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# Conservation and genomic diversity of a rare tree, *Eucalyptus imlayensis* (Myrtaceae), regenerating after wildfire

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## Abstract

Rare species confined to small populations are frequently targeted for conservation because they are prone to reduced fitness stemming from inbreeding depression, loss of diversity and genetic drift resulting in an increased extinction risk. In this study, we used genome-wide scans to quantify clonal diversity and plant relatedness in the only known population of *Eucalyptus imlayensis* which was regenerating after wildfire burnt the entire population in summer 2019/2020. Samples taken from resprouting lignotubers were used to generate a dataset of single nucleotide polymorphisms (SNPs) and contained 406 loci across 51 stems and 5 technical replicates. Clonality was found to be extensive, with the entire population of *E. imlayensis* composed of only 17 distinct genotypes. Ten genotypes were confirmed as clonal with the remaining 7 genotypes each recovered only once. Kinship analysis revealed two unique genotypes with a first-degree relationship to one of the clonal genotypes, and five samples with a second-degree or beyond relationship to any clonal groups. This information will form the basis for the development of an *ex situ* collection and manipulated crosses to produce diverse seed for population enhancement, as well as to establish a seed store for conservation.

***Keywords*:** clonality, single nucleotide polymorphism, conservation genetics, relatedness

## Introduction

Fire has been a significant evolutionary force for much of the Australian flora, with adaptations that include the ability to re-sprout from below-ground meristems and soil-stored seedbanks. Such adaptations enable some species to withstand the wholesale consumption of the plants above-ground by fire (Burrows 2013). On a global scale, climate change has precipitated shifts in fire regimes, resulting in longer fire seasons with more frequent and more destructive fires (Jones *et al*. 2022). The extent and severity of these fires impact areas not previously exposed to frequent or intense burning


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(Filkov *et al.* 2020). This general trend is reflected in the fire history of south-eastern Australia over recent decades (Dutta *et al.* 2016; Boer *et al.* 2020), most notably in 2019/2020, where fires burnt more than 7 million ha of dry sclerophyll forest dominated by re-sprouting eucalypts (Filkov *et al.* 2020; Gibson & Hislop 2022). The evolution of re-sprouting is linked to the rise of flammable sclerophyll biomes in Australia from the early Cenozoic (60–62 mya), and in eucalypts the anatomy of epicormic and basal sprouting from lignotubers has been preserved despite changes to habitat and response to fire (Crisp *et al.* 2011).

Mount Imlay National Park (Figure 1), in south-eastern New South Wales (NSW), incorporates Mount Imlay, an isolated and culturally significant peak that rises to 886 m above sea level (a.s.l.) and which is known to local peoples as Balawan (Department of Agriculture, Water and the Environment 2022a). Mount Imlay and the surrounding park were burnt extensively when a wildfire moved rapidly from the south on New Year's Eve 2019, affecting typically moist vegetation that had become drier during several preceding years of drought. Mount Imlay is considered an Asset of Intergenerational Significance for two endemic plants species, *Eucalyptus imlayensis* Crisp & Brooker and *Hibbertia circinata* K.L.McDougall & G.T.Wright, under the amended NSW *National Parks and Wildlife Act 1974*. There is one other endemic plant species on Mt Imlay (*Boronia imlayensis* Duretto) and several other significant plant species, most of which are threatened by the pathogen *Phytophthora cinnamomi* (McDougall *et al.* 2023).

Mallees such as *Eucalyptus imlayensis* are usually found in semi-arid, fire-prone regions of southern Australia but in high rainfall areas, they are often confined to 'difficult' sites characterised by shallow soils over bedrock (Lacey & Johnston 1990). The only known population of *E. imlayensis* occurs just below the summit on the eastern face of Mt Imlay at 850 m a.s.l. and is confined to a site with rocky, shallow soils. The area receives an average annual rainfall of 750 mm. Plants have a characteristic mallee growth form with multiple stems to 7 m tall originating from a lignotuber, a dense canopy and smooth bark that sheds in ribbons (Crisp & Brooker 1980).

In 2022, *Eucalyptus imlayensis* was identified as one of 100 priority threatened species in the Commonwealth

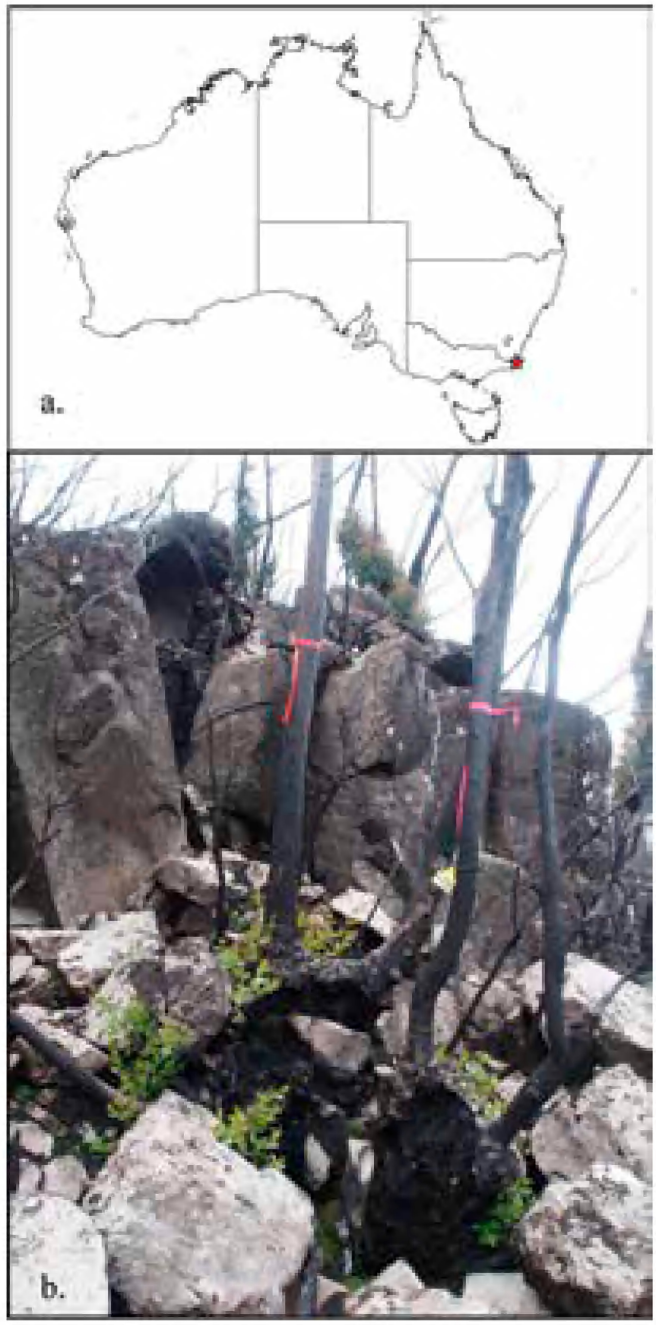


**Figure 1**. a. Location of Mt Imlay in south-eastern New South Wales (asterisk) where the only population of *Eucalyptus imlayensis* is situated, and b. re-sprouting lignotubers nestled amongst granite boulders as recorded on 6 November 2020 approximately 10 months after above-ground stems were killed by fire (image: G. Wright).

of Australia's Threatened Species Strategy Action Plan 2022-2032 (Department of Climate Change, Energy, the Environment and Water 2022). It is listed as Critically Endangered under the New South Wales *Biodiversity Conservation Act 2016* and the federal *Environment Protection and Biodiversity Conservation Act 1999* (NSW Government 2016, AWE 1999) and managed under the

NSW government Saving Our Species (SOS) program. The high-intensity wildfire of 2019/2020 consumed all vegetation within the habitat of *E. imlayensis* and highlighted the vulnerability of this highly restricted species to a single high intensity fire. Seed production from fire-impacted plants is not expected to commence for at least 10 years, based on the absence of flowers on seed-grown plants planted *in situ* in 2011. As a result, *E. imlayensis* is listed as requiring urgent post-fire assessment (Gallagher 2020), with the establishment of an *ex situ* conservation collection developed from asexual material (cuttings) part of urgent conservation action post-fire (DPE 2022).

Due to its very small area of occupancy (0.3 ha), *Eucalyptus imlayensis* has been of high conservation concern since it was first described (Crisp & Brooker 1980). Extensive surveys were undertaken between 1998 and 2003 to monitor the health and size of the population which was estimated to comprise approximately 80 multi-stemmed plants (James & McDougall 2007). Separate lignotubers were considered to represent distinct plants, each of which were tagged and mapped on site. No juvenile or seedling plants have been observed within the population in the past 20 years, and seed production is limited and sporadic. Genetic studies in 2007 identified five genotypes from 27 *E. imlayensis* samples analysed, indicating fewer genetic individuals than lignotubers genotyped and a high level of clonality (James & McDougall 2007). However, it was recognised at the time that there may be additional undetected plants because the steep terrain and dense growth of *Leptospermum scoparium* limits accessibility at the site (James & McDougall 2007). The absence of dense vegetation in the post-fire landscape aided surveys and accurate mapping of the population in May 2020. Despite above-ground stems being killed, most *E. imlayensis* adult plants survived the wildfire and were re-sprouting from lignotubers (Figure 1).

Identifying the genetic signature of plants and populations and the relationships between them have long been recognised as vital for genetically representative seed banking, *ex situ* conservation, and conservation-focussed breeding programs (Griffith *et al.* 2021). In this study, a comprehensive genetic assessment of all surviving plants within the population of *Eucalyptus imlayensis* was undertaken. Genome-wide scans were utilised to identify the extent of clonality, kinship and genotypic diversity. This information provides the basis for future conservation efforts enabling reproductive variability and genotype survival to be tracked over time. Here, we aim to 1) determine extent of clonality in the population; 2) identify the number and size of clones; 3) map the spatial distribution of genotypes; and 4) assess the relatedness between genotypes to develop a breeding strategy for *ex situ* seed production and potential population augmentation.

## Methods

### Fieldwork and sampling

Field work was undertaken by the NSW Department of Planning and Environment, Biodiversity Conservation staff in November 2020, as part of the annual SOS monitoring program. During this survey work, 48 *Eucalyptus imlayensis* plants were re-tagged and geolocated. A 'plant' was defined as a group of stems that appeared to belong to the same lignotuber, and a 'stem' was defined as a single shoot or trunk from a lignotuber. The removal of vegetation by the fire 11 months prior to the survey enabled a more accurate assessment of the number of plants because previously unseen associations between lignotubers were visible and reduced the plant count from the 80 estimated in 2006 (James & McDougall 2007); some plants had also died since 2006. Not all resprouting plants could be reconciled with previously mapped plants because tags were burnt, and not all known plants could be found post-fire. However, the ability to locate all re-sprouting plants enabled the first accurate count and genetic assessment of the population.

Although most re-sprouting lignotubers surveyed in 2020 were presumed to correspond to previously tagged plants, a new number was assigned to each apparent individual to avoid confusion. It was not possible to determine visually whether stems were clonemates or belonged to distinct genets based on proximity in the field because physical connections between lignotubers may have been lost over time. All live stems were re-shooting from existing lignotubers with no evidence of newly recruited seedlings in the population. To identify the number of genets, assess relatedness between plants (genets) and identify clonemates (ramets), in

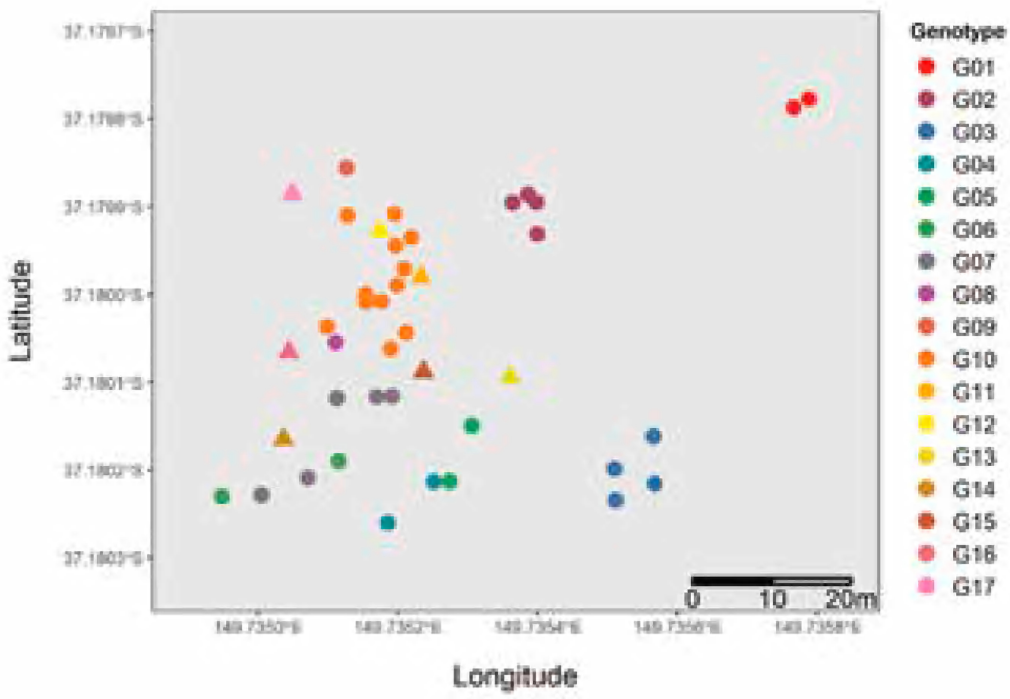


**Figure 2**. Site map showing spatial distribution of sampled *Eucalyptus imlayensis* stems within the site, colour-coded for genotype. Genotypes *G1–G10* were recovered multiple times and are represented by circles while *G11–G17* were recovered only once and are represented by triangles.

November 2020, leaf samples for genetic analysis were collected from one stem of each of the 48 newly tagged plants likely to be a mix of genets and ramets. The location of each sample was georeferenced with a handheld GPS. A duplicate sample was collected from each of three tagged plants from a second stem on the same lignotuber and within 1 m of the primary sample giving a total of 51 samples. The duplicate samples were annotated with 'a' to differentiate them from the paired sample of the same lignotuber or plant (*103,103a*; *115, 115a*; *117, 117a*). Samples were stored in silica gel and sent to Royal Botanic Gardens Victoria for preparation. The locations of samples used in the final analyses are shown in Figure 2.

## Molecular data collection

Approximately 15 mg from each of the 51 leaf samples, plus 5 technical repeats (56 in total), were subsequently sent to a commercial genotyping service, Diversity Arrays Technology ('DArT', Canberra, Australia) for DNA extraction and DArTseq™ analysis, which is a reduced representation sequencing method proven for use in eucalypts (Sansaloni *et al.* 2010; Kilian *et al.* 2012; Cruz *et al.* 2013). DNA was extracted at DArT™ using the Nucleo Mag Plant C-Bead kit (Machery Nagel, Germany) on a Tecan 100 platform per the manufacturer's protocols. Library preparation involved DNA digestion and ligation using methylation-sensitive restriction enzymes PstI and HpaII and uniquely barcoded adaptors. Following PCR and quantification, the samples were standardised and pooled for sequencing in a single lane of an Illumina HiSeq2500. Filtering of poor-quality sequences (Phred score <30) and read assembly were undertaken by DArT™'s proprietary primary analytical pipeline and the filtered, assembled data were then used in a proprietary secondary pipeline for SNP calling (DarTsoft14), producing 18 427 binary SNPs from 55 samples as one individual did not yield sequences.

## Molecular data analysis

Clonality was investigated within 55 samples made up of 47 *Eucalyptus imlayensis* plants from tagged lignotubers (no data from *eim124*), the paired samples from three different lignotubers, and 5 technical repeats, following the methods of Bradbury *et al.* (2021). Variation is known to occur among clonemates due to genotyping errors and somatic mutations and can lead to over-estimation of genotypic diversity. To minimise this, the average sequencing error rate in the unfiltered dataset (18.86 ±1.17 SD) was estimated by calculating the average genetic distance between known identical samples (our technical replicates) using the R package dartR (Gruber *et al.* 2018). To enable an accurate estimate of clonality, a threshold of genetic differentiation is typically applied

to determine the degree of variation that is acceptable between ramets (Douhovnikoff & Dodd 2003; O'Brien *et al.* 2014). Therefore, a pairwise individual Euclidean genetic distance matrix was constructed with the expectation that distance values between genuinely distinct genotypes (genets) versus near-identical genotypes (ramets) should clearly differ. From our dataset, pairwise samples with a distance value ≤37.72 (i.e., twice the average error rate, to account for deviation from the mean) were considered ramets of a clonal genotype. The mean distance between clonal samples (ramets) of 24.85 (±5.89 S.D.) was clearly distinct from the mean distance among all other stems (91.71±12.15 S.D.), giving us high confidence in clone identification and a low risk of over-estimating genotypic diversity. From these analyses, we identified 17 genotypes with varying numbers of ramets recovered per genotype.

To verify the relationship of the clonal stems, the technical replicates were removed, data were filtered using the R packages dartR (Gruber *et al.* 2018), poppr (Kamvar *et al.* 2014, 2015) and SNPRelate (Zheng *et al.* 2012) in R (R Core Team 2019) to a locus and individual call rate of 85% and 100% (respectively), a reproducibility score of 1, a Hardy-Weinberg equilibrium with a 5% level of significance, a minor allele frequency greater than 5%, removal of monomorphic loci, and finally filtered on Hamming distance to remove potential paralogues. The resultant dataset contained 2980 SNPs across 50 individuals.

Pairwise genetic relatedness (kinship coefficient, $\phi$) was calculated between individuals using the identity by descent maximum likelihood estimation method in the R package SNPRelate (Zheng *et al.* 2012; Manichaikul *et al.* 2010). Kinship values of 0.50 represent a monozygotic twin, $\phi$=0.25 and higher indicate a first-degree relationship (full siblings, parent-child etc.) and $\phi$=0.125-0.25 indicate a second-degree relationship (i.e., half-siblings, grandparent-grandchild etc). Unrelated pairs are expected to have values close to $\phi$=0 (Manichaikul *et al.* 2010). A kinship threshold of k=0.45 was used to define ramets (Bragg *et al.* 2021).

To visualise relationships among plants and genotypes, a principal components analysis (PCA) was undertaken, implemented in the adegenet package (Jombart 2008; Jombart & Ahmed 2011). PCA does not rely on an evolutionary model and is therefore free of the assumptions made by other clustering methods (Jombart *et al.* 2009). A neighbour-joining tree was plotted using the ape package in R (Paradis & Schliep 2019). The R packages pophelper (Francis 2017), ggplot2 (Wickham 2016) and FigTree v1.4.4 (Rambaut 2018) were used to visualise results, and individuals were mapped on to the site layout according to their genotype.

Finally, a third dataset was assembled using one representative of each genotype selected at random and filtered as described above. The dataset contained 3165 variable SNPs from 17 individuals. Individual heterozygosities and inbreeding coefficients were calculated for each genotype. A genomic relatedness matrix for the genotypes was calculated with the gl.grm function and plotted as a heatmap in dartR (Gruber *et al.* 2018). An individual genetic distance matrix (Euclidean) was produced in the R package dartR (Gruber *et al.* 2018) and a pairwise genetic relatedness (kinship coefficient, $\phi$) was calculated between genotypes as described above, in the R package SNPRelate (Zheng *et al.* 2012; Manichaikul *et al.* 2010).

## Results

Analyses on the unfiltered dataset identified 17 genotypes composed of ten recurrent (clonal) genotypes (*G01 – G10*) and seven genotypes (*G11– G17*) recovered only once (unique). Individual Euclidean genetic distances (GD) and kinship coefficients ($\phi$) between the 17 *Eucalyptus imlayensis* genotypes were calculated based on 3165 SNPs and ranged from GD = 24-59 and $\phi$ = 0-0.37 (Table 1; *see also* Suppl. files 1 & 2). Within each clonal genotype, $\phi$ values among ramets ranged from 0.47 to 0.49 which is within the threshold of 0.50 expected for monozygotic twins (Suppl. file 2). Individual observed heterozygosity ranged from 0.221 (*G11*) to 0.474 (*G05*; *see* Suppl. file 3) and inbreeding coefficients ranged from -0.272 (*G05*) to 0.406 (*G11*).

Clonal genotypes ranged from having two distinct lignotubers (*G1*, *G8*) to having 13 lignotubers (*G10*) excluding duplicate samples. Ramets of each genotype are, on the whole, spatially clustered (Figure 2). Genotype *G10* occupies the largest area and appears to have become fragmented after expansion. Two unique plants, *G11* and *G12,* are located within the geographic spread of *G10* with kinship coefficients indicative of a

| | G01 | G02 | G03 | G04 | G05 | G06 | G07 | G08 | G09 | G10 | G11 | G12 | G13 | G14 | G15 | G16 | G17 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **G01** | | 0.08 | 0.18 | 0 | 0 | 0 | 0 | 0 | 0.09 | 0 | 0 | 0 | 0 | 0.04 | 0 | 0 | 0 |
| **G02** | 44 | | 0.00 | 0 | 0 | 0 | 0 | 0 | 0.17 | 0 | 0 | 0 | 0 | 0.01 | 0 | 0 | 0.03 |
| **G03** | 38 | 47 | | 0 | 0 | 0.05 | 0.05 | 0 | 0.06 | 0 | 0 | 0 | 0 | 0.02 | 0 | 0 | 0.06 |
| **G04** | 52 | 53 | 48 | | 0.05 | 0.02 | 0.14 | 0 | 0 | 0 | 0 | 0 | 0.14 | 0.03 | 0.13 | 0.09 | 0 |
| **G05** | 52 | 52 | 54 | 45 | | 0.03 | 0 | 0.01 | 0 | 0.05 | 0.01 | 0.02 | 0.00 | 0 | 0.07 | 0 | 0 |
| **G06** | 51 | 53 | 45 | 48 | 49 | | 0.12 | 0 | 0 | 0 | 0 | 0 | 0.06 | 0.06 | 0 | 0 | 0 |
| **G07** | 49 | 51 | 42 | 39 | 50 | 42 | | 0 | 0 | 0 | 0 | 0 | 0.15 | 0.16 | 0 | 0.14 | 0.00 |
| **G08** | 53 | 54 | 48 | 52 | 48 | 53 | 52 | | 0 | 0.17 | 0.09 | 0.09 | 0 | 0 | 0 | 0 | 0.05 |
| **G09** | 42 | 39 | 41 | 50 | 59 | 53 | 52 | 48 | | 0.01 | 0 | 0.00 | 0 | 0 | 0.01 | 0 | 0.08 |
| **G10** | 50 | 49 | 50 | 49 | 44 | 53 | 50 | 38 | 43 | | 0.33 | 0.37 | 0 | 0 | 0.01 | 0 | 0 |
| **G11** | 56 | 55 | 56 | 55 | 50 | 59 | 57 | 46 | 49 | 27 | | 0.29 | 0 | 0 | 0 | 0 | 0 |
| **G12** | 55 | 54 | 55 | 54 | 49 | 58 | 55 | 45 | 48 | 24 | 34 | | 0 | 0 | 0 | 0 | 0 |
| **G13** | 50 | 49 | 45 | 39 | 47 | 44 | 38 | 51 | 48 | 50 | 56 | 55 | | 0.06 | 0.09 | 0.11 | 0 |
| **G14** | 46 | 50 | 45 | 46 | 52 | 47 | 39 | 55 | 50 | 53 | 59 | 58 | 43 | | 0 | 0.01 | 0 |
| **G15** | 50 | 48 | 49 | 40 | 44 | 52 | 51 | 51 | 44 | 45 | 51 | 50 | 41 | 51 | | 0.09 | 0 |
| **G16** | 50 | 50 | 46 | 41 | 50 | 53 | 39 | 48 | 46 | 46 | 53 | 51 | 40 | 46 | 41 | | 0 |
| **G17** | 49 | 47 | 42 | 50 | 52 | 50 | 46 | 46 | 42 | 48 | 54 | 53 | 47 | 50 | 48 | 49 | |

**Table 1**. Matrix of individual Euclidean genetic distance (bottom) and kinship coefficient ϕ (top) between the 17 *Eucalyptus imlayensis* genotypes (3165 SNPs, 17 samples). Darker shaded genetic distance values indicate greatest differences, while darker shaded kinship coefficients indicate closer relationships between genotypes.

first-degree relationship between them. Paired samples collected as separate stems from the same lignotubers (*103, 103a; 115, 115a; 117, 117a*) belong to the same genotype as their pairs (*G2*, *G5*, *G7*, respectively). This confirms that stems from the same lignotuber and assessed as the same plant in the field were clonemates. However, some ramets were identified from apparently separate lignotubers so the extent of each clone cannot be determined visually.

Variation across the dataset and the genetic clustering of samples (genets and ramets) are depicted in the PCA (Figure 3), with axes 1 and 2 accounting for 29.62% and 18.45% of the variation in the dataset respectively. Clusters indicate clonemates whereas samples distanced away from clusters are unique genotypes of varying kinship. Two unique genotypes, *G11* and *G12*, had a first-degree relationship to genotype *G10* and to each other, indicating full sibling or parent-child (Table 1), with ϕ values ranging 0.29–0.37 and genetic distance values ranging 24–34. The neighbour-joining tree also presents visually the relationships between the colour-coded clonal groups and unique genotypes shown in black (*see* Suppl. file 4). The nesting of genotypes *G11* and *G12* within the group of samples assigned to *G10* in the neighbour-joining tree and in the PCA displays the close relationship between these genotypes. Eight genotype pairs had a second-degree (half-siblings, grandparent-grandchild), and all remaining pairs had a more distant relationship to any other genotype (Table 1) with ϕ values of 0 and genetic distance values generally >45 for distantly related pairs. The probability of Identity by Descent is shown for all 17 *E. imlayensis* genotypes and provides a visual overview of the relationships among genotypes that could be used for breeding selection (Figure 4). Genotypes that are closer to red have a higher probability of similarity, supporting the relationships shown in the neighbour-joining tree, such as the similarity between *G10*, *G11* and *G12*.

## Discussion

Extensive clonality has been substantiated in *Eucalyptus imlayensis,* a rare mallee species, with only 17 unique genets identified for the species from the 48 tagged plants. This represents a sizable increase from the five genotypes identified in an earlier study where only part of the population was accessible (James & McDougall 2007). There was no evidence of seedling recruitment after the fire of 2019/20 suggesting a lack of soil-stored seed. The removal of dense vegetation by fire enabled comprehensive sampling across the entire site and the collection of reliable baseline data on the extent of the population and its genetic composition.

Measures of genetic diversity can be used as a proxy for a species' evolutionary potential, which is likely to be a decisive factor in determining its ability to adapt and persist (Sgrò *et al.* 2011). Over time, changing climate can create suboptimal environmental conditions and affect a species' resilience, including reduced fecundity with a shift in reproductive strategies (Silvertown

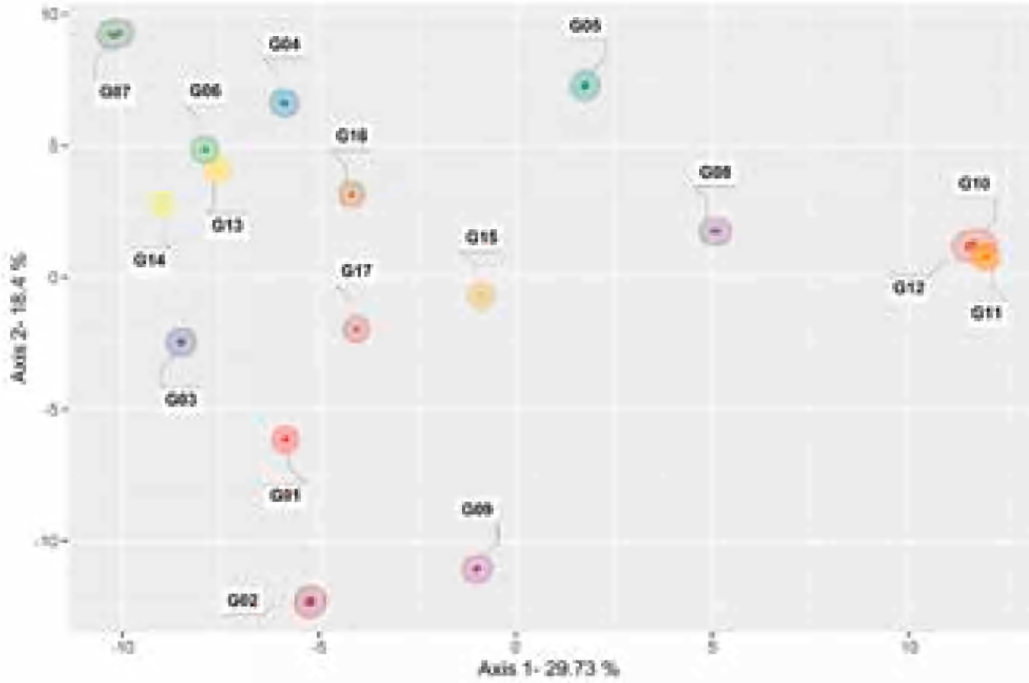


**Figure 3**. Principal components analysis of genetic differentiation for the set of 2980 SNPs for 50 *Eucalyptus imlayensis* individuals, colour-coded by genotype, with recurrent genotypes displayed with a circle and unique genotypes displayed with a triangle. See Suppl. file 2 for samples assigned to each genotype.

2008) and a tendency to succumb to environmental stressors resulting in the loss of intraspecific lineages (Levin 2019). Species restricted to a single population or few populations with few genotypes are consistent with remnant species rather than with neospecies, as suggested by Crisp and Brooker (1980) when describing *E. imlayensis*. A multi-species multi-population phylogenetic analysis to evaluate the relationship of *E. imlayensis* with both narrow-ranged and widespread relatives would provide context for developing conservation strategies within a wider biodiversity framework (Rossetto *et al*. 2021) but was beyond the scope of this study.

While many of the *Eucalyptus imlayensis* ramets

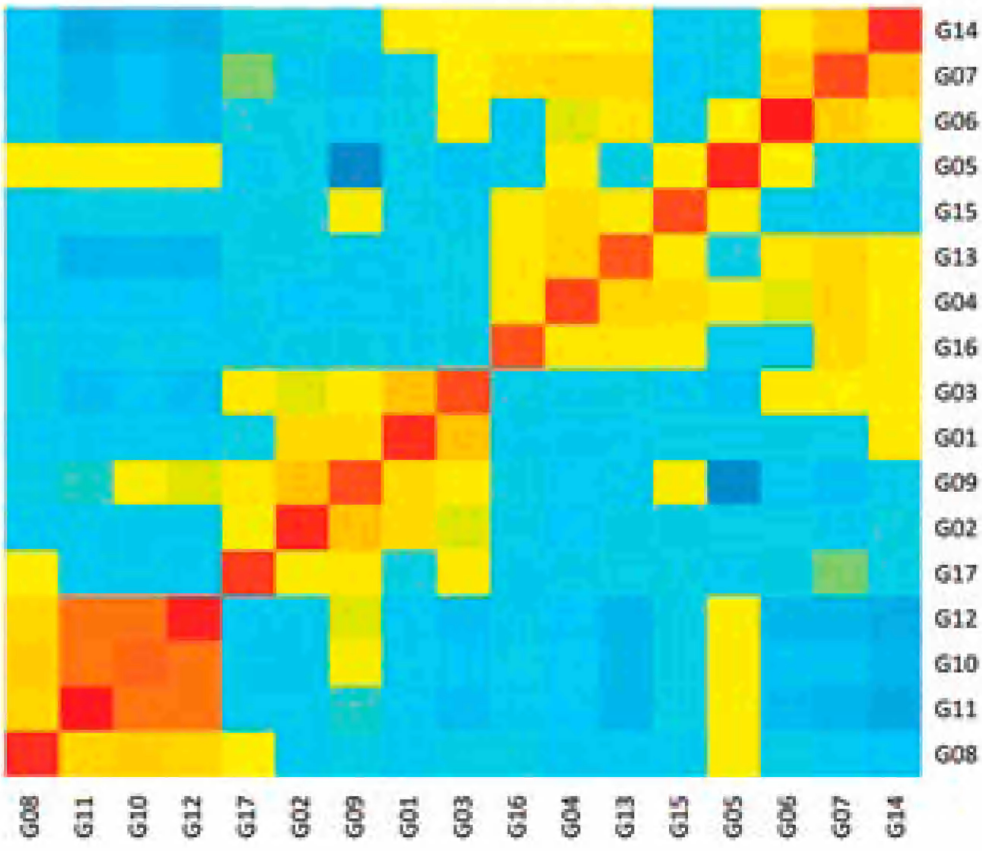


**Figure 4.** Heatmap showing probability of Identity by Descent for the 17 *E. imlayensis* genotypes (3165 SNPs, 17 samples). Each small square in the IBS distance matrix represents the genetic distance value between two pairs of genotypes. Higher probability values are shaded in red, indicating closer relationships between genotypes, while blue indicates lower probability and less closely related genotypes.

identified in this study corresponded to previously tagged clones, loss of a genet since 2007 is consistent with *E. imlayensis* being a relict species reliant on clonal growth and impacted by the loss of genotypic diversity over time. All sampled stems were resprouting from lignotubers and in some cases, stems from apparently separate lignotubers were found also to be clonemates. Clones varied in size with the largest (*G10*) extending over an area of 236 $m^2$. Size difference may be due to differences in the ages of clones but competitive advantage may also favour the expansion of some clones over others. Clone *G10* appears to have become fragmented because lignotubers with ramets belonging to clone *G10* were no longer obviously connected physically, or necessarily even the closest. Clones of woody species such as eucalypts are difficult to age because the lignotubers expand and fragment over time, losing obvious signs of previous physical connection. Several estimates of clone age in mallee eucalypts suggest that individual genets can survive for thousands of years (*E. curtisii*, Smith *et al.* 2003; *E. argutifolia*, Kennington & James 1997; *E. phylacis*, Rossetto *et al.* 1999; *E. absita,* Bradbury *et al.* 2016). Our findings are in keeping with what has been found in other rare, clonal eucalypts and clones of *E. imlayensis* are likely to be of a similar age.

Clonal growth confers some resistance to disturbances such as fire by enabling persistence when sexual reproduction is infrequent (Bond & Midgley 2003, Gross & Caddy 2006). The combined effects of drought, fire severity and frequency, and plant growth characteristics are likely to be elements in the persistence of fire-affected plants but their impact on the effectiveness of resprouting is understudied in Australian temperate forests (Bendall *et al.* 2022). Fire frequency is known to reduce the ability of eucalypts to resprout from lignotubers if the interval between fires is too short for energy reserves to be replenished (Fairman *et al.* 2019). Shorter fire intervals can be detrimental to seed production and seedling establishment leading to increased reliance on vegetative reproduction. Historically, fire occurred infrequently on Mt Imlay (McDougall *et al*. 2023), reflected in the high number of obligate seeders present. The lignotubers of *E. imlayensis* clearly provided a level of fire resilience to the 2019/2020 fires but greater mortality may occur if climate change driven increases in fire frequency and severity reduces a plant's ability to re-sprout (Fairman *et al.* 2019; Bendall et al 2022).

At present, very little seed of *Eucalyptus imlayensis* is stored in conservation seedbanks due to often unreliable natural seed production. To improve the conservation value of future seed collections, information about seed viability and paternity of open pollinated progeny arrays is still needed to determine whether viability and seedling vigour are affected by parentage. Separate storage of seed lots is vital for maintaining maternal lines and biparental crosses in conservation germplasm collections (Commander et al. 2018), and this is particularly true for mallee eucalypts, where seed production is low (Wellington & Noble 1985), as well as for many other predominantly clonal species (Bond & Midgley 2003). Factors contributing to reduced fecundity could include the differential effects of inbreeding and self-incompatibility found in some eucalypt species (Horsley & Johnson 2010; Nicklas *et al.* 2019) or an accumulation of somatic mutations that affect sexual but not vegetative reproduction (Gross *et al.* 2012). Lignotubers may also be a growth characteristic that enables species with low fecundity to persist where site conditions are rarely conducive to seedling establishment even when some viable seed is produced. The viability rates of seed from *E. imlayensis* is not known, but seedlings have been grown successfully *ex situ*. Natural seed production at the remnant site has been observed to be infrequent, low in number, and sporadic, and time to flowering and hence seed production in the population post-fire has been estimated to be at least 10 years (McDougall et al. 2023).

The small number of *E. imlayensis* genotypes means that it is feasible for all to be represented in *ex situ* conservation collections and these would provide a valuable genetic resource for breeding or seed production programs (Amor *et al*. 2020). *Ex situ* collections are ideal for maintaining control over genotype, creating insurance populations, and providing germplasm, including pollen, for experimentation and seed production in the field and off-site. However, eucalypts are often difficult to propagate vegetatively and previous attempts to graft *E. imlayensis* onto related species (e.g. *E. dalrympleana*) have not been successful (Peter Bredell, Australian National Botanic Gardens,

pers. comm. 2022). Micropropagation methods that have been successful for the multiplication of eucalypt germplasm (Trueman *et al.* 2018) may provide a better option, although the lead time for micropropagated plants to flower is unknown.

Despite the presence of 17 genotypes, many individuals are closely related which could affect the success of a breeding program and there are still many biological unknowns about the species. For sexually reproducing species, erosion of genetic diversity is a risk when populations are limited. Where sexual reproduction is compromised there may be little scope for genetic recombination or to maintain existing genetic diversity. The historical loss of intraspecific lineages of *E. imlayensis* resulting in closely related extant individuals may be a factor in a compromised mating system. Identification of *E. imlayensis* genotypes and measures of their relatedness provides a genetic basis for examining breeding and pollination systems. Studies of widespread eucalypts have shown a bias towards outcrossed seed or germinants and a deficit of homozygotes. This is thought to be due to the purging of individuals resulting from self-fertilisation in open pollinations containing mixed pollen loads (Horsley & Johnson 2010, Nickolas *et al.* 2019). Little is known of the seed viability in *E. imlayensis*, and whether selection against homozygosity post seed production occurs as it does for other rare clonal mallees (Kennington & James 1997).

Genetic interventions may be warranted for conservation targets using genetically optimised population augmentation or introduction (Sampson & Byrne 2016), manipulated pollinations to minimise genetic erosion (Cook & Sgrò 2019) or translocations from *ex situ* collections (Thomas *et al.* 2021; Rutherford *et al.* 2022). A workflow, adaptable for other species, was designed by Bragg *et al.* (2021) to ensure that *ex situ* collections of an endangered rare eucalypt, *Eucalyptus* sp. Cattai, were genetically optimised for conservation by manipulating the genetic diversity represented. With additional research, keeping in mind that the available gene pool is highly restricted, a pollination program could be implemented for *E. imlayensis* that minimises inbreeding while maximising the retention of genetic diversity. Self-fertilisation and biparental inbreeding should be avoided. Seed production from crosses between the most genetically distinct lineages of *E. imlayensis* (pairs of genotypes represented by lighter colours in Table 1) should minimise the loss of diversity and reduce the risks associated with inbreeding. New plants grown from seed, each of a unique genotype, have the potential to contribute to improving genetic resilience due to recombination during sexual reproduction. Even so, with so few extant *E. imlayensis* genotypes, it may be necessary to perform controlled pollinations between any genotypes of plants flowering concurrently rather than the ideal of plants of greatest genetic distance.

The conservation of a species restricted to a single, small population provides both opportunities and novel management challenges, including a restricted gene pool. Competition and site conditions are likely to limit seedling establishment and options for augmentation or translocation. While the remnant site is not considered suitable for population enhancement due to its to rocky profile and shallow soil, 40 tubestock seedlings grown *ex situ* were successfully established near the remnant site on Mt Imlay in 2011 and 2018 (McDougall *et al.* 2023). Thirty-seven of these plants survived until they were killed in the 2019/20 fires. This outcome highlights the vulnerability of plants that have not yet developed sufficient protection for buds or the ability to resprout after high intensity fire. However, the assisted establishment of seedlings suggests that additional translocation enhancements may be a feasible conservation option if sufficient seed is available, and the chance of survival is deemed to outweigh the risk of fire impacting the site before plants have established lignotubers.

The future persistence of *E. imlayensis* is likely to be affected by various factors, not all of which can be controlled, including ongoing climate change, increasing fire frequency and intensity, competition from other species such as *Leptospermum scoparium*, and potentially the impact of disease-causing agents such as *Phytophthora cinnamomi* (Department of Environment and Energy 2018). The practicalities of establishing a genetically representative *ex situ* population, success of cross-pollinations and seed production, feasibility of storing viable pollen and the cost effectiveness of their implementation are still to be evaluated by conservation managers, but the high priority conservation actions for *E. imlayensis* are those

that are most likely to be achievable and to contribute to the species future persistence. They include a genetic focus on collecting seed from the wild, testing the genetics and viability of seedlots, and expansion of the genetic representation and number of stored seeds from both natural and controlled pollinations. Additionally, an *ex situ* collection is not only valuable as insurance against repeated fires but will provide an experimental laboratory for undertaking breeding system studies and support on-ground application of a genetically supported conservation program for *E. imlayensis*.

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## Supplementary files

1. Unfiltered matrix genetic distance, unfiltered, all samples (55, including technical replicates)
2. Matrix of kinship values filtered (50 samples, no technical replicates)
3. Heterozygosity and fixation indices for 17 individuals (one representative of each of 17 genotypes)
4. Neighbour-joining tree showing clusters of *Eucalyptus imlayensis* stems based on genetic similarity (50 dataset). Samples eim131 and eim142 (G11 and G12, respectively) were identified as first-degree relatives of G10 clonemates and are embedded with them. Samples eim107, eim120, eim125, eim140 and, eim148 are unique genotypes G13, G14, G15, G16 and G17, respectively.

Supplementary files 1–4 are appended to the online listing of this paper, accessible via: https://www.rbg.vic.gov.au/science/journal/