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# DNA fingerprinting of *Eucalyptus graniticola*: a critically endangered relict species or a rare hybrid?

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*Eucalyptus graniticola* is known from a single plant located on a granite outcrop south-east of Perth in Western Australia. Since its discovery in 1987, it has been uncertain whether this eucalypt is a relict species or a hybrid and, consequently, further study is required in order to devise appropriate conservation strategies. The similarity of features, such as leaf, bud and fruit morphology, to those of *E. rudis*, a common tree found in the vicinity, suggested that *E. graniticola* is a hybrid. This study uses random amplified polymorphic DNA (RAPD) analysis to demonstrate the additive inheritance of DNA markers from *E. rudis* and *E. drummondii*, the putative parent species, in *E. graniticola*. All the markers detected for *E. graniticola* using nine primers were shared with either *E. rudis* (40 per cent), *E. drummondii* (35 per cent) or both parent species (25 per cent). The DNA fingerprinting results, combined with other factors, such as the segregation of cotyledon morphology, demonstrate the hybrid origin of *E. graniticola*. As a result, conservation of this rare eucalypt should rely more on *ex situ* propagation and storage than on active management.

**Keywords:** conservation genetics, *Eucalyptus*, hybrid, RAPD, rare flora.

## Introduction

It has been estimated that up to 60 000 of the 250 000 plant species in the world could be extinct within the next 50 years (Holsinger & Gottlieb, 1991). Critically endangered species are at the sharp end of today's global extinction crisis. These are species judged most likely to become extinct in the immediate future unless remedial action is taken.

The south-west of Western Australia is a region especially rich in biodiversity and endemism. Of an estimated 8000 species of flowering plants, some 2000 may be of conservation concern (Hopper *et al.*, 1990), with 280 of these being in danger of extinction in the near future (Department of Conservation and Land Management, 1995). Natural (intrinsic) rarity underpins much of this problem. However, the more floristically diverse areas of Western Australia have undergone substantial anthropogenic change, resulting in high levels of endangerment (extrinsic rarity).

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Causes of rarity and critical endangerment are diverse, ranging from direct human destruction of wild ecosystems to processes such as recent evolutionary origin or reproductive failure of relict species under changed environmental circumstances (Fiedler & Ahouse, 1992; Pate & Hopper, 1993). A knowledge of such causes is vital for conservation managers interested in preventing extinction.

In this paper, we investigate an extreme case of a taxon, *Eucalyptus graniticola* Hopper ined., known from a solitary wild individual discovered in 1987 along the Darling Scarp to the south of Perth (Fig. 1). The eucalypt is a lignotuberous mallee 7 m tall and 8 m across with some 45 trunks. It occurs in dense heath in a shallow soil pocket on a granite outcrop surrounded by *E. marginata* forest (Fig. 2a).

Although buds and fruits of *E. graniticola* are reminiscent of those of *E. rudis*, they are smaller (Fig. 2b). Moreover, *E. graniticola* is a mallee with smooth white bark, whereas *E. rudis* is a tree with a stocking of rough, grey bark (Brooker & Kleinig, 1990). The apparent rarity of *E. graniticola* suggests that it is either a relict undescribed species or a rare

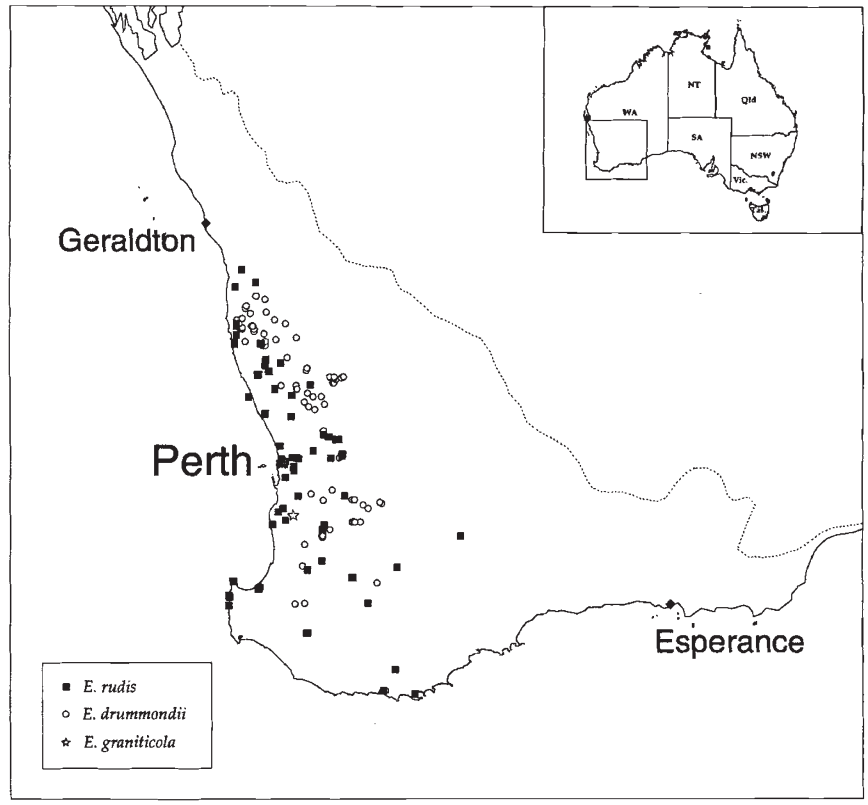


**Fig. 1** Geographical distribution of *Eucalyptus drummondii* and *E. rudis* showing the areas of overlap for the two species and the location of the *E. graniticola* site (data obtained from the WA Herbarium). The demarcation line indicates the south-west botanical province.

hybrid. The latter hypothesis requires the presence of likely parental species, and none is evident in the area immediately surrounding the granite outcrop on which *E. graniticola* occurs.

A few anomalous mallee eucalypts related to the smooth-barked, small tree, *E. drummondii*, were also discovered on steep granite slopes 5 km north of the *E. graniticola* location. This new population (referred to as 'small-fruited *E. drummondii*') had smaller buds and fruits than typical *E. drummondii* and showed some similarities to *E. graniticola*. Other species encountered within a 10 km radius of *E. graniticola* included *E. marginata*, *E. patens*, *E. rudis* and *Corymbia calophylla*, but *E. rudis* and *E. drummondii* appeared closest morphologically to *E. graniticola*.

On the available evidence, clear resolution of the identity of *E. graniticola* as a relict species or a rare hybrid was not possible. In any event, with only one individual known, the taxon was considered as critically endangered and requiring further study.

If relictual, *E. graniticola* would be expected to have unique DNA markers and to produce uniform progeny. If a hybrid, *E. graniticola* progeny would display segregation in characters such as cotyledon shape and additive inheritance of unique DNA markers from each parental taxon.

DNA fingerprinting relies on the detection of distinctive, parent-specific DNA markers within the progeny DNA profile. One of the techniques previously used for such a task is random amplified polymorphic DNA (RAPD; Williams *et al.*, 1990), a simple polymerase chain reaction (PCR)-derived technique capable of analysing a large number of loci inherited in a Mendelian fashion. It has been used successfully for detecting the hybrid origin of plant (Crawford *et al.*, 1993; Xu *et al.*, 1993; Rieseberg & Gerber, 1995) and animal species (Shoemaker *et al.*, 1994; Chu *et al.*, 1995).

This study applies RAPD analysis, progeny trials and fertility tests to resolve the identity of *E. graniticola*. A better understanding of the evolutionary origin of this eucalypt aims to provide basic information crucial for the development of appropriate conservation strategies.

## Materials and methods

### *Ecological survey*

Extensive searches for *E. graniticola* were undertaken from 1987 to 1996 on more than 100 granite outcrops throughout the ranges of *E. rudis* and *E. drummondii*. When populations were encountered, a

full inventory of associated species was made and the microhabitat occupied was recorded.

### *Plant material*

Voucher material of *E. graniticola* has been deposited at the Western Australian Herbarium.

Leaf material for DNA extraction was collected from *E. graniticola* and the closest known populations of the putative parents.

In order to test whether the only known *E. graniticola* mallee was a single plant, samples were collected from two opposite ends of the clump (g1 and g2).

Samples were collected from six distinct *E. drummondii* plants (d1–d6) at two sites on Mount Wells (the closest known population) approximately 25 km north-east of the *E. graniticola* site (Fig. 1).

Samples from four distinct small-fruited *E. drummondii* plants (sf1–sf4) were collected approximately 5 km north of the *E. graniticola* site.

Samples from four distinct *E. rudis* plants (r1–r4) were collected 7 km north of the *E. graniticola* site and a fifth sample (r5) at Mount Wells (approximately 4 km east of the *E. drummondii* site).

### *Seed material and pollen fertility*

Seed capsules for progeny trials and fertility tests were collected from populations of *E. graniticola*, *E. rudis* and *E. drummondii* (a minimum of 50 capsules each) in late March 1996 and dried in paper bags.

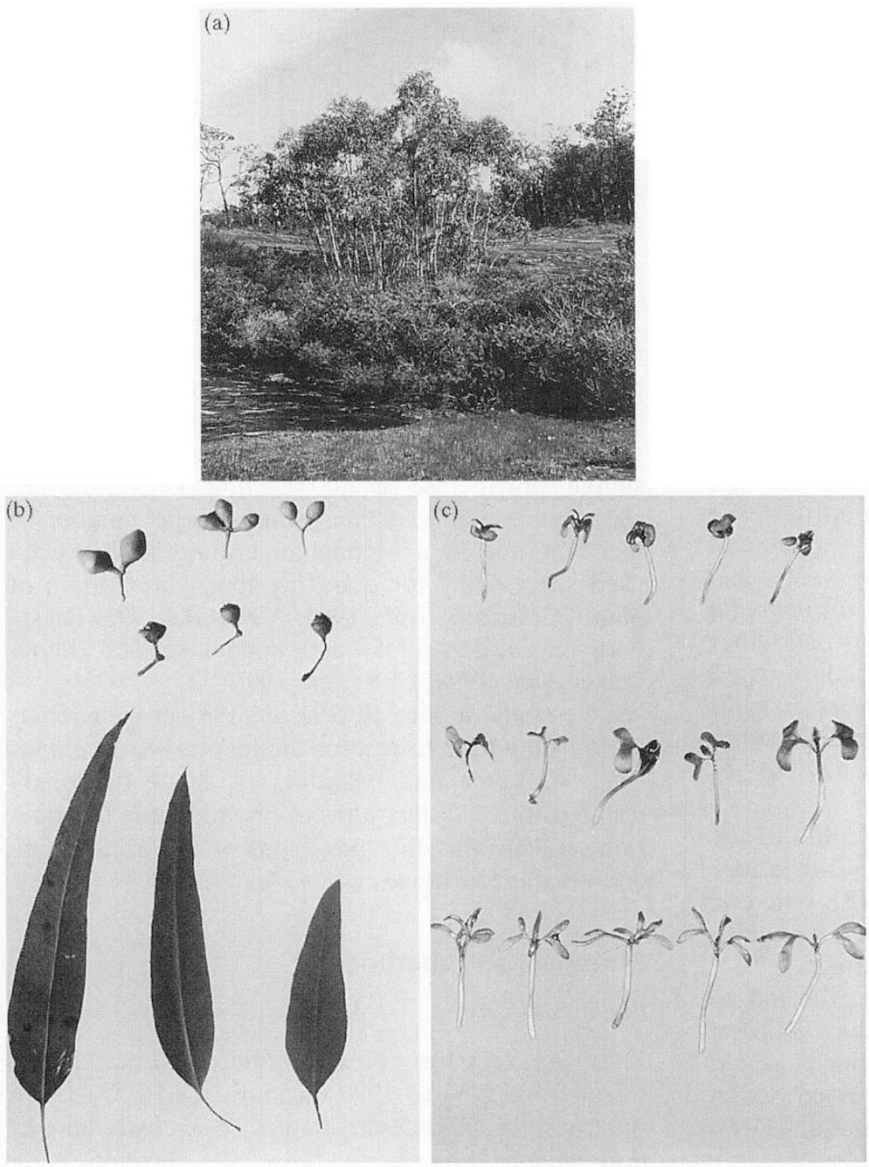


**Fig. 2** (a) The only known *Eucalyptus graniticola* plant in the wild. (b) Comparison of flower bud, fruit and leaf morphology for *E. drummondii* (right), *E. graniticola* (centre) and *E. rudis* (left). (c) Seedlings showing different cotyledon morphology for *E. rudis* (top), *E. graniticola* (centre) and *E. drummondii* (bottom).

Seeds were counted and subsequently germinated in the dark on moist filter paper. After germination, they were transferred to the light and eventually transferred to soil in a glasshouse. Number of seeds, germination success and cotyledon morphology were recorded.

Fresh anthers were squashed in a drop of aceto-carmine on a microscope slide, and a minimum of 200 grains were scanned for stainability and shape as indicators of viability.

### *Polymerase chain reaction*

DNA was extracted using a sodium dodecyl sulphate (SDS) protocol described by Rossetto *et al.* (1995). PCR was performed in a 12.5 $\mu$L total volume containing 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1 per cent Triton X-100, 2.0 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 units of Tth DNA polymerase (Biotech International), 50 ng of primer, 10 ng of DNA and DNA-free water. Each reaction mix was overlaid with 20 $\mu$L of PCR-grade paraffin oil. In order to test reproducibility, duplicate reactions were run and, if the banding patterns obtained were not reproducible, the primers were not used for the full course of the experiment. After selection, six 9-bp primers and three 10-bp primers were used (Table 1). PCR reactions were performed on a Hybaid OmniGene thermocycler programmed for an initial melting step at 94°C for 5 min, followed by 35 cycles each at 94°C for 15 s, 34°C for 45 s, 72°C for 1 min. A final extension step at 72°C for 10 min was performed after the 35 cycles. A negative control reaction, in which DNA was omitted, was included with every PCR run in order to verify the absence of contamination.

### *Data analysis*

Amplification products were resolved electrophoretically on 2 per cent agarose gels run at 60 V in TBE and visualized by staining with ethidium bromide. Lambda DNA cut with *Eco*RI/*Hin*dIII was used as a size marker. The gel images were digitized directly from the UV transluminator through a Sony SSC-M370 SE high resolution, black and white video camera. The images were then analysed using CREAM for Windows (Kementec) image analysis software.

The presence/absence data were used to obtain an estimate of similarity using Nei & Li's (1979) similarity index, calculated as $F = 2m_{xy}/(m_x + m_y)$, where $m_{xy}$ is the number of shared markers between two samples and $m_x$ and $m_y$ the number of markers for each sample. Group estimates were calculated by averaging interindividual similarities obtained for each group considered.

UPGMA analysis was carried out on the matrix of genetic similarity calculated using Nei & Li's (1979) index, and a dendrogram representing similarities between the 17 samples representing three taxa was obtained from it.

### *Hybrid band homology assessment*

Assessment to test homology of parental/hybrid bands was performed using the two *E. graniticola* samples, four *E. drummondii* samples and four *E.*

**Table 1** Summary of data obtained by RAPD analysis for six 9-bp-long primers and three 10-bp-long primers for the three eucalypts

| Primer sequence 5′ to 3′ | No. of bands for *E. graniticola* | No. of bands for *E. drummondii* | No. of bands for *E. rudis* | Approx. band size range (bp) |
|---|---|---|---|---|
| CCCACCAAC | 5 | 7 | 5 | 500–1250 |
| CCCTCCTTC | 5 | 7 | 4 | 530–1080 |
| GGGTTGTGG | 5 | 8 | 7 | 270–990 |
| GGGAGGAAG | 4 | 6 | 6 | 270–1150 |
| GGGTGGTTG | 8 | 8 | 8 | 370–940 |
| GGGTGTTGG | 5 | 7 | 6 | 260–1110 |
| TGAGCGGACA | 3 | 3 | 5 | 640–1500 |
| CAGGCGCACA | 4 | 4 | 3 | 680–1510 |
| CGGTGGCGAA | 4 | 7 | 6 | 600–1820 |
| Total | 43 | 57 | 50 | 260–1820 |

The table shows primer sequence, number of markers obtained with each primer for each taxon and the approximate size range of the markers detected.

*rudis* samples. The 640-bp fragment detected for *E. graniticola* and *E. rudis* and the 1210-bp fragment detected for *E. graniticola* and *E. drummondii* using primer TGAGCGGACA were tested using Southern hybridization. Amplification products were transferred to an Amersham Hybond $N^+$ nylon membrane and hybridized according to the manufacturer's instructions. Putative homologous fragments were isolated from an agarose gel with a Qia ex II (Qiagen) gel purification procedure and labelled with [$\gamma$-$^{32}$P]dATP. Hybridization protocols were similar to those of Byrne *et al.* (1993), except hybridizations and stringent washes were carried out at 50°C.

## Results

### *Ecological study*

*Eucalyptus graniticola* was only found at its type location, which is seasonally wet as a result of catchment from the surrounding sheet rock. It occurs in a 20-m-diameter soil pocket, with species favouring damp soils with high runoff, such as *Calothamnus quadrifidus* (Myrtaceae), *Acacia oncinophylla* (Mimosaceae), *Grevillea bipinnatifida* (Proteaceae) and *Lepidosperma* spp. (Cyperaceae).

*Eucalyptus rudis* and *E. drummondii* were rarely found on granite outcrops, at two and three sites, respectively. *Eucalyptus rudis* is a riparian or wet-site tree growing to 15 m, usually in waterlogged soils. When on granite, *E. rudis* is more abundant along adjacent creeklines, with just a few plants colonizing soil pockets upslope, growing with species of *Calothamnus*, *Lepidosperma* and *Hypocalymma* (Myrtaceae).

*Eucalyptus drummondii* is a small to medium tree growing to 5 m and usually occurs in drier, well-drained sites on the slopes and crests of hills and lateritic breakaways. On granite, it grows in shallow soils on dry north-facing slopes, with species such as *Gastrolobium spinosum* (Fabaceae), *Xanthorrhoea preissii* (Xanthorrhoeaceae) and *Daviesia horrida* (Fabaceae). These sites are usually upslope from large areas of sheet rock and do not enjoy significant runoff or waterlogging. Their dry soils are characterized by the absence of species of *Lepidosperma* and *Calothamnus*.

### *Morphological study*

*Eucalyptus graniticola* is an erect-stemmed mallee growing to 4 m high with smooth, creamy and powdery bark, dull leaves, 11 cm long by 2.5 cm wide, globular buds up to 9 mm long by 6 mm in diameter with a conical operculum, and a small subcampanulate fruit 6 mm long by 7 mm in diameter.

The cotyledon morphology of germinated *E. graniticola* seeds showed clear segregation between that of *E. rudis* (flat-kidney shaped) and that of *E. drummondii* (Y-shaped) (Fig. 2c).

Similarly, leaf length, bud and fruit shape of *E. graniticola* were intermediate between those of the putative parent species (Fig. 2b).

### *Seed and pollen viability*

The number of viable seeds was lower in *E. graniticola* (0.2 seeds per fruit) than in *E. drummondii* (1.1 seeds per fruit) and *E. rudis* (0.8 seeds per fruit).

Similarly, pollen fertility was estimated at 23 per cent for *E. graniticola* compared with 82 per cent or more for both *E. drummondii* and *E. rudis*.

### *Marker description and distribution*

Table 1 summarizes the banding pattern obtained with the nine primers for the three eucalypts studied. A total of 96 scorable markers were detected for all the samples tested with nine primers. The 9-bp-long primers consistently produced a greater number of markers (average of 6.2 per species) than the 10-bp-long primers (average of 4.3 per species). *Eucalyptus drummondii* produced the greatest number of markers (average of 6.3 per primer), followed by *E. rudis* (average of 5.6 per primer) and *E. graniticola* (average of 4.8 per primer).

Figure 3a illustrates the type of banding pattern obtained for the three taxa with one of the primers used (TGAGCGGACA). The gel shows that all the markers visualized for *E. graniticola* with the primer are shared either with *E. rudis* or with *E. drummondii*. Figure 3 also shows a clear negative control, which was the case for all the reactions used in this study.

Table 2 shows that all the markers identified for *E. graniticola* were found in either *E. rudis* (40 per cent), *E. drummondii* (35 per cent) or both (25 per cent). In other words, *E. graniticola* has no exclusive and specific markers. *Eucalyptus drummondii* and *E. rudis* share 25 per cent and 28 per cent of their markers with each other and possess 49 per cent and 38 per cent exclusive markers, respectively.

Southern hybridization showed homology for the fragments selected to represent parental markers within the fingerprint of the putative hybrid (Fig. 3b).

### *Estimate of similarity*

Table 3 shows the estimate of similarity within each taxon and the comparisons of similarity between taxa. The two samples obtained from opposite ends of the *E. graniticola* clump showed identical fingerprints. Within-species similarity ranged from 78 per cent to 88 per cent for the other eucalypts. If the *E. drummondii* sample and the small-fruited *E. drummondii* sample are grouped together, the similarity within these 10 plants is 79 per cent.

Table 3 shows high within-species similarity, even though it must be taken into account that the individuals tested represent only a small sample of the species. However, between-species similarity is low between the putative parents (26 per cent). This is not the case for *E. graniticola*, which shows high similarity to *E. rudis* (65 per cent) and to *E. drummondii* (52 per cent), as well as to the small-fruited *E. drummondii* (57 per cent). The small-fruited *E. drummondii* sample is highly similar to *E. drummondii* (75 per cent), but not to *E. rudis* (26 per cent).

The dendrogram in Fig. 4 illustrates the similarities between the 17 individuals tested, based on similarity results obtained by RAPD analysis. The major taxa are clustered, whereas *E. rudis* and *E. drummondii* are distant (with the small-fruited *E. drummondii* within the *E. drummondii* clump), whereas *E. graniticola* is intermediate between the two potential parent species.

## Discussion

### *Evidence for hybrid derivation of* E. graniticola

RAPD analysis has clearly demonstrated the additive inheritance of DNA markers in *E. graniticola* arising from both *E. rudis* and *E. drummondii*. On this evidence, the identity of *E. graniticola* as a rare hybrid of these parental taxa is strongly supported.

The theory behind the detection of parent species-specific markers in a hybrid by RAPD analysis is that first-generation hybrids contain a set of chromosomes from each of the parent species and will thus share all their markers with one or the other parent species. As no specific markers to *E. graniticola* were detected, this implies that the plant is a first-generation hybrid. If no segregation or backcrossing with

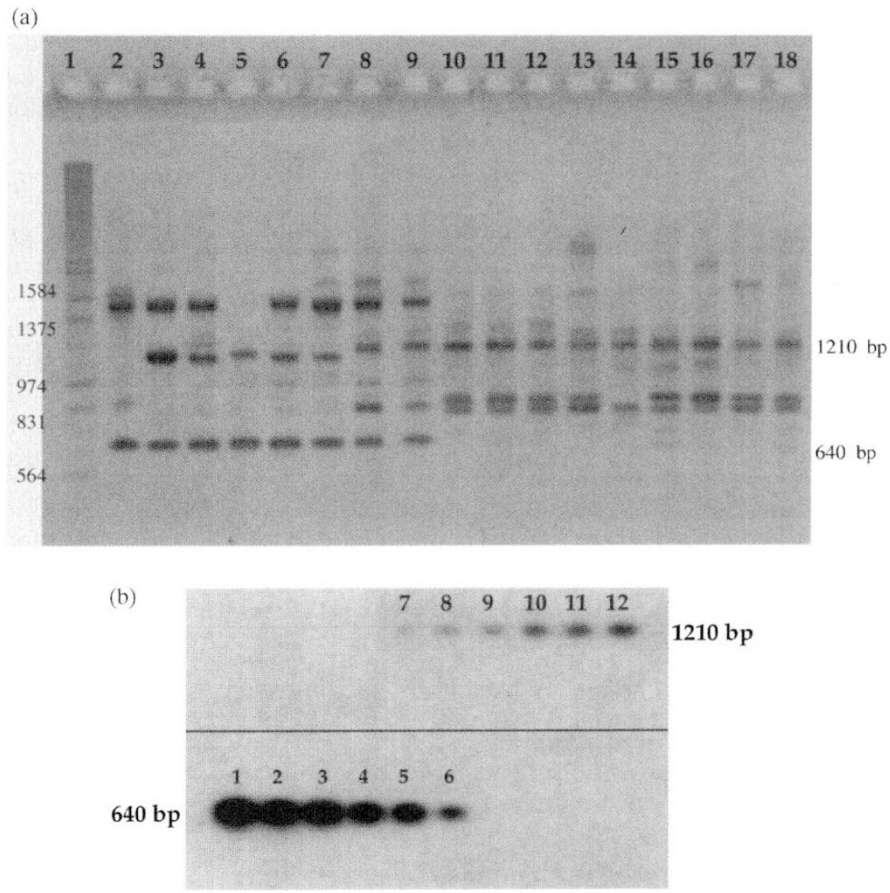


**Fig. 3** (a) Agarose gel showing the results obtained using primer TGAGCGGACA. Lane 1 is a size marker, lanes 2–7 are *Eucalyptus rudis* samples, lanes 8 and 9 are *E. graniticola* samples, lanes 10–18 are *E. drummondii* samples. The 1210-bp fragment and the 640-bp fragment, from the same primer and used for hybridization, are also indicated. (b) Results from the hybridization of the two fragments indicated in Fig. 3a. Lanes 1–4, *E. rudis*; lanes 5–6 and 7–8, *E. graniticola*; lanes 9–12, *E. drummondii*.

**Table 2** Percentage of the 43 markers detected for *Eucalyptus graniticola* with nine primers, which were exclusive to *E. graniticola*, shared with *E. rudis*, shared with *E. drummondii* and shared with both species

| Markers recorded for *E. graniticola* | Percentage (*n*) |
|---|---|
| Exclusive to *E. graniticola* | 0 (0) |
| Shared with *E. rudis* | 40 (17) |
| Shared with *E. drummondii* | 35 (15) |
| Shared with *E. rudis* and *E. drummondii* | 25 (11) |

the parent species has occurred, no new markers are expected, unless they are derived from mutations (but the probability of detecting these is low). The results from RAPD analysis (Table 2) show that *E. graniticola* does not possess any exclusive and distinctive markers, because all its markers were detected in either or both parent species.

RAPD analysis has been challenged as a reliable technique owing to the production of nonparental markers in progeny fingerprints (Riedy *et al.*, 1992). However, this was not the case in this study, and it seems to be a relatively rare event. For instance, Scott *et al.* (1992) only found 0.002 per cent (from 824 reactions) nonparental markers in *Fragaria vesca* and 0.012 per cent (from 1450 reactions) in *Nicrophorus tomentosus*. Similarly, the lack of homology in co-migrating fragments has sometimes been raised as a potential problem. For example, in an extreme case, Rieseberg (1996) detected up to 20 per cent nonhomologous co-migrating fragments in three species of *Helianthus*. However, in this study, the lack of exclusive *E. graniticola* markers and the verification of homology for the selected co-migrating fragments (Fig. 3b) confirm the results obtained by RAPD analysis.

Corroboration of this result is provided by the similarity analysis. Similarity between the two different species (26 per cent) is much lower than that between the hybrid and its parent species (65 per cent with *E. rudis* and 52 per cent with *E. drummondii*).

The segregation of cotyledon characters in the progeny of *E. graniticola* (Fig. 2c), intermediate leaf, bud and fruit morphology (Fig. 2b), low seed and pollen viability and occupancy of an intermediate habitat strongly support the proposed hybrid origin of *E. graniticola* (Hopper, 1995). However, it is important to realize that reliable recognition of hybrids cannot always depend on morphological similarities alone as these can have other origins (Hopper, 1995).

*Eucalyptus* is the genus with the highest number of recorded interspecific hybrids in Western Australia (Hopper, 1995). Yet the formation of a hybrid between *E. rudis* and *E. drummondii* is a surprising event, as these species are not closely related.

RAPD analysis was also able to demonstrate for *E. graniticola* that it is likely that all 45 stems arising from ground level were genetically identical and likely to have arisen from a single mallee rootstock. The similarity data also indicate that the small-fruited eucalypt found in the vicinity of the rare hybrid is *E. drummondii* and not another hybrid or a new species. In order to determine whether the small-fruited eucalypt is simply a form or a distinct subspecies, a study including the entire *E. drummondii* distribution should be carried out.

### *Origin of* E. graniticola

*Eucalyptus graniticola* is a hybrid that originated when *E. rudis* and *E. drummondii* occurred at that same site.

The two parent species are still found in the near vicinity and granite outcrops represent a rare juxtaposition of their habitats. *Eucalyptus rudis* and *E. drummondii* are not commonly found in the same area and this, plus the distant taxonomic relation-

**Table 3** Genetic similarity within and between *Eucalyptus* taxa, calculated as for Nei & Li (1979) $F = 2m_{xy}/(m_x + m_y)$, using the presence/absence data obtained from RAPD analysis

| | *E. rudis* (*n* = 5) | *E. graniticola* (*n* = 2) | *E. drummondii* (*n* = 6) | Small-fruited *E. drummondii* (*n* = 4) |
|---|---|---|---|---|
| *E. rudis* | 0.78 | | | |
| *E. graniticola* | 0.65 | 1.00 | | |
| *E. drummondii* | 0.26 | 0.52 | 0.82 | |
| Small-fruited *E.d.* | 0.26 | 0.57 | 0.75 | 0.88 |

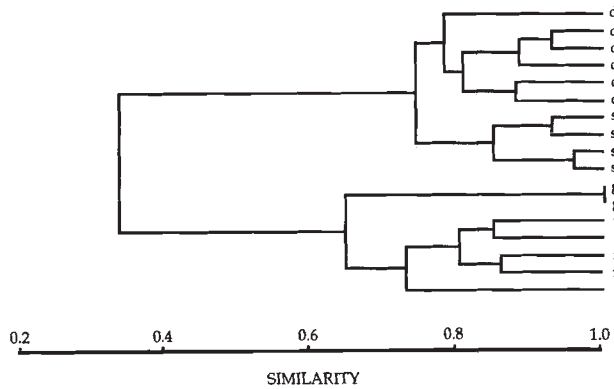


**Fig. 4** Dendrogram showing the relationships between the taxa tested. d1–d6, *Eucalyptus drummondii* samples; sf1–sf4, small-fruited *E. drummondii* samples; g1 and g2, *E. graniticola* samples; r1–r5, *E. rudis* samples.

ship between them, may explain why this hybrid is rare.

The distribution of *E. rudis* and *E. drummondii* could have been reduced by Quaternary climatic fluctuations and/or by recent disturbances, such as changes in fire frequency and logging-related pressures. Thus, *E. graniticola* appears to be a relict of past reproductive interaction, signalling populations of the parent species now extinct. If, as the molecular data seem to indicate, *E. graniticola* is a first-generation hybrid, this reproductive interaction could be more recent than previously anticipated.

Similarly, the small-fruited *E. drummondii* could potentially be a remnant of a once more widespread subspecies.

### *Conservation strategy*

*Eucalyptus graniticola* is a natural hybrid representing a unique combination of genetic material that may justify its conservation.

Simply determining the parentage of rare hybrids is not sufficient; the conservation consequences of such findings need to be understood. There are traditional prejudices and reservations against the conservation of natural hybrids (e.g. O'Brien & Mayr, 1991), and conservation biologists and managers may need further information before deciding on conservation actions. For instance, when determining the parentage of a rare hybrid mahogany, Rieseberg & Gerber (1995) were concerned that outbreeding depression could occur in the remaining *Cercocarpus traskiae* plants (one of the parent species), resulting in reduced seed-set and eventually driving the species to extinction. In addition, the risk existed of genetic assimilation of the rare species by hybridization, especially in such a restricted and disturbed habitat (Catalina island). As a result, although recognizing the conservation value of the hybrid, the authors recommended its transplantation to a location where no parent species are found.

*Eucalyptus graniticola* is restricted to one potentially aged individual and is not ecologically aggressive, as the distances involved mitigate against backcrossing to the parental taxa and seed production is low. Furthermore, both parent species are common and widespread and, therefore, not in danger of genetic swamping. Being the only known representative of a natural hybrid between *E. rudis* and *E. drummondii*, *E. graniticola* represents a valuable genetic package of great scientific and some horticultural interest and is, therefore, worthy of conservation attention. Furthermore, being a mixture of two such distant taxa might have produced some adaptive advantages for such a large mallee to survive in the difficult granite rock environment where dry/wet extremes can be expected.

Finding the hybrid origin of *E. graniticola* helps in defining the conservation strategy to be adopted for this taxon. If it were a relict species, decreasing in number because of anthropomorphic pressure, reinforcement of the existing population (i.e. plant) would have been seen as one of the main options for the species recovery. However, being a long-lived resprouting hybrid, adequately protected and under no immediate danger, reinforcement is not recommended for this eucalypt, and the main conservation objective with this taxon is *ex situ* conservation. A programme to establish the species in tissue culture and cryopreservation has been successfully undertaken at Kings Park and Botanic Garden (Rossetto *et al.*, 1993; D. H. Touchell, unpublished observations) so that the species is conserved *ex situ*.

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