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Short Communication

Extent of clonality, genetic diversity and decline in the endangered mallee *Eucalyptus imlayensis*

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Abstract. *Eucalyptus imlayensis* Crisp & Brooker is a rare mallee known from one location in south-eastern Australia. Discovered in 1977, the population has declined in number and health of stems since 1998. Inter-simple sequence repeat (ISSR) markers were used to assess genetic variation and clonality. Only five multilocus genotypes were distinguished from 27 samples and the proximity of like genotypes within the population is consistent with the population being largely clonal. This means that the species has a much lower number of genetic individuals than is suggested from a census of the number of stems present. The implications of this finding for *ex situ* conservation of the species are discussed.

Introduction

Clonal reproduction is common in many plant species, and retention of sexual reproduction by clonal species has been reported by many authors (Ellstrand and Roose 1987; Eriksson 1993; Pandit and Babu 2003). A combination of sexual and asexual modes of reproduction is thought to enhance a species' ability to persist in the landscape. Sexual reproduction produces novel genetic combinations that may be an advantage under changing environmental conditions (Stearns 1987), whereas asexual reproduction maintains well adapted genotypes and population size (Pandit and Babu 2003). The dominant reproductive mode can influence the extinction risk for clonal species, depending on environmental factors. On one hand, predominantly clonal species are potentially at risk because they lack the adaptability enabled by sexual reproduction, while on the other hand, they have a lower risk of extinction than non-clonal species because vegetative growth can maintain successful genotypes and expand population size when conditions are unsuitable for seed production and seedling recruitment.

Asexual reproduction can increase relative to sexual reproduction under unfavourable environmental conditions such as habitat fragmentation (Rossetto *et al.* 2004) or grazing (Kleijn and Steinger 2002) and such a shift can be temporary or permanent (Honnay and Bossuyt 2005). The relative importance of sexual *v.* clonal recruitment can vary widely also among populations within species and has been demonstrated, for example, in *Acacia anomala* (Coates 1988), *Grevillea rhizomatosa* (Caddy and Gross 2006) and *Decodon virticillatus* (Eckert *et al*. 1999) where some populations appear to be maintained entirely asexually while others rely on seed production. Where the relative importance of reproductive modes differs between populations of species capable of asexual reproduction, census data can provide erroneous estimates of the number of individuals present (Coates 1988; Kennington *et al*. 1996; Warburton *et al*. 2000) and detrimentally influence the actions taken for conservation.

Eucalyptus is the largest genus in the family Myrtaceae, with most species occurring only in Australia (Ladiges 1997). Several rare eucalypt species are mallees, multi-stemmed trees often capable of clonal reproduction. New stems are derived from a specialised underground organ, the lignotuber, and clonal growth occurs by expansion of the lignotuber and/or peripheral production of stems that subsequently develop independent root systems and lose their connection to the original lignotuber (Kennington *et al*. 1996).

Eucalyptus imlayensis is a mallee with smooth grey, salmon, orange or green bark that sheds in long ribbons (Hill 1991). Plants grow from large lignotubers to ~7 m tall. *E. imlayensis* has been recorded only on the eastern face of Mount Imlay, an isolated peak (886 m above sea level) in South East Forests National Park, in the south-eastern corner of mainland Australia (Fig. 1). The species is listed as *Endangered* in the NSW *Threatened Species Conservation Act 1995.*

Despite its close proximity to a popular walking track, there have been no reports of *E. imlayensis* population size and structure since the discovery of the species in 1977. This is perhaps mainly due to the steepness and rockiness of the site. In 1998, at the time of the construction of a telecommunications tower on Mount Imlay, one *E. imlayensis* plant visible from the track was reported to be in poor health. A closer inspection of the site suggested that the total population size was small and some stems were apparently dead.

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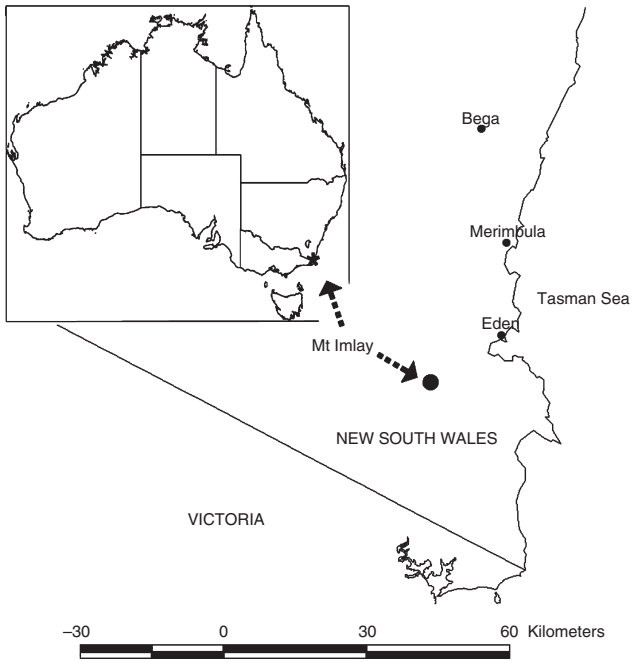

Fig. 1. Location map of Mount Imlay in the south-eastern corner of Australia.

This paper describes the single *E. imlayensis* population and its habitat, and assesses potential clonality. Because of its small population size and isolation, a future recovery action for *E. imlayensis* may involve collection of material for *ex situ* conservation. Therefore, one of the aims of this project was to provide information on the level and structure of neutral genetic variation present in the population.

Materials and methods

Species ecology

Between December 1998 and May 2003, observations were made of flowering, fruit production, plant health, recruitment and habitat characteristics in the *Eucalyptus imlayensis* population. The positions of all *E. imlayensis* plants were recorded in March 2001. Because of the dense canopy of shrubs on the site it was not possible to obtain accurate GPS locations. Instead, the relative position of plants was determined with a tape measure and compass. For this study, we define a 'stem' as a single shoot or trunk and a 'plant' as a group of stems that appeared to belong to the same lignotuber. Plants were given a number and tagged for future monitoring. The number of stems on each individual was counted and an estimate was made of the percentage of dead branchlets on each stem. Part of the population (23 plants) was reassessed in April 2002.

Genetic analysis

Inter-simple sequence repeats (ISSRs) were used to identify genetic variation in *E. imlayensis*. This method is suitable for species where little or no information is available on the genome (Zietkiewicz *et al*. 1994), a situation common with rare species. Generally, 1–3 ISSR primers are sufficient for identifying individuals (Wolfe *et al*. 1998).

Young leaf material was collected from 27 plants of *E. imlayensis* in April 2003, placed in separate vials containing silica gel and allowed to dry. Sampling was restricted to plants where foliage was accessible with minimal damage to the steep, rocky site. DNA was isolated from 20–30 mg of ground, dried material by using QIAGEN plant DNeasy minikits according to the manufacturer's protocol.

Nine primers were screened for their ability to consistently amplify DNA fragments. Primers were obtained either from Life Technologies by using published sequences ($AW3 = (GT)_6RG$; $AW7 = (CT)_8RG$; $17\,889 = BDB(AC)_7$; $17\,902 = (GT)_6AY$, where R = A or G; Y = C or T; B = C, G or T; D = not C) or from Rebecca Ramsden (Institute of Food and Land Resources, University of Melbourne, Vic.) (BDB, HB, 99A, NHA8, RGA4). Sequences for the last five primers are not available because of patent applications; however, some have been found useful for distinguishing individual *Eucalyptus* genotypes (R. Ramsden, unpubl. data). The primer used in this study is probably a modification of primer $BDB(ACA)_5$ used by Van der Nest *et al*. (2000) to amplify microsatellite regions in *Eucalyptus grandis*.

DNA was amplified in 20-μL reactions containing 10 μL QIAGEN HotStart Master Mix, 8.6 μL dH_2O, 0.4 μM primer and 1 μL (5–20 ng) of template DNA. Polymerase chain reactions (PCR) were performed in an Eppendorf MasterCycler gradient thermal cycler (Eppendorf, Hamburg, Germany), with the following profile: 95°C for 15 min (1 cycle); 94°C for 45 s, 44°C for 45 s, 72°C for 1 min (35 cycles); final extension step of 72°C for 10 min; 4°C soak.

PCR products were visualised on 2.0% agarose gels stained with ethidium bromide and viewed under UV light. Band presence/absence was scored manually.

Data analysis

In species where vegetative reproduction is likely, the analysis of genetic-diversity data can be flawed because a genet can be sampled several times but each sample treated as a separate individual. Where numerous individuals of the same genotype are clustered, it can be difficult to find a statistical basis for deciding whether individuals are asexual descendants of a single ancestral zygote or whether, by chance, this same genotype was produced independently many times via sexual reproduction (Parks and Werth 1993; Widén *et al*. 1994).

One approach is to calculate the probability, P (Eqn 1), once a particular genotype is found, of obtaining that same genotype, assuming sexual reproduction, in $(n-1)$ subsequently sampled individuals (Parks and Werth 1993; Sydes and Peakall 1998), as follows:

$$P = (P_{gen})^{n-1}, \quad (1)$$

where n is the total number of individuals with the same multilocus genotype and P_{gen} = probability of obtaining the observed multilocus genotype via sexual reproduction. If P is small, it can be concluded that the most likely explanation for the observed cluster of individuals of the same genotype is asexual reproduction.

For dominant markers such as ISSRs where only two phenotypes are possible (presence or absence of a particular fragment), P_{gen}, represented as P_{dgen} (Sydes and Peakall 1998), is calculated by using Eqn 2 as follows:

$$P_{dgen} = \Pi x_i, \quad (2)$$

where x_i is the frequency of whichever phenotype (band presence or absence) was observed at locus i in the individual being considered. This approach has been used by several authors to analyse data from suspected clonal species (Parks and Werth 1993; Widén *et al*. 1994; Sydes and Peakall 1998; Warburton *et al*. 2000).

As *E. imlayensis* has been observed to have multiple stems arising from a single lignotuber, analysis of ISSR data was carried out according to the above formulae to give the probability of genotypes having arisen independently more than once (Table 1). In addition, the position and multilocus genotype of each sample have been superimposed onto the site map (Fig. 2).

Results

Species ecology

Approximately 80 *E. imlayensis* plants were recorded in an area of ~1000 m^2. The imprecision of the count is the result of the difficult terrain and the close proximity of some individuals, which may have been one or several plants. The population occurs on a steep, east-facing slope near the summit of Mount Imlay, at an altitude of 850–870 m. The vegetation is a shrubland ~3 m tall, dominated by *Leptospermum scoparium*. Other common species at the site are *Boronia imlayensis*, *Cassytha pubescens*, *Derwentia perfoliata*, *Dianella tasmanica*, *Doodia media*, *Lomandra longifolia*, *Melaleuca squarrosa*, *Oxylobium ellipticum* and *Prostanthera walteri*. The ground layer is dominated by mosses.

Establishment from seed is probably a rare event. No seedlings or immature plants were located. Plants produced flowers and buds annually but only two fruits were observed and these were present at the commencement of monitoring. None of the seed from the two fruits germinated when placed in pots in a temperature-controlled glasshouse. Most flowering occurred in mid-summer, although one plant had numerous buds in May 2002.

Abundant psyllid leaf galls were observed in autumn on most plants on two occasions but not when leaf samples were collected for genetic analysis. The species of psyllid is unknown. It does not appear to affect the other eucalypt species adjoining the site. The soil-borne pathogen *Phytophthora cinnamomi* has been isolated from soil at the summit of Mount Imlay and from the roots of a dying *Banskia spinulosa* subsp. *cunninghamii* plant adjoining the *E. imlayensis* population on the steep slope (McDougall and Summerell 2003). However, the susceptibility of *E. imlayensis* to infection could not be ascertained. No lesions were detected in dying *E. imlayensis* stems and, because of the rockiness of the site, it has not been possible to obtain root samples for testing.

In March 2001, 9.7% of the total *E. imlayensis* stems had no foliage. Four plants had no foliage at all. These plants still had their ultimate branchlets and had not shed their bark so had presumably died recently. The percentage of branchlets without foliage on each stem ranged from 0 to 100% with a mean (±s.e.) of 46 ± 3%. Almost one-third of plants were estimated to have more than 50% crown death. Of the 23 plants resampled in April 2002, two additional plants had no foliage (i.e. 100% dead branchlets). The mean proportion of dead branchlets on stems had increased significantly ($P < 0.01$) from 45 ± 7% to 56 ± 6%, as determined by a paired *t*-test of angular transformed estimates. Despite the apparent poor condition of many plants in the population, one plant, which had almost no crown foliage when first observed in November 1998, had produced numerous epicormic shoots when inspected in 2002. Regeneration from the base had occurred on one stem that had lost its crown foliage between the two surveys.

Although two fires have been recorded near the summit of Mount Imlay since 1949 (Bushfire and Environmental Services 2000), the population of *E. imlayensis* does not appear to have been burnt in recent times, judging from the lack of burn marks on trees on the eastern fall.

Genetic analysis

All ISSR primers tested amplified DNA samples from *E. imlayensis*. Primers BDB, 99A and NHA8 produced profiles that were clear and easy to score, whereas primer RGA4 did not detect any variation. In total, 20 bands were scored. Ten fragments were polymorphic. Five distinct multilocus genotypes (G1–G5) were found in the 27 samples tested.

Each sample was assigned a multilocus genotype on the basis of the combined fragment patterns of the three primers which were used to calculate the probability of genotypes having arisen independently more than once (Table 1). Multilocus genotypes differed from each other by a minimum of two and a maximum of nine ISSR fragments (Table 2). Samples with the same

Table 1. Multilocus ISSR genotypes, number of stems sampled, genotype-specific bands, distribution of polymorphic bands and the probability that the genotypes have arisen independently

Multilocus genotype	No. of stems sampled (% total population)	Genotype-specific bands	No. of polymorphic bands (% total no. bands in each clone)	Probability of genotype occurring	Probability of (P^{n-1}) being present *n* times
G1	15 (19.2)	0	4 (28.6)	0.289	2.84×10^{-8}
G2	2 (2.6)	2	4 (28.6)	0.003	2.70×10^{-3}
G3	2 (2.6)	0	2 (16.7)	0.589	5.89×10^{-1}
G4	2 (2.6)	0	3 (23.1)	0.652	6.52×10^{-1}
G5	6 (7.7)	4	5 (33.3)	0.002	6.66×10^{-14}

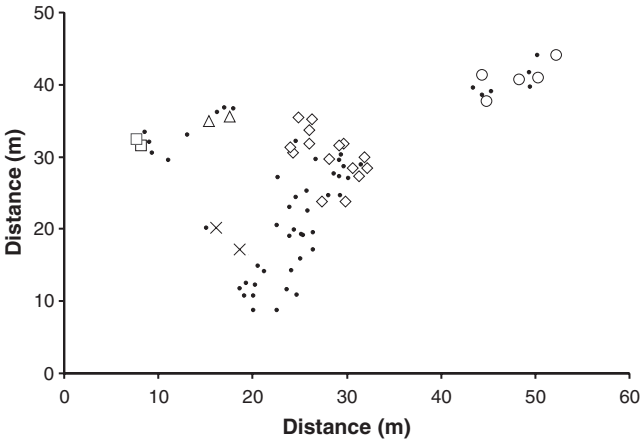

Fig. 2. Position of sampled plants with multilocus genotype indicated, G1 (◇); G2 (□); G3 (×); G4 (△); G5 (○); plants not sampled (●). Distance is measured from an arbitrary origin point.

Table 2. Number of band differences among the five ISSR multilocus genotypes identified

Clone	G1	G2	G3	G4	G5
G1	–				
G2	4	–			
G3	2	6	–		
G4	2	3	3	–	
G5	7	9	5	6	–

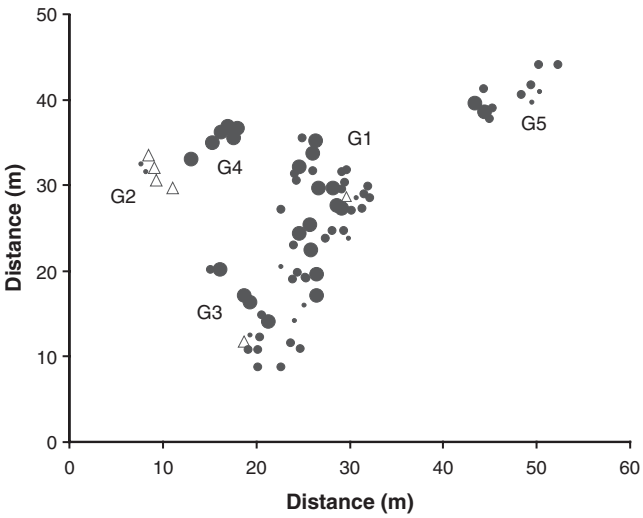

Fig. 3. Plant health (as indicated by the mean percentage estimate of branchlets without foliage), ≤1/3 of branchlets dead (small circles); >1/3 and ≤2/3 branchlets dead (medium circles); >2/3 and less than 100% branchlets dead (large circles); 100% branchlets dead (triangles). Distance is measured from an arbitrary origin point.

multilocus genotype clustered together and clone sizes for G1 and G5 are estimated to be a minimum of 114 and 50 m^2, respectively (Fig. 2). All of the plants in the vicinity of genotype G2 were in poor health or apparently dead whereas those in the vicinity of genotype G4 were in the healthiest class (Fig. 3). Plants in the other genotype clusters were of a range of health classes.

Two genotypes, G2 and G5, contained ISSR fragments that were not found in any other genotypes. These two genotypes also had the lowest probabilities of occurring ($P_{dgen} = 0.003$ and 0.002, respectively) given the frequencies of individual bands. However, given that they were present, the probability that two individuals with the same genotype had arisen by independent sexual reproduction is extremely small. In contrast, the probability of genotype G1 occurring was quite high ($P_{dgen} = 0.289$) but the probability of 14 additional individuals being present with the same genotype is extremely small (2.84×10^{-8}). The frequencies of polymorphic bands in genotypes G3 and G4 were high and as the genotypes were only sampled twice, the possibility that the two plants arose independently with the same genotype cannot be ruled out. On the basis of the proximity of the two samples, it is likely that they are clonal; however, additional ISSR data are required to resolve it statistically.

Discussion

Poor plant health combined with low number of genets makes active management a priority for the conservation of *E. imlayensis*. Five distinct multilocus genotypes were identified for 27 plants of *E. imlayensis* by using three ISSR primers, providing evidence that the genetic structure of *E. imlayensis* is largely the result of asexual reproduction.

The proximity of plants of *E. imlayensis* with genotypes that are indistinguishable using ISSRs is consistent with the population comprising a limited number of distinct plants with most stems arising from one of a few genets. Kennington *et al*. (1996) similarly found clusters of *E. argutifolia* plants with identical allozyme multilocus genotypes. They considered that the clustering was most likely a reflection of the clonal habit of the species rather than the result of limited seed dispersal of allozymically uniform individuals. As with *E. argutifolia*, there are considerably fewer genetically distinct individuals of *E. imlayensis* than is indicated by a census of populations.

The importance of assessing genetic variation in rare species capable of clonal reproduction is highlighted by the different patterns obtained in studies of rare species including *Eucalyptus*. For example, although *E. phylacis* and *E. dolorosa* are both known from single locations, *E. phylacis* comprises a single genet whereas *E. dolorosa* consists of several stands (Rossetto *et al*. 1999). In contrast, genetic assessment of another rare species, *E. graniticola*, showed it to be a single long-lived hybrid plant capable of resprouting, rather than a relict species (Rossetto *et al*. 1997). There has been no suggestion in the taxonomic literature that *E. imlayensis* is a hybrid.

Clonal plants are generally considered to exhibit genotypic diversity comparable to sexually reproducing species (Eckert 1999), largely based on reviews by Ellstrand and Roose (1987) and Widén *et al*. (1994). However, Honnay and Bossuyt (2005) argued that the conclusion should be moderated, given that 46.6% of species reported had at least one population occurring as a monoclonal patch (Eckert 1999; Eckert *et al*. 1999; Honnay and Bossuyt 2005). Limited empirical evidence (Warburton *et al*. 2000; Bartolome *et al*. 2002) supports a

tendency towards monoclonal patches. The exact mechanisms generating diversity in clonal species are poorly understood. However, if seed production was a major recruitment strategy early in a population's history, even low numbers of immigrants or rare sexual reproduction (Eriksson 1993) in long-lived species may be sufficient to maintain comparatively high levels of diversity.

Seed production can be inconsistent across and within rare clonal plants, and for some species, there is a strong association between seed fertility and genotypic diversity (Eckert and Barrett 1993; Dorken and Eckert 2001). Establishment of *E. imlayensis* plants from seed is probably a rare event. Although plants have produced viable seeds that have been used for propagation at the Australian National Botanic Gardens, Canberra they appear to do so rarely as none was observed during the 4 years of this study. The Australian species, *Elaeocarpus williamsianus*, consists of a single genet at most sites and sterile fruit is common, possibly a result of fragmentation disrupting a balance between vegetative and sexual reproduction in the species (Rossetto *et al*. 2004). Viable seed, thought to be the result of outcrossing, was recovered from one stand of *E. dolorosa* but none has been collected for *E. phylacis*, suggesting that there could be different mating systems operating. *E. imlayensis* may be an example of a species that is undergoing a shift in reliance from sexual to asexual reproduction.

There is thought to be a strong association between longevity and clonality, especially in woody species (Cook 1983). Honnay and Bossuyt (2005) suggested that even though widespread species may show no evidence of decline despite prolonged clonal growth, they may not be as persistent in the long-term because prolonged suppression of sexual reproduction and a shift to reliance on clonal growth can lead to monoclonal populations of species as expanding ramets of better adapted genotypes outcompete less adapted clones (Eriksson 1989). If the number of genotypes drops, local sexual extinction can result because of the lack of compatible mating types. For relict species with long-lived clones, it is conceivable that environmental conditions have changed so that in the current habitat, clonal rather than sexual reproduction is dominant. The restriction of *E. imlayensis* to the highest peak in the region could reflect historical climatic change.

It is of concern that viable seed production in *E. imlayensis* has not occurred recently. *Ex situ* conservation and testing of the species for susceptibility to infection by *Phytophthora cinnamomi* would be greatly aided by the propagation of plants from seed. Determining the mating system would be useful because even occasional mating between genets and subsequent seedling establishment could provide an important mechanism for maintaining genetic variation. The timing and extent of flowering, presence of incompatibility alleles or poor pollen viability caused by reasons other than genetics may all be contributing to the lack of seed production. Intervention to favour seed production and seedling establishment could be considered in the conservation management of the species.

Population decline, lack of reliable seed production, unknown success of seedling establishment, limited genotypic variation and restricted distributions are common threads in the conservation of rare clonal species. For an *ex situ* collection of *E. imlayensis* it will be essential to include each of the genotypes identified in this study. As the G2 genotype appears to be close to extinction, there is some urgency in commencing this collection. Further genetic testing of stems lower down the slope, if accessible in the future, might reveal additional genotypes. Given the small number of genetically distinct individuals identified so far, any additional genotypes would be valuable components of an *ex situ* population. In addition to being used to conserve the genotypes present in the population now, *ex situ* plants could be used to produce seed and so increase both the genotypic diversity and the number of plants. Once established, any new genotypes may well contribute to the diversity for many decades and so assist in the long-term viability of the species.

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