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**Genetic analysis
to inform management of
Mallee Box, *Eucalyptus cuprea***

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**Department of
Environment and Conservation**

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(412) Genetic analysis to inform management
AM of mallee box, *Eucalyptus cuprea*

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Background

Eucalyptus cuprea Brooker & Hopper was declared as Rare Flora in 1987 and ranked as Critically Endangered in 1998. It is known from 4 disjunct population groups from north of Galena to south of Northampton. Field and anecdotal evidence suggests that *E. cuprea* resprouts after disturbance from a persistent lignotuber. It has a typical mallee-form and there is contention about the number of genetically distinct individuals and the size of populations. Some plants occur on nature reserves but the majority are on road reserves or private property. Investigation of genetic diversity of the species is required to confirm the presence of genetic clones within populations and to describe the genetic structure as a basis for management.

In general, members of the genus *Eucalyptus* are animal-pollinated and self-compatible but produce predominantly cross-pollinated (outcrossed) seeds. Seeds that are outcrossed can be half siblings (different male parents) or full siblings (same male parent). Field evidence for *E. cuprea* shows low fruit production and a lack of seedling recruitment. Low plant numbers, increased distance between populations because of vegetation clearing and variable flowering time and density may increase the level of inbreeding in remnant populations and are a key issue for consideration in conservation strategies.

The Interim Recovery Plan for *E. cuprea* identified a requirement for information on population genetic structure, reproductive strategies and mating system to support recovery actions. This project identified suitable genetic markers for *E. cuprea* and undertook genetic analysis to determine whether clumps of stems within populations were genetically identical, to describe the distribution of genetic variation among populations and to determine the level of outcrossing within seed crops.

Activity

Undertake genetic analysis of *E. cuprea* to determine clonality within populations, genetic structure among populations and mating system.

Methods

Collections of leaf material were made from individual stems from populations in each of the locations as in Table 1. The term population is used here to refer to all separate stands of *E. cuprea* whether they consist of one or numerous individuals. GPS locations were taken of each stem sampled to enable identification of proximate stems with the same genotype. These collections represent the majority of *E. cuprea* populations however some populations on private property were not sampled because permission was not able to be obtained from the landholders at the time of sampling. Mature seed capsules were collected from seed crops when present, which resulted in 10 seed samples, although three of these were subsequently found to contain no viable seed.

DNA was extracted from all plants using Qiagen mini-kits. 30 microsatellite primers for eucalypts were tested and the most informative 12 loci were used to genotype all samples of *E. cuprea*. Genotypes were scored from the raw data using GENEMAPPER version 4.0 and

statistical analyses undertaken using PowerMarker V3.25, STRUCTURE 2.2, MEGA4 and GenAlEx (v. 6).

Seeds from seven plants were germinated and DNA extracted from 24 seedlings per plant by a small-scale version of the method of Doyle & Doyle (1990) with the addition of 0.1M sodium sulphite to the extraction buffer. The DNA was assayed for 6 microsatellite loci using eucalypt primers. Genotypes were scored from the raw data using GENEMAPPER version 4.0 (Applied Biosystems) and statistical analysis undertaken using GENETICSTUDIO. Outcrossed seedlings were identified by the presence of non-maternal alleles.

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Table 1 Details of populations sampled for genetic analysis

Location	Site	No. stems sampled	No. of genotypes	Seed collections	Comments
Southern populations					
Howtharra Road	Nature Reserve	4	1	1 (no viable seed)	All stem groups sampled
Bella Vista	Nature Reserve	6	1 & 1*		All stem groups sampled
South western populations					
Stannich Clump P	Private property	5	2		One of several small populations within 1-2 km ²
Stannich Clump U	Private property.	3	3	1	One of several small populations within 1-2 km ²
Stannich Clump O	Private property	3	2		One of several small populations within 1-2 km ²
Stannich Clump Q	Private property	2	2		One of several small populations within 1-2 km ²
Stannich Clump PC	Private property	2	2		One of several small populations within 1-2 km ² . Identity uncertain, possibly not <i>E. cuprea</i>
Stannich Clump R	Private property	3	1		One of several small populations within 1-2 km ²
Chillamony	Nature Reserve	3	1		
Le Steere 1 (2B)	Private property	5	3	2 (1 - no viable seed)	
Le Steere 2 (2A)	Private property	8	5		Collected by Gemma Phelan, individual GPS coordinates not collected
South eastern populations					
East Ogilvie road verge	Road verge	3	3*		Identity uncertain, possibly <i>E. cuprea</i>
East Ogilvie Johnson granite	Private property	7	6	2 (1 - very little seed)	Several clumps scattered over sheet granite
East Ogilvie Johnson road verge	Private property	8	7		Several clumps adjacent to granite population
Northern population					
Mary Springs	Private property	28	19	4	Disjunct population

* Probably not *E. cuprea*

Results

Genetic diversity

The microsatellite loci showed moderately high allelic diversity with 153 alleles over the 12 loci and allowed reliable identification of clonality and population structure within *E. cuprea*.

There are some plants where identity is uncertain. This includes a small tree away from the main clump in Bella Vista Nature Reserve, stems in a cluster on Stanich property (named Stanich PC) and three trees on a road verge in East Ogilvie. The samples from the tree at Bella Vista and two of the road verge trees had genotypes that were not consistent with those of the rest of the *E. cuprea* individuals (see Figure 1 and 2) and these trees are probably some other species. One of the road verge trees had a genotype that was intermediate between the non-*E. cuprea* individuals and the *E. cuprea* trees and may represent a hybrid. The two samples from Stanich PC showed genotypes that were consistent with being *E. cuprea*. Taxonomic confirmation of the identity of these trees should be made.

The presence of clonality in stem clumps is identified by the same genotypes in stems from the same clumps. Generally stems from the same clump showed identical genotypes indicating one clone in each clump, except for clumps in Stannich U and Le Steere (2B). Some clonal clumps were quite large covering linear distances of up to 25m. Each of the clumps present in the three nature reserves were clonal clumps therefore, of the 13 individual stems sampled from nature reserves, there were only three distinct *E. cuprea* individuals found, one in each reserve.

Assessment of genetic structure in *E. cuprea* was made by combining sample sites into four groups based on geographic proximity; northern (Mary Springs), south eastern (East Ogilvie), south western (Stanich, Chillamony, Le Steere) and southern (Howatharra, Bella Vista). Overall, genetic differentiation was high (diversity = 0.256) and 27% of this variation was distributed between groups. Populations had moderate to high levels of observed heterozygosity ranging from 0.488 to 0.792 and expected heterozygosity ranging from 0.250 to 0.660.

Analysis of genetic relationships between individuals by a principal co-ordinates analysis also illustrates the diversity of these groups (Figure 1). Individuals did not cluster into discrete groups however there was separation of the northern Mary Springs population from southern, south western and south eastern populations. South western and south eastern populations tended to separate from each other. The separation of the non-*E. cuprea* individuals and the one intermediate individual can be seen in the PCA.

A Bayesian analysis in which individuals are assigned to clusters on the basis of their genotype produced similar results to that of the PCA (Figure 2). The putative non-*E. cuprea* individuals from Bella Vista Nature Reserve and East Ogilvie Road Verge were clearly different to *E. cuprea* (1a) while the third plant in the East Ogilvie Road verge stand is probably a hybrid. Two plants of questionable identity collected from Stannich Clump PC had genotypes like *E. cuprea* (1b). Four groups were found among the remaining plants; the Stannich populations and Le Steere populations in the south west, East Ogilvie and the northern Mary Springs population.

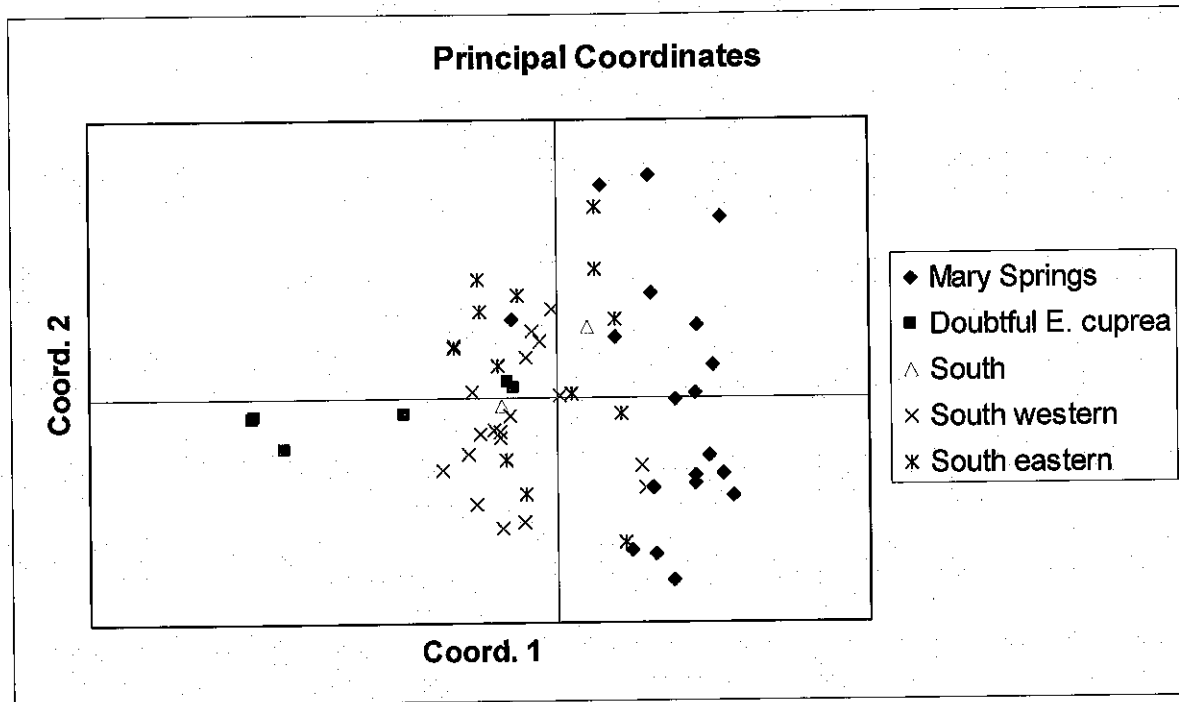


Figure 1 PCA analysis of the relationships between genotypes.

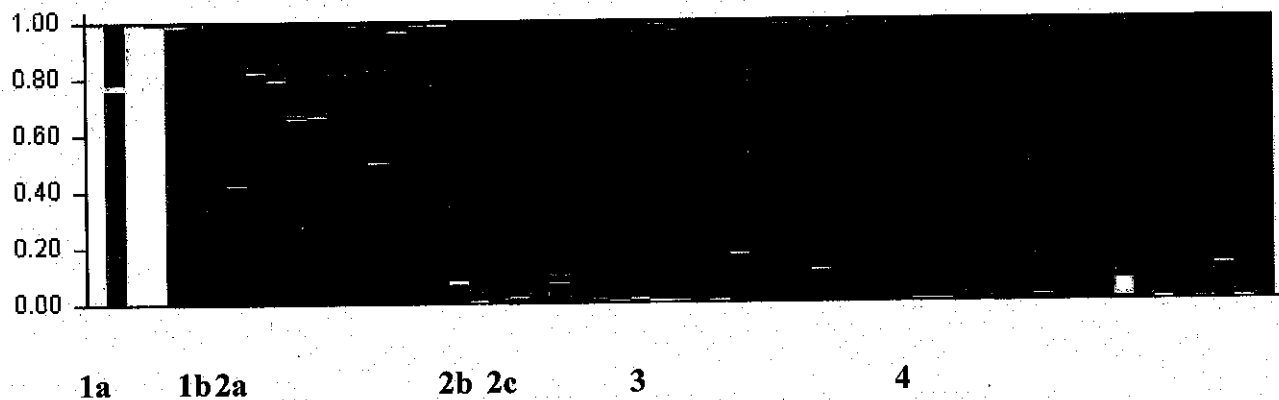


Figure 2 Results of assignment analysis for individuals of *E. cuprea*. Plots show show assignment of individuals to different genetic groups shown by different colours. Each individual is represented as a vertical bar broken into coloured segments representing components of different genetic groups. Numbers below the plot indicate bars that represent individuals from groups: **1**, doubtful *E. cuprea* from **(1a)** Bella Vista Nature Reserve and East Ogilvie Road Verge and **(1b)** Stannich PC; **2**, South western stands divided into **(2a)** Stannich, **(2b)** Chillamony NR and **(2c)** Le Steere; **3**, South eastern stands; **4**, Mary Springs.

Mating system

Very few (~14%) of *E. cuprea* plants sampled had capsules present and of these two had no viable seed and one had only 8 shrunken seeds. Viable seed was obtained from 7 individual trees.

The level of outcrossing among the plants sampled ranged from 46.2%, which is at the low end of the range for eucalypts, to predominant outcrossing of 90.9%. Such a range reflects the plasticity of the eucalypt breeding system which is affected by a range of ecological factors such as flowering density and phenology and the availability of pollinators.

Both low and high individual outcrossing rates were found in plants from small and larger populations. In the south western populations, at least half of the pollen fertilizing ovules surveyed came from outside the population. Potential sources of this pollen were identified in populations within 500m and 5km of two mother plants indicating that, rather than being isolated, these small populations form neighbourhoods of interbreeding plants connected by pollen transfer over significant distances.

In contrast, there was no evidence of pollen from outside the population fertilizing ovules in the more isolated Mary Springs or East Ogilvie Johnson granite populations. This is most likely because of the large distance to other populations. Two plants in the Mary Springs population had low outcrossing rates although this was a relatively large population with more potential male parents. A TWOGENER comparison of the pollen pools sampled by plants in the Mary Springs population shows that they were significantly different from each other ($\Phi_{ft} = 0.138$, $P < 0.001$). This is probably because all the plants do not flower at the same time and to the same extent so that the pollen available to one plant is very different to that of another.

There was evidence that many seeds on individual mother plants also shared the same male parent and this correlation of paternity may be a significant source of inbreeding. At Mary Springs, analyses suggest three to four male parents are found among seed from individual plants. At East Ogilvie Johnson Granite, the only plant that produced seeds was highly outcrossed but one adjacent plant was the source of 70.6% of the successful pollination. Therefore most of the seeds produced by this tree are full siblings and don't represent an outcrossed diverse seed collection. Thus there are two significant sources of inbreeding in *E. cuprea*. Firstly inbreeding arising from self-pollination and secondly, from outcrossing to relatively few male parents.

Table 2 Estimates of the proportion of seeds produced from outcross pollination of individual plants of *E. cuprea*

Location	Minimum % of seeds outcrossed per plant	Minimum % of outcrossed seed cross-pollinated by plants from outside the population	Comment
Stannich Clump U	90.9	55.0	Possible male parents of seeds crossed outside the population found within 500m
Le Steere 1 (2B)	46.2	50.0	Possible male parents of seeds crossed outside the population found within 5km
East Ogilvie Johnson granite	85.0	0	70.6% of outcrossed seed are full siblings
Mary Springs	78.3 57.1 87.5 47.8	0 0 0 0	Average number of male parents represented in seeds from individuals mother plants is 3.6

Outcome

Genetic analysis confirms that *E. cuprea* is clonal and that the number of genetically different individuals in the remnants is less than the number of stems. Only one genotype is represented in each of the three small remnants in nature reserves. In general each clump of stems represents a mallee individual and therefore a single genotype. Some single genotypes can be quite large such as those at Howatharra and Bella Vista Nature Reserves

Several individuals that were considered to be *E. cuprea* are not *E. cuprea* and require definitive identification.

Remnant populations of *E. cuprea* are differentiated from each other and can be grouped as (1) northern Mary Springs, (2) Howatharra Road Nature Reserve, Bella Vista Nature Reserve, Stannich populations, (3) Le Steere populations and (4) the south eastern East Ogilvie populations. Unsourced plants on private property are likely to represent further diversity. Conservation of plants from each of these areas would be necessary for representative genetic diversity.

Microsatellite genotypes confirm that outcrossing occurs in *E. cuprea* but the amount varies between plants and is not directly related to population size so that plants in larger populations may contain significant proportions of inbred seed. Dispersal of genes through the transfer of pollen does occur among clusters of populations and was detected up to 5km. There was no evidence of gene dispersal into the isolated populations of Mary Springs and East Ogilvie Johnson from other more distant populations. Seed from plants, particularly in isolated populations, have few male parents and this correlation of paternity is a form of inbreeding that should be considered in any seed collection for the purpose of conservation.