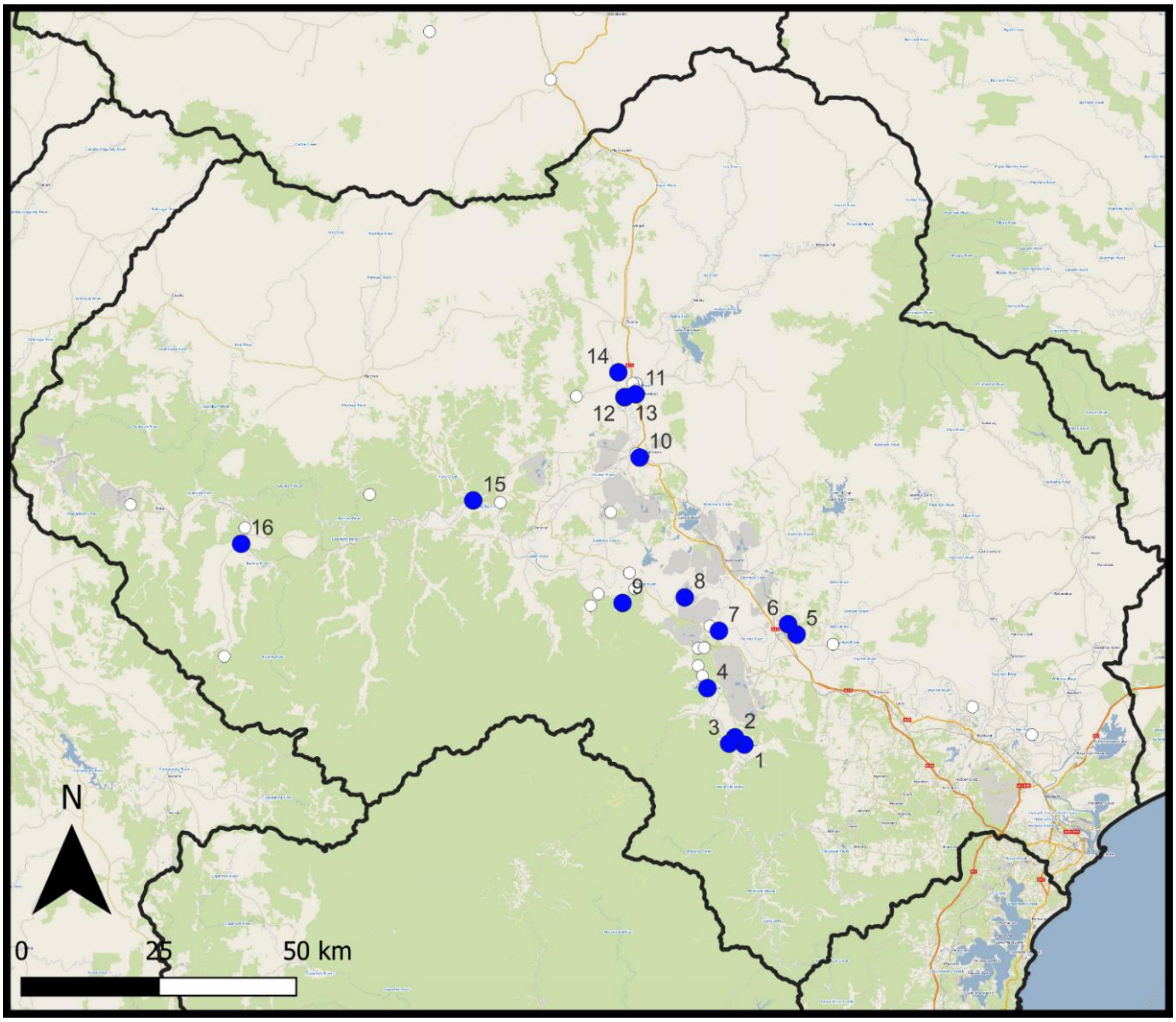

Figure 2: Map of *E. camaldulensis* subsp. *camaldulensis* stands, including two planted stands (3 and 13) in the Hunter Catchment genotyped for this study (blue points), with site numbering corresponding to that used in Table 1. Also plotted as white points are the records of E. camaldulensis occurrences downloaded from the Atlas of Living Australia (2024) and the boundaries of the major river catchments in NSW.

3. Results and discussion

High quality SNP data generated for *E. camaldulensis* and related Red Gum species were analysed and we report the results here:

- Hybridisation with other Red Gum species is common across NSW, including in the Hunter, where both historic introgression and recent hybridisation with *E. amplifolai* was recorded along Wollombi Brook.
- Only one subspecies of *E. camaldulensis*, subspecies *camaldulensis,* naturally occurs in the Hunter.
- Stands previously suggested to be *E. camaldulensis* subsp. *acuta* within the Hunter were found to include *E. camaldulensis* subsp. *camaldulensis* x *E. tereticornis* hybrids.
- The Hunter *E. camaldulensis* population is closely related to stands across the Darling River catchment, with seed movement from the Namoi River Catchment the likely route of colonisation of the Hunter for this species.
- The genetic diversity of mature remnant stands in the Hunter largely reflects processes prior to widespread land clearing, and thus standing diversity is high.
- The small number of mature trees and narrow geographic spread at many remnant stands has led to elevated kinship between individuals in these populations. This does not affect the health of the standing trees but may impact recruitment and hybridisation rates.
- The smaller stands of *E. camaldulensis* in the Hunter should be avoided as sources for seed for restoration plantings, with seed from larger remaining stands along the main rivers likely to be more genetic ally diverse and include less hybrids than seed from smaller stands on tributaries of the Hunter River.

- Seed from catchments from the north-west of the Hunter including the Namoi, Gwydir and Macintyre catchments can be used to increase the genetic diversity and adaptability in the face of climate change of the restored and supplemented stands of *E. camaldulensis* subsp. *camaldulensis* in the Hunter without impacting natural genetic patterns or increased risk of outbreeding depressions.
- As all mature trees along Wollombi Brook are descendants of an *E. amplifolia* x *E. camaldulensis* hybrid, seed from this waterway should only be used along the same waterway and not in the broader Hunter region.
- A combination of 5 maternal lines from 4 stands (20 total maternal lines) within the Hunter catchment guarantee >90% of common alleles would be captured in a seed collection. This increased to 5 maternal lines from 5 stands (25 total maternal lines) to capture the same proportion across the Hunter, Namoi, Gwydir and Macintyre catchments.

3.1 Genetic findings on *Eucalyptus camaldulensis* across NSW

Hybrids between *E. camaldulensis* subsp. *camaldulensis* and several other Red Gum species were observed in the genetic data, with these hybrids more commonly being identified as *E. camaldulensis* in the field rather than the other hybridising parent (Figure 3). The most frequently observed hybrid combination was *E. camaldulensis* subsp. *camaldulensis* x *E. blakelyi*, which is expected as these two taxa are co-distributed across the inland tablelands and slopes of NSW. Hybrids with *E. tereticornis* were also observed in the Hunter and at the QLD border where this species' distribution overlaps with that of *E. camaldulensis*. Likewise, in the only location where their distributions overlap, the lower Hunter catchment, *E. camaldulensis* subsp. *camaldulensis* x *E. amplifolia* hybrids were also recorded (Figure 4). Further specific discussion of hybridisation in the Hunter Valley is presented in the following section dealing with that region.

Overall, the observed frequency of hybridisation highlights how care needs to be taken when selecting sites from which to source *E. camaldulensis* subsp. *camaldulensis* seed, as seed from sites where *E. camaldulensis* and any other red gum species occur in proximity is likely to include a proportion of hybrids. Such hybrid seed should be avoided when restoring the Hunter catchment *E. camaldulensis* population to ensure restored stands are true *E. camaldulensis* and avoid the dilution of an already narrowed local genetic pool. Seed morphology checks provide additional control against the use of hybrids, as the seed of *E. camaldulensis* is smooth and brown to yellow in colour while the seed of all the other discussed species is black with toothed edges.

We find strong genetic support for the three subspecies of *Eucalyptus camaldulensis* known to occur in NSW as being distinct taxa (Figure 5 and 6), namely *E. camaldulensis* subsp. *camaldulensis* from permanent waterways in the Murray-Darling Basin, *E. camaldulensis* subsp. *arida* from irregularly flowing waterways in the arid north-west corner of the state and throughout central and western Australia, and *E. camaldulensis* subsp. *acuta,* known only from a small area between Baradine and Moree in north-east NSW, but otherwise widespread in Queensland. While it has previously been suggested that *E. camaldulensis* subsp. *acuta* may be present in the Hunter, we find no evidence to support this hypothesis and conclude only *E. camaldulensis* subsp. *camaldulensis* is native to this catchment. As discussed in the following section, these putative *E. camaldulensis* subsp. *acuta* stands in the Hunter are shown to include a high proportion of *E. camaldulensis* subsp. *camaldulensis* x *E. tereticornis* hybrids (Figure 4), explaining their unusual morphology.

We find that genetic diversity is high across *E. camaldulensis* subsp. *camaldulensis* distribution in NSW, with low genetic structuring (i.e., low genetic differentiation between populations) (Figure 6). There was evidence for limitations to natural geneflow between the Darling River catchment and the Murray River catchment (Figure 6). Given *E. camaldulensis* seed is primarily dispersed via waterways and flooding events, this genetic divergence between catchments is possibly due to limited water flow between them upstream of their confluence. However, the genetic separation between the catchments was not complete, with pollen flow likely occurring between the Lachlan and Macquarie catchments. While outside the Murray-Darling Basin, the Hunter catchment population of *E. camaldulensis* subsp. *camaldulensis* are closely related to those in the Darling River catchment, particularly stands in the Namoi River catchment (Figure 6).

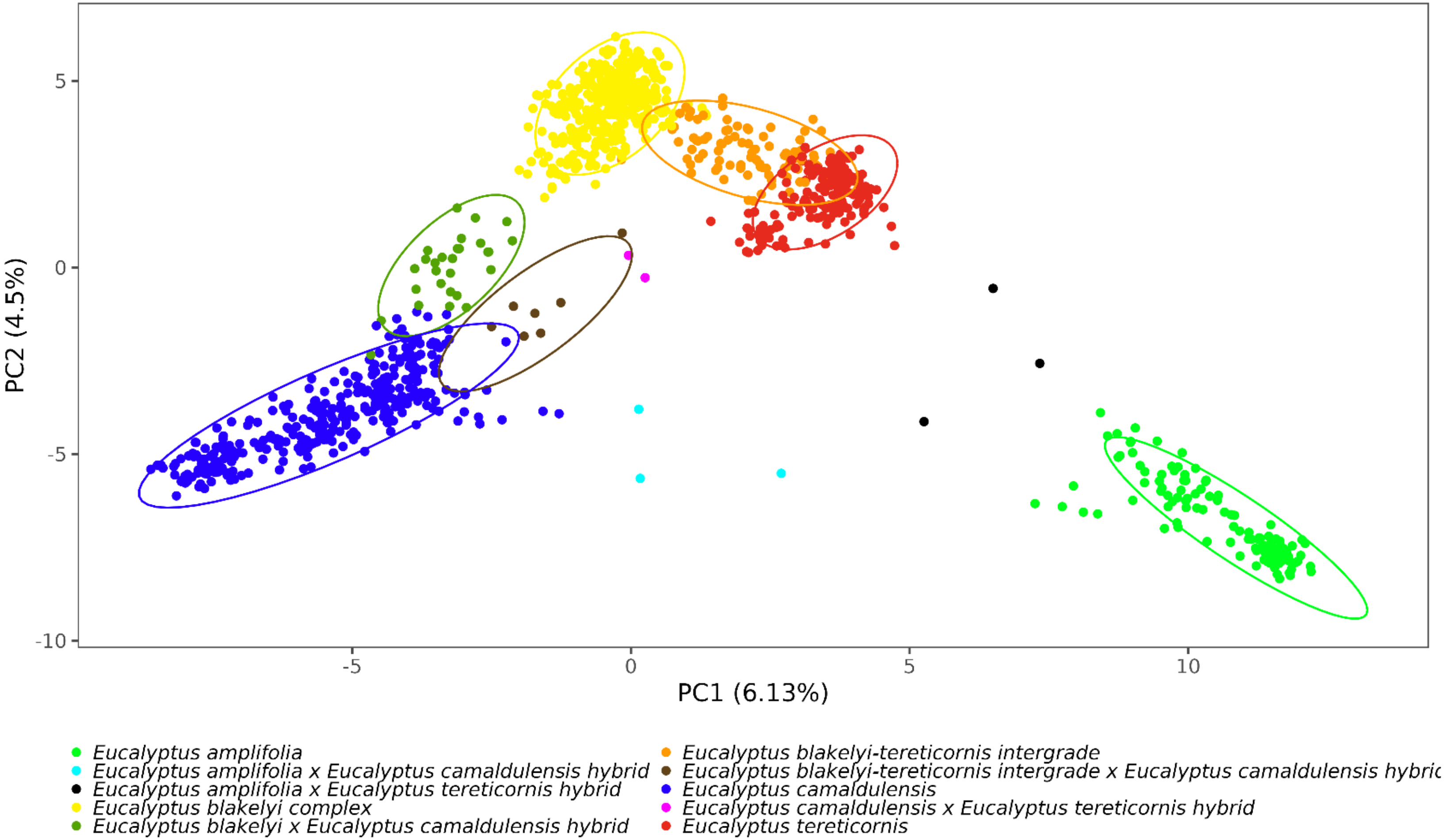

Figure 3: Plot showing first two axes of variation in the genetic dataset including both E. camaldulensis and related Red Gum taxa. Points represent individual samples and are coloured to correspond to their genetic ID and the relative clustering of points corresponds to the genetic relatedness of the sampled individuals. There are many samples that represent *E. camaldulensis* x *E. blakelyi* and *E. tereticornis* hybrids which originate from many sites across NSW, indicating these species will frequently hybridise where they co-occur. In contrast there is very few *E. camaldulensis* x *amplifolia* hybrids, all of which are from Wollombi Brook in the Hunter catchment as this is the one of the very few locations the distribution of these two species overlaps.

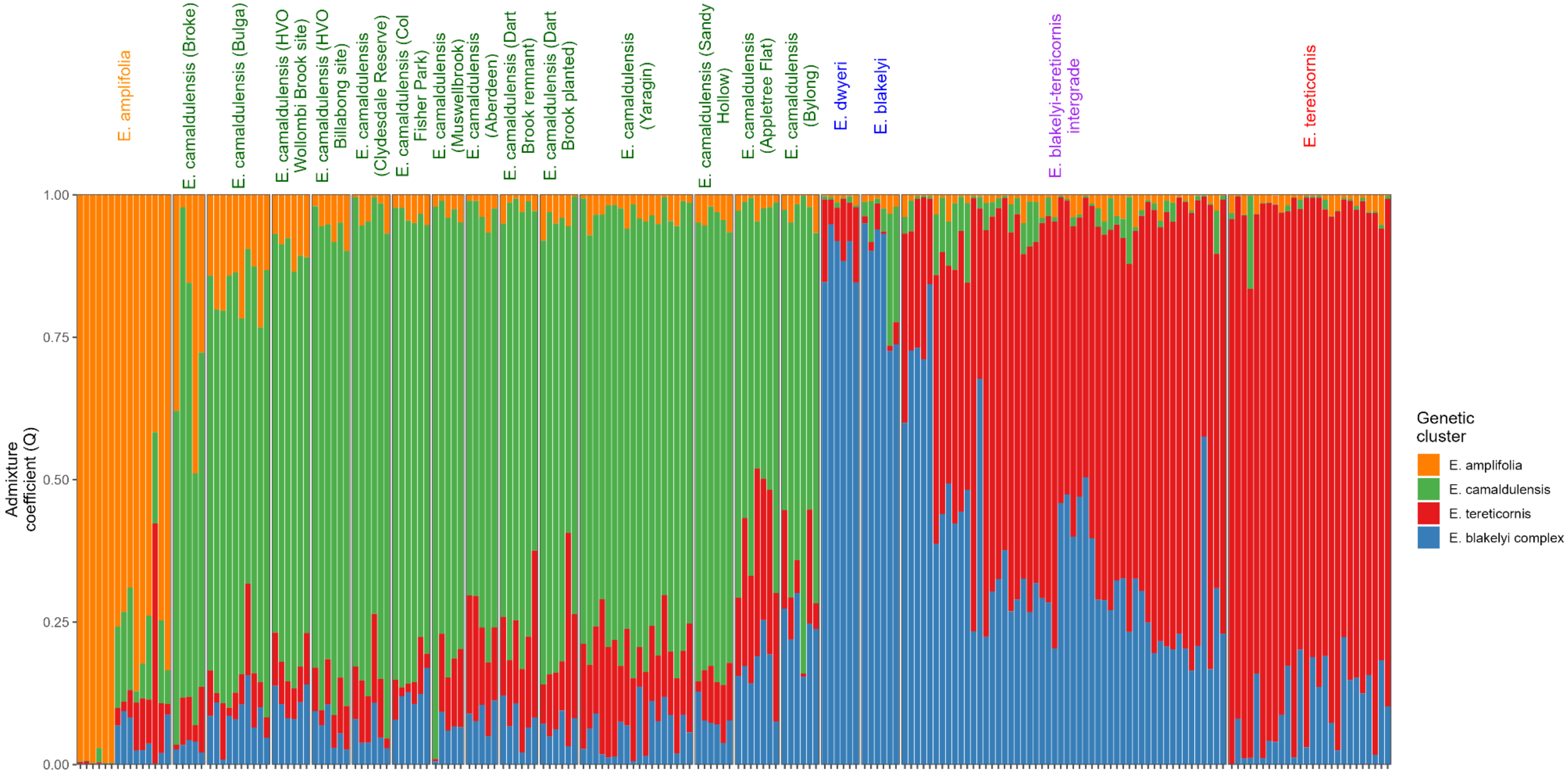

Figure 4: Results of structure analysis showing the assignment of Red Gum individuals from the Hunter Valley to the four genetic clusters that best match the genetic data. This analysis was carried out on all Red Gum samples genotyped by ReCER but only results for samples collected within the Hunter catchment are shown here. Note the increased levels of assignment to the *E. amplifolia* cluster of samples collected along Wollombi Brook (at Broke, Bulga, and the HVO Wollombi Brook site) compared to all other E. camaldulensis samples from the Hunter. The same pattern can be seen for samples collected at the Apple Tree Flat and Bylong sites, as well as select individuals from other sites, but with *E. blakelyi* and *E. tereticornis* rather than *E. amplifolia.*

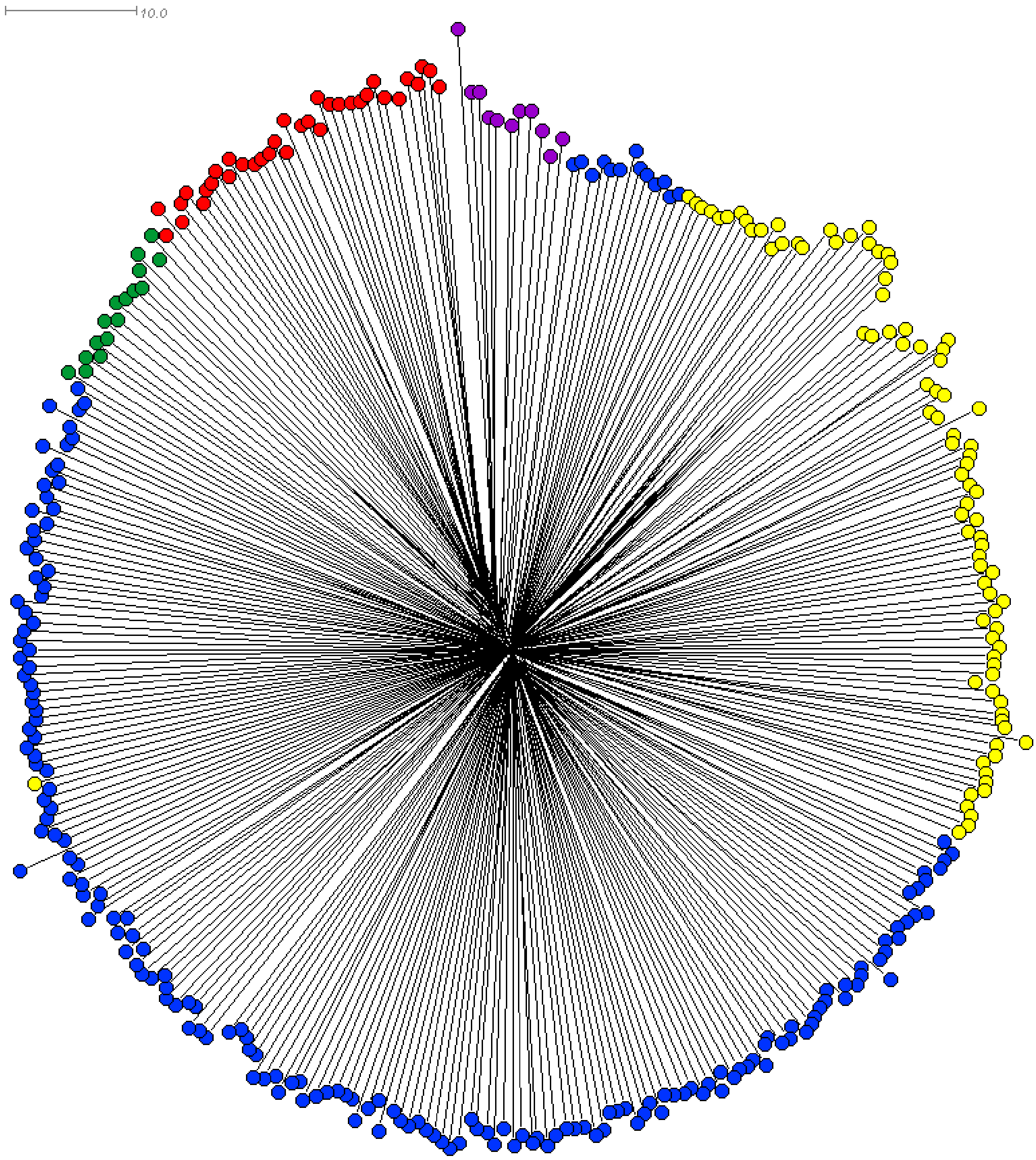

Figure 5: Phylogenetic network of all genotyped *E. camaldulensis* samples determined to not be hybrids. Coloured tips represent individual sampled plants, with colour corresponding to subspecies id, with yellow being samples from the Hunter Catchment (subsp. camaldulensis), blue being subsp. *camaldulensis*, green being subsp. *acuta*, red being subsp. *arida* and purple being individuals intermediate between subsp. *camaldulensis* and subsp. *arida*. The closer samples cluster the more closely related they are to one another, and thus it can be seen that subsp. *arida* is the most genetically different subspecies, with subsp. *acuta* being more closely related to subsp. *camaldulensis*.

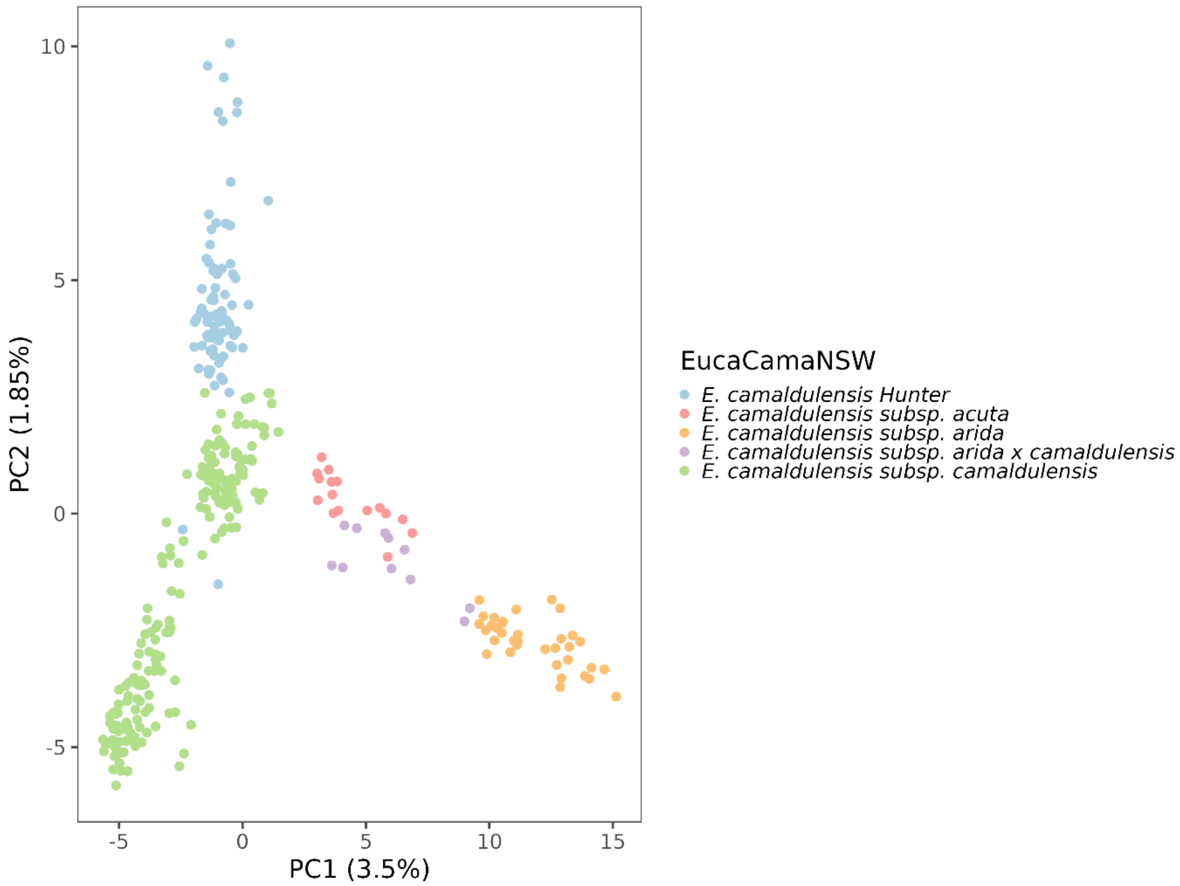

Figure 6: Plot showing the placement of all genotyped *E. camaldulensis* samples on the first two axes of variation of PCA analysis. Points representing individuals are coloured according to subspecies identification, with blue being subsp. *camaldulensis* samples from the Hunter Catchment, green being subsp. *camaldulensis* from outside the Hunter Catchment, red being subsp. *acuta*, orange being subsp. *arida* and purple being individuals intermediate between subsp. *camaldulensis* and subsp. *arida*. Variation explained by each axis is low (< 5%), suggesting much genetic diversity cannot be represented on two axes, a common occurrence in *Eucalyptus* due to the high genetic diversity at individual sites and extensive gene flow across the landscape. Despite this the three subspecies separate well on axis 1, while axis 2 separates *E. camaldulensis* subsp. *camaldulensis* (blue and green points) into two loose clusters corresponding to sites in the Murray River catchment at the top, and those in the Darling and Hunter River catchments at the middle and top.

3.2 Genetic findings on *Eucalyptus camaldulensis* stands in the Hunter

As mentioned in the previous section, hybridisation between *E. camaldulensis* subsp. *camaldulensis* and other red gum species is particularly prevalent in the Hunter Catchment. This included two of the six samples from the Bylong River at Bylong stand being identified as *E. blakelyi* hybrids, four of seven sampled individuals from Apple Tree Creek at Apple Tree Flat, one planted and one natural individual on Dart Brook west of Aberdeen being hybrid with local *E. blakelyi-tereticornis* intergrades, and three individuals from Wollombi Brook west of Broke being hybrid with *E. amplifolia* (Figure 4). The Apple Tree Creek stand was putatively identified as *E. camaldulensis* subsp. *acuta*, and the hybridisation with *E. blakelyi-tereticornis* integrates seems to be the cause of the bud morphology at this site approaching that of subsp. *acuta* despite the stand having no genetic relationship to this subspecies.

In addition to the true *E. camaldulensis* subsp. *camaldulensis* x *E. amplifolia* hybrids on Wollombi Brook, stands along this waterway all appear to have some genetic material originating from *E. amplifolia* in their genomes (Figure 4), making them more genetically divergent from other stands of subsp. *camaldulensis* than would otherwise be expected. This process, termed introgression, occurs when a hybridisation event has occurred many generations ago and the hybrid individuals have mated with one parent species, in this case *E. camaldulensis* subsp. *camaldulensis*, in subsequent generations. Given these introgressed individuals are mature and the hybridisation event happened many generations ago, it can be assumed this was a natural process rather than being driven by human activity. However, given the observation of three F1 hybrid individuals out of five total, non-planted samples genotyped on Wollombi Brook at Broke, it may be the case that the frequency of hybridisation increases upstream on Wollombi Brook, possibly due to the increased prevalence of *E. amplifolia* and smaller number of *E. camaldulensis* leading to significantly more *E. amplifolia* pollen being available than *E. camaldulensis*. Human induced population reduction and fragmentation can be expected to exacerbate this, leading to increased rates of hybrid seed being produced in the small stands along Wollombi Brook, and potentially the Hunter Catchment more broadly.

In terms of levels of standing genetic diversity in the Hunter Catchment, there was a general pattern of decreasing genetic diversity moving downstream on the Hunter River (Table 2; Figure 7). The highest genetic diversity was observed in stands around Aberdeen, including on Dart Brook. Notably low genetic diversity was observed in stands at Bulga on Wollombi Brook and at Sandy Hollow on the Goulburn River. Stands between Muswellbrook and Singleton showed moderate genetic diversity that was consistent with the range observed at stands across the Murray-Darling Basin. This pattern suggests that colonisation of the Hunter Catchment occurred along the Hunter River itself resulting in high genetic diversity there, with subsequent dispersal up its smaller tributaries resulting in lower genetic diversity in stands on these waterways. The higher diversity around Aberdeen is in line with the remnant stands in this area being larger than those in other parts of the catchment. There was a no difference in genetic diversity between the three generations sampled on the property Yaragin (Table 2; Figure 7), which is a promising sign suggesting this population is not experiencing an ongoing loss of genetic diversity. It also indicates seed sourced from this stand should be genetically diverse and lack high levels of hybrid seed, which were not observed at this site at all. Overall, these patterns of genetic diversity suggest sourcing seed from the largest possible stands on or near the main course of the Hunter or Goulburn rivers is the best option for local material to be used in the restoration of the Hunter Valley population of *Eucalyptus camaldulensis* subsp. *camaldulensis*.

3.3 Pilot seed collections by Broke Bulga Landcare

Seed collecting for this pilot project was undertaken prior to the initiation of the genetic project. Based on the results from the genotyping (outlined above) it was identified that seed were sourced from sub-optimal sites that would not be recommended for seed sourcing. All seed sourcing sites were along Wollombi Brook where the *E. camaldulensis* stands have experienced introgression from *E. amplifolia*, however as the seed is only to be used at plantings along Wollombi Brook, this reflects natural genetic patterns in the area. In terms of the maternal trees from which seed was sourced, one was a full *E. camaldulensis* x *amplifolia* hybrid (mother tree NSW1151619 from stand 3 – Wollombi Brook west of Broke), and therefore seed from this tree was not planted in the restoration site. Seedlings propagated from this seed were disposed of. Only a single seedling, from stand 2 at Broke, was confirmed as a hybrid, in this case with *E. amplifolia*. A further potential hybrid seedling was recorded from stand 4 at Bulga, although the parent species could not be confirmed. All other seedlings showed no further genetic relatedness to *E. amplifolia* beyond the signal of historical genetic introgression.

Seed from stands 1, 2 and 3 near Broke showed low levels of genetic diversity and elevated average kinship values between seed from the same mother (Table 3), indicative of high rates of inbreeding. The maternal tree at stand 1 (Wollombi Brook in Broke) was identified as a planted individual due to its high kinship values with individuals from the Muswellbrook stand (stand 10), the likely original seed collection location of this planted individual. While this is not a reason to avoid sourcing seed from this tree in general, due to its isolation from other members of the species, the seed coming from this tree were highly inbred, with a very high average kinship value (Table 3), even compared to the nearby stands 2 and 3. This is the result of much of the seed resulting from self-pollination of the mother tree, meaning the resultant individuals are likely to show stunted growth rates and low survival due to inbreeding depression which is common in *Eucalyptus* (Hardner and Potts 1997, Costa e Silva et al. 2010, Breed et al. 2014, Nickolas et al. 2019, Pupin et al. 2019) including the closely related Red Gum *E. tereticornis* (Ginwal 2010). Anecdotal observations in the nursery suggest these seed germinated later and less vigorously than other seed with lower kinship values. These genetic diversity and kinship results demonstrate why isolated individuals and small stands of the species should be avoided when seed is being sourced.

Seedlings from the seven maternal lines sourced from the Bulga site (stand 4) showed significantly higher genetic diversity and significantly lower average kinship values than the other three Wollombi Brook source stands. The proportion of seed that resulted from self-pollination at stand 4 was within the range that has been observed for other *Eucalyptus* species in natural, open pollinated systems (McDonald *et al.* 2003, Byrne 2008, Costa e Silva *et al.* 2010, Breed *et al.* 2014). This indicates the site is an appropriate stand from which to source genetically healthy seed from for restoration along Wollombi Brook, although the introgression of *E. amplifolia* genetic material into this entire stand means its seed should not be used for restoration plantings on other waterways in the Hunter catchment.

3.4 Optimisation of seed sourcing strategies

Our simulations suggest that 5 maternal lines from each of 4 sites within the Hunter Catchment (20 maternal lines total), with the additional of one site from the Macintyre Catchment if external provenances are to be used, is sufficient to guarantee 90% of common alleles are captured in the planting, meeting genetic diversity targets. Our optimisation analyses showed that the number of source sites and/or maternal lines represented in plantings is the single biggest factor influencing the genetic diversity and health of a restored stand. This in turn means the most powerful tool in the arsenal of restoration practitioners is to make sure the minimum number of maternal lines needed to capture significant genetic diversity is reached in seed collections. This will maximise the chances that plantings establish self-sustainability stands over the long term. The impact of the specific choice of seed source locations on genetic diversity and health of plantings is marginal compared to the number of maternal lines used. Therefore, the main considerations for seed sourcing strategies should be finding enough stands separated by such a distance that they do not commonly exchange genetic material (e.g., 10 km apart) but are also large and on main waterways (to minimize the proportion of inbred and hybrid seed). The source sites and maternal lines used in different plantings should be varied to capture different genetic variation at each site to avoid homogenising the Hunter Catchment *E. camaldulensis* population, a negative conservation outcome.

3.5 Climate change ready provenances

As can be seen in Figure 9a, the large climate gradient that exists along the Hunter Valley results in *E. camaldulensis* stands in the catchment experiencing a very broad climatic range, which results in large regions of south-eastern Australia matching this climatic range. This climate matched area includes large parts of the tablelands and inland slopes across the entirety of NSW, regions where *E. camaldulensis* currently occurs, but also regions outside the distribution of the species such as the eastern Northern Tablelands, parts of the Sydney Basin and South Coast. This suggests that climate is not a major factor influencing the species distribution, which is far more influenced by its habitat in riparian and floodplain ecosystems where water availability is higher than the surrounding landscape.

Under future climate projections (Figure 9b), many of the Hunter Catchment stands may experience climatic conditions that match current conditions experienced by the species in the catchment, with the notable exceptions of stands along the Goulburn and Bylong rivers in the east of the catchment. This suggests both that climate change is not a major threatening process for *E. camaldulensis* in the Hunter and that seed sourced from within the Hunter should be adapted to future climate conditions. Outside the Hunter, there is an overall smaller area that matches the future climate of the Hunter than its present climate, with predominately parts of the northern inland slopes being climate matched. This further strengthens the case for supplementation of the Hunter Catchment population with seed from the Namoi, Gwydir and Macintyre catchments to increase genetic diversity and climate adaptation.

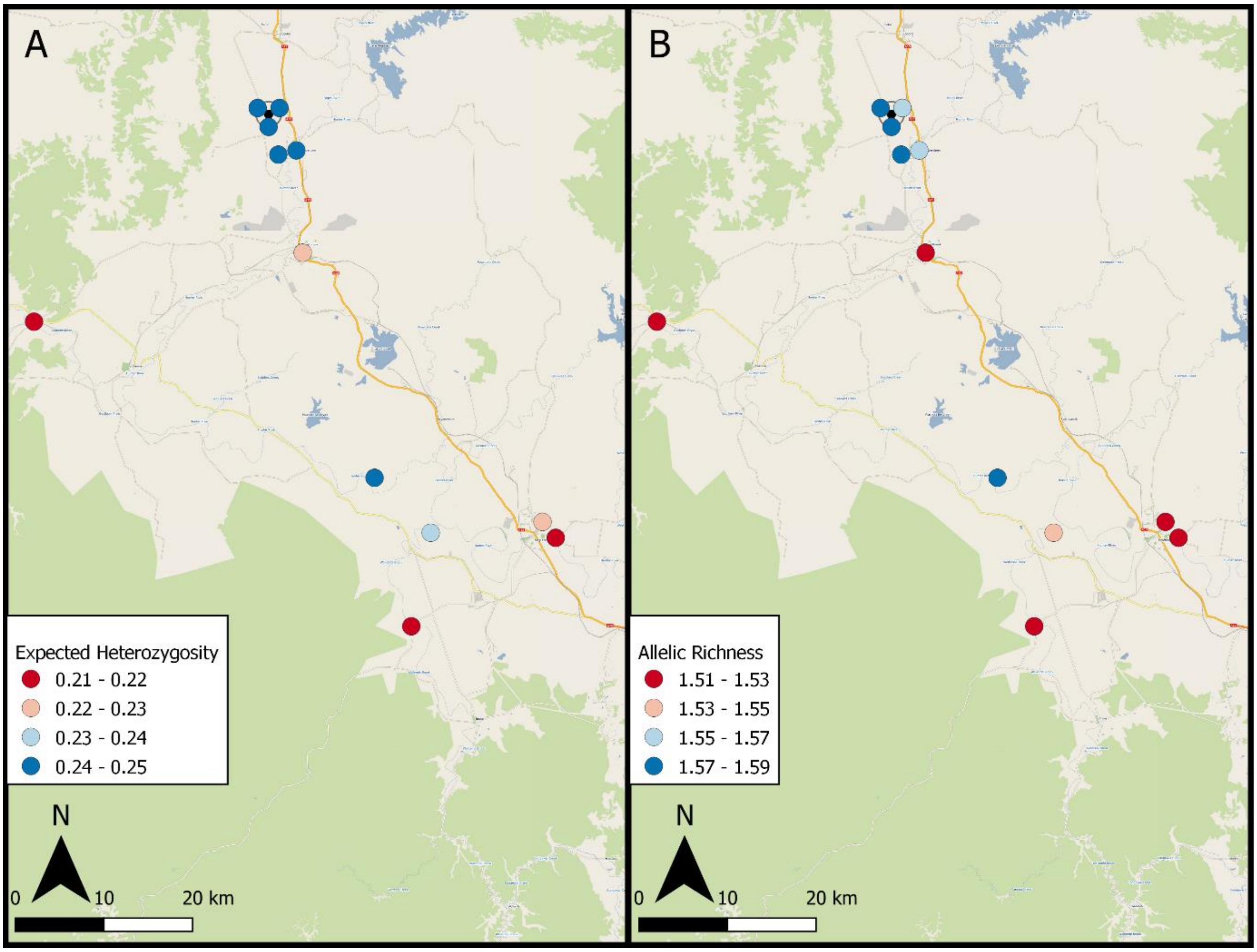

Figure 7: Map showing the genetic diversity of *E. camaldulensis* subsp. *camaldulensis* stands in the Hunter Valley, with more blue circles representing higher diversity and redder circles representing sites with lower genetic diversity. A) shows expected heterozygosity values and B) shows allelic richness, two separate measures of genetic diversity. As at least 5 successfully genotyped, non-hybrid samples are needed to reliably estimate these parameters not all sampled stands are shown. Note that the three known generations at the property Yaragin north of Aberdeen are shown by linked circles.

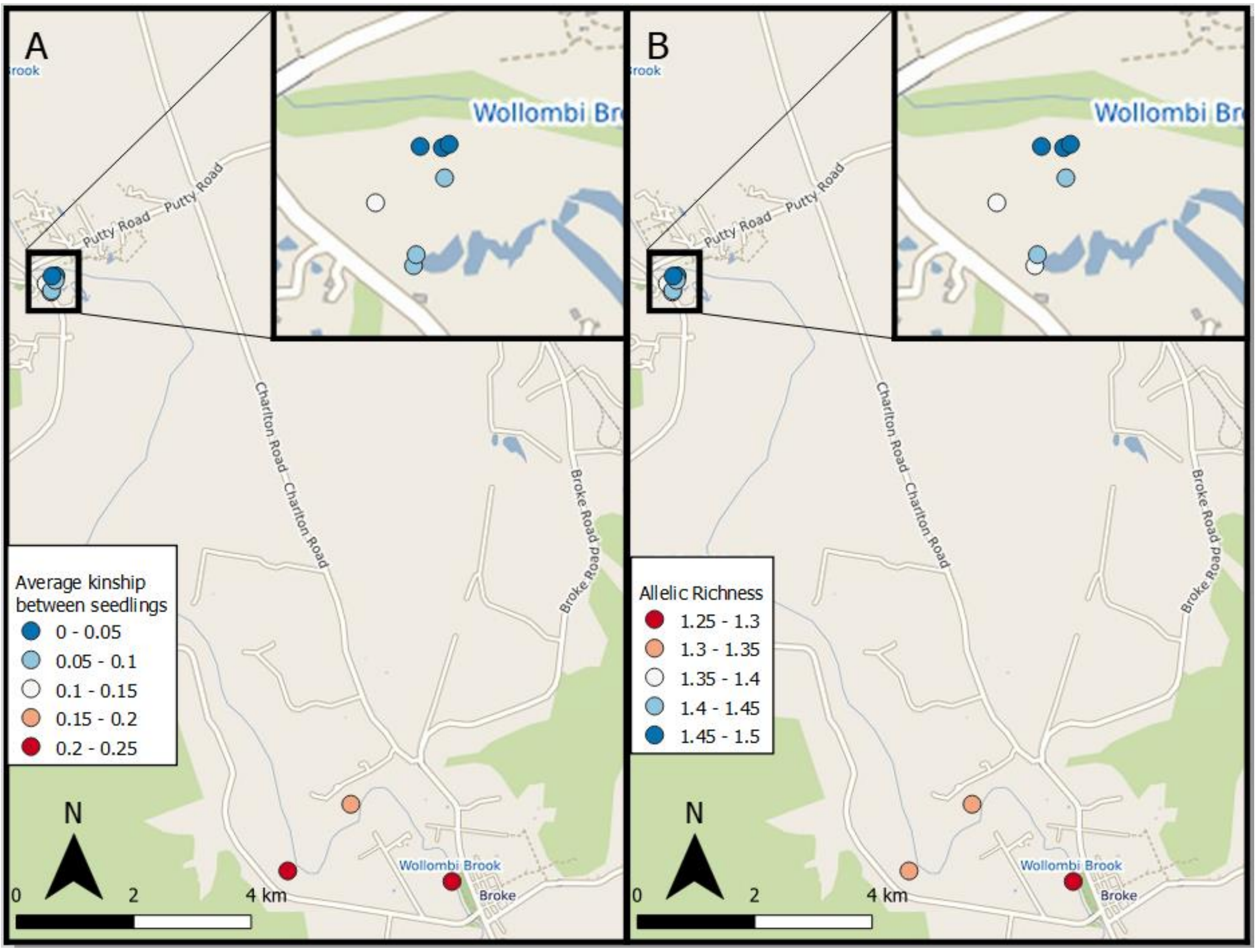

Figure 8: Map showing the key genetic measures for *E. camaldulensis* subsp. *camaldulensis* seed collected for Broke Bulga Landcare's plantings, with circles representing seed from a single maternal tree plotted at the coordinates of that mother tree. A) shows the average kinship between seedlings within the maternal line, a measure of how diverse the pollen was that lead to the seed, with lower values (represented by bluer circles) being more diverse seed lines, and higher values (represented by redder circles) being less diverse seed lines. B) shows the allelic richness of the seedlings, a measure of genetic diversity, with higher values (represented by bluer circles) being more diverse seed lines, and lower values (represented by redder circles) being less diverse seed lines.

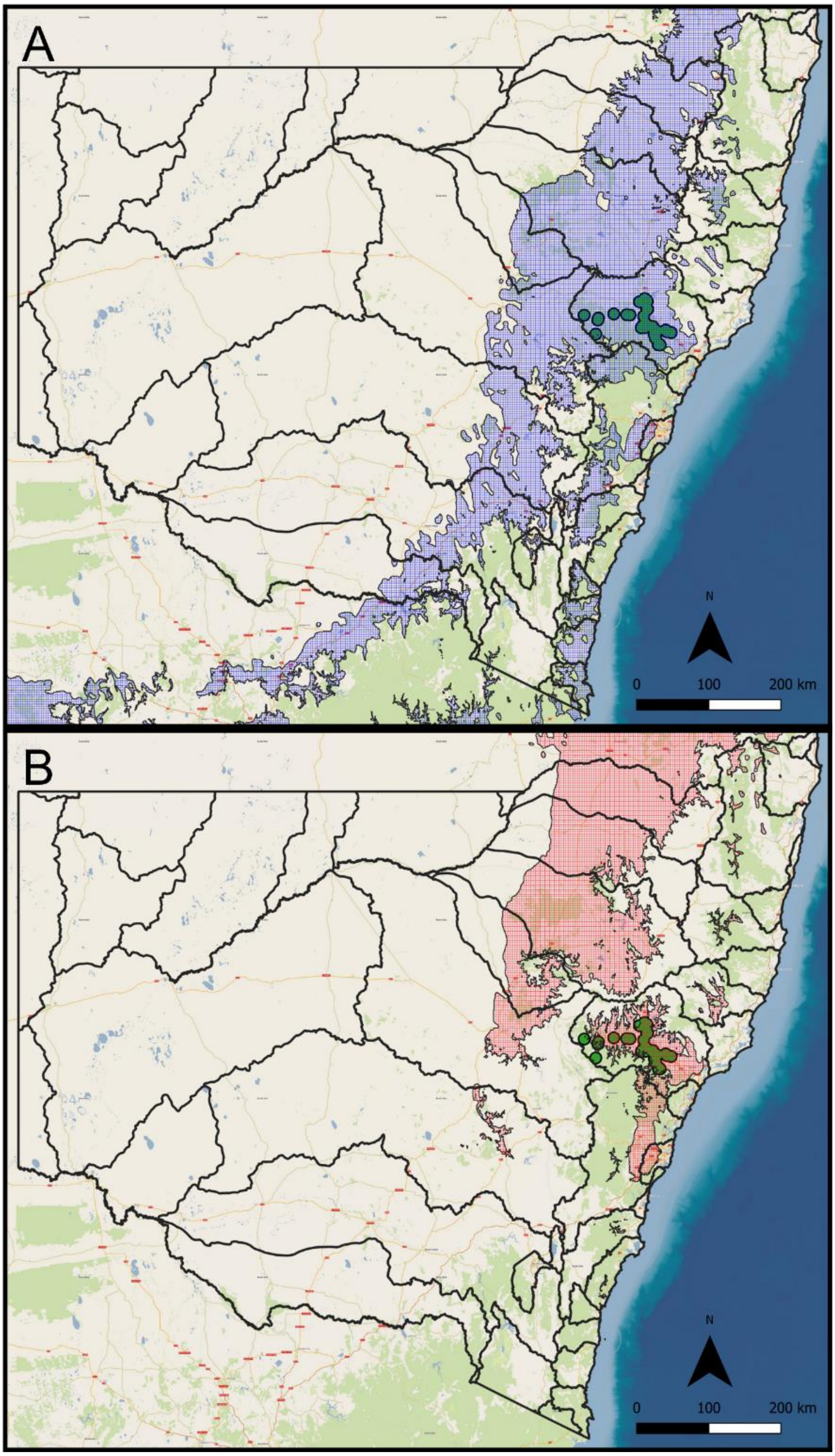

Figure 9: Maps showing the results of regional climate matching analyses. A) Blue hashing shows the areas which match the current climate experienced by *E. camaldulensis* stands in the Hunter (as determined from the area of occurrence marked in green), while the red hashing in B) shows areas that match the predicted future climate that the Hunter *E. camaldulensis* stands may experience in the future (2070). Note that due to the strong climate gradient along the Hunter Valley, much of the catchment will not move outside the range of current conditions under which *E. camaldulensis* currently grows. Also clear is that catchments to the north-west currently experience climatic conditions within the range predicted for the Hunter in the future and are thus good places to source seed to supplement the Hunter catchment stands.

3.6 Conservation and restoration recommendations and conclusion

3.6.1 Seed sourcing within the Hunter

As outlined in the previous sections, our recommendations for undertaking genetically informed seed sourcing within the Hunter catchment for *E. camaldulensis* are as follows:

- Choose the stands with largest possible number of mature *E. camaldulensis* individuals as seed collection sites.
- Preferentially collect seed from stands along main waterways (Hunter and Goulburn rivers), however it is better to include seed from more source stands than to avoid suboptimal source stands.
- Avoid stands growing near other red gum species native to the Hunter (*E. amplifolia*, *E. blakelyi, and* E*. tereticornis* predominately) to limit the use of hybrid seed in restoration plantings.
- Undertake quality control checks of seed and seedling morphology to help identify hybrids before planting in restoration sites.
- Seed sourced from Wollombi Brook should only be used for restoration along Wollombi Brook to avoid introducing *E. amplifolia* genetic material into the wider Hunter population. However, we recommend that Wollombi Brook restoration plantings are supplemented with material from outside the Wollombi Brook area.
- Low number of mature individuals in the Hunter and their clustering into a small number of stands means introducing new genetic material may be improve the success of restored populations in the long term.
- If seed is collected from 5 unrelated maternal trees from each of 4 different stands across the Hunter Catchment, then we predict >90% of the genetic diversity of the Hunter catchment population should be captured.
- Note that the maternal lines used from each site should be varied between restoration plantings, to avoid homogenizing and reducing the diversity of *E. camaldulensis* families present in the Hunter Catchment. For example, the Muswellbrook and Broke restoration plantings could use seed from the same 4 Hunter catchment stands but would ideally use seed from different mother trees.

3.6.2 Seed sourcing outside the Hunter

Given the above recommendation to introduce seed from outside the Hunter catchment to assist in increasing genetic diversity and adaption to climate change, the following recommendations for genetically informed sourcing of seed from outside the Hunter catchment for use with the catchment are provided:

- The same general recommendations apply as when sourcing seed within the Hunter: namely choose the largest stands possible and avoid those co-occurring with other Red Gum species to minimise the risk of collecting hybrid seed.
- Given the close relationship between the Hunter catchment population and populations to the northwest of the region, namely those in the Namoi, Gwydir and Macintyre catchments, there is limited risk associated with introducing seed from these regions, thus making these ideal seed sources.
- If seed is collected from 5 unrelated maternal trees from 4 different stands within the Hunter, than the addition of seed from 5 maternal lines from a single site from outside the region is enough to capture >90% of genetic diversity across the Hunter, Namoi, Gwydir and Macintyre catchments.
  - While stands in the Namoi catchment are most closely related to Hunter populations, seed sourced from stands in the Macintyre catchment, being more geographically removed from the Hunter catchment, would introduce a greater amount of new genetic diversity. Parts of the Macintyre Catchment also currently experiences conditions similar to those predicted for the Hunter in 2070 when restored trees will be mature and reproducing.

3.6.3 Supplementation of planting sites

- Optimisation of seed sourcing the Muswellbrook stand, and the Broke planting showed the same strategy can be used for both:
  - three sites within the Hunter (achieving the target of four source sites when combined with the existing plants at the site) and one site from the Macintyre catchment maximised genetic diversity and minimised the number of sites from which seed collection is needed.
  - The three most complimentary stands to both in the Hunter were:

- Stand 6 – Col Fisher Park Singleton
- Stand 11 – Hunter River at Aberdeen
- Stand 12 – Dart Brook west of Aberdeen

However, the source for seeds should be varied between different restoration plantings, whether by using seed from different mother trees at the same sites or using a different set of source sites for the different plantings.

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