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# ESTIMATION OF GENETIC DIVERSITY IN *EUCALYPTUS MICROTHECA* POPULATIONS WITH DOMINANT AFLP MARKERS

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**LI, C. 2000. Estimation of genetic diversity in *Eucalyptus microtheca* populations with dominant AFLP markers.** The use of amplified fragment length polymorphism (AFLP) to study genetic variability in *Eucalyptus microtheca* (family Myrtaceae), a native Australian species grown in the arid and semi-arid zones, was demonstrated. Nine natural populations of *E. microtheca* were studied based on seed collected from widely-separated locations in Australia. Molecular markers were used to calculate similarity coefficients, which were then used for determining genetic distances between the populations. Based on genetic distances, a dendrogram was constructed. AFLP amplification of genomic DNA revealed the low level of variation among the different populations. Although the results were in general agreement with the conventional taxonomy, it also highlighted discrepancies in the classification.

Key words: AFLP - *Eucalyptus microtheca* - genetic variability - molecular markers

**LI, C. 2000. Anggaran kepelbagaian gen dalam populasi *Eucalyptus microtheca* dengan penanda AFLP yang dominan.** Penggunaan *amplified fragment length polymorphism* (AFLP) untuk mengkaji perubahan gen dalam *Eucalyptus microtheca* (famili Myrtaceae), satu spesies asli Australia yang tumbuh di zon gersang dan separa gersang, telah ditunjukkan. Sembilan populasi semula jadi *E. microtheca* dikaji berdasarkan kutipan biji benih dari lokasi yang berjauhan di Australia. Penanda molekul digunakan untuk mengira pekali persamaan, yang kemudiannya digunakan untuk menentukan jarak genetik antara populasi tersebut. Berdasarkan jarak genetik, satu dendrogram dibina. Pembesaran AFLP bagi genom DNA menunjukkan tahap perubahan yang rendah di kalangan populasi yang berbeza. Walaupun keputusan tersebut secara umumnya sama dengan taksonomi konvensional, ia juga menonjolkan perbezaan dalam pengkelasan.

## Introduction

*Eucalyptus microtheca* F. Muell. is characteristically a species of open woodlands which has a wide and patchy natural distribution in the arid and semi-arid zones of Australia (Boland *et al.* 1984). It has been demonstrated that different environmental conditions in its native habitats, such as seasonal changes in water availability, may have resulted in large intra-specific variation in physiology and growth performance (Tuomela *et al.* 1993, Johansson & Tuomela 1996, Li 1998).

Currently, the taxonomy and nomenclature of *E. microtheca* are subject to controversial debate, with at least three different classifications presented (Boland *et al.* 1984, Brooker & Kleinig 1994, Hill and Johnson 1994). Compared with the conventional taxonomy (Boland *et al.* 1984), Brooker and Kleinig (1994) divide *E. microtheca* into three species as follows: *E. microtheca* F. Muell., comprising only the northern Australian populations; *E. victrix* Johnson and Hill, growing on western sites; and *E. coolibah* Blakely and Jacobs, mainly covering eastern sites. In the third classification of Hill and Johnson (1994), *E. microtheca* is split into eight different species and two subspecies. However, in all classifications, numerous intergrades are found along the border lines of different populations, and the fact that the taxonomic classification is based solely on visual observations of specimens makes the taxonomic classifications of the species inconclusive. Therefore, a rapid and reliable method to determine the taxonomic status of *E. microtheca* populations should be of significance.

Genetic analysis of isozyme variations has been used in a wide range of *Eucalyptus* (Moran & Bell 1983, House & Bell 1994, Byrne *et al.* 1995). DNA markers, such as RAPD (random amplified polymorphic DNA) and RFLP (restriction fragment length polymorphism), have been also used in genetic information of *Eucalyptus* (Byrne *et al.* 1994, Grattapaglia & Sederoff 1994, Keil & Griffin 1994, Nesbitt *et al.* 1997). Recently, AFLP (amplified fragment length polymorphism) markers provide an extensive amount of variation leading to clear identification of genotypes. It is being used as a advanced tool for studying genetic relationships in many plants (Hill *et al.* 1996, Maughan *et al.* 1996, Schondelmaier *et al.* 1996, Tohme *et al.* 1996, Gaiotto *et al.* 1997, Hartl & Seefelder 1998). Unfortunately, so far the use of these molecular markers to determine the genetic diversity of *E. microtheca* populations has been little studied.

The objectives of the present study were to estimate the discriminatory power of AFLP markers to describe genetic variation in nine natural populations of *E. microtheca,* and to assess the use of AFLP in the taxonomic study of *E. microtheca.*

## Materials and methods

### *Plant materials*

Nine populations of *E. microtheca* were selected for this study (Table 1). Seeds were collected from natural stands. Each population contained seeds from at least ten maternal trees. Seeds were soaked in water overnight, then placed on filter paper for germination. When the radicles had grown to about 5 mm in length, the germinating seeds were transplanted into plastic pots containing 80% peat and 20% sand (v/v). The seedlings were grown in a growth chamber. The temperature and relative humidity were maintained at 20–30 °C and 70–80% respectively. The photoperiod was maintained at 12/12 hours daily dark/light period and photosynthetic light intensity was maintained at 800–1000 μmol $m^{-2}s^{-1}$ on the seedling canopy. Juvenile leaves from seedlings about 5 months old were

used for DNA isolation. From each population, a number of samples were randomly selected for the present study, resulting in a total of 193 individual plants (Table 1).

**Table 1.** Origin of nine *Eucalyptus microtheca* populations used in the study

| Seedlot No. | Location | Latitude | Longitude | Alt. (m) | No. of individuals analysed |
|---|---|---|---|---|---|
| 15074 | Newcastle Waters | 17° 00' | 134° 45' | 160 | 21 |
| 15073 | West Kimberleys | 18° 00' | 125° 00' | 110 | 20 |
| 15322 | Richmond | 20° 43' | 142° 47' | 195 | 25 |
| 15070 | Hamersleys/Pilbara | 22° 40' | 118° 05' | 550 | 22 |
| 12827 | Alice Springs | 23° 51' | 133° 48' | 520 | 19 |
| 15430 | Murchison River | 27° 22' | 115° 54' | 300 | 23 |
| 12821 | Marree | 29° 40' | 137° 39' | 85 | 21 |
| 12813 | Bridge Nwbourke | 29° 53' | 145° 53' | 90 | 20 |
| 12854 | Walgett | 30° 15' | 148° 07' | 130 | 22 |

## *DNA isolation*

DNA was extracted from 300 mg of leaf material from each individual using a method modified from Doyle and Doyle (1990). The modifications were as follows: 10% insoluble PVP and 0.4% β-mercaptoethanol were used in the extraction buffer; the first precipitation was with ethanol rather than isopropanol; after the first precipitation the DNA was washed in 76% ethanol/0.2 M Na-acetate; finally before the final precipitation the salt concentration was adjusted to 2.0 M by the addition of an equal volume of 4 M NaCl (Fang *et al.* 1992, Nesbitt *et al.* 1995). DNA concentration was estimated from ethidium bromide stained gels, and all samples were diluted to 20 ng $\mu l^{-1}$.

## *AFLP procedures*

AFLP technique was used essentially following the procedures described by Vos *et al.* (1995) employing *Eco*RI and *Mse*I as rare- and frequent-DNA cutting enzymes respectively. An optimisation for the species of *Eucalyptus* was necessary, mainly for the pre-amplication step, resulting in higher reproducibility in the final amplication step (Gaiotto *et al.* 1997). According to the original programme (Vos *et al.* 1995), the annealing temperature started at 65 °C and decreased 0.7 °C in every cycle, until it was kept constant at 56 °C in the last 23 cycles. Comparing the pre-amplification products on agarose gels, the modified cycle clearly generated a more consistent spread of bands than the original cycle. The following seven primer combinations were used for the estimation of genetic similarity: E37/M47, E37/M61, E38/M51, E38/M55, E38/M59, E41/M51, E41/M60.

### *Data analysis*

AFLP markers are typically dominant, and scoring of bands was done considering only two possible alleles: band presence or band absence. A binary data matrix reflecting the presence or absence of specific AFLPs was generated for the set of *E. microtheca* populations. It was assumed that each band of different size reflects a single locus. Only unambiguously scored bands were used in the matrix. The genetic similarities (GS) between A and B were estimated using the formula of Nei and Li (1979) as $GS_{AB} = 2 \times$ the number of shared bands / (the number of bands in A + the number of bands in B). An UPGMA analysis (unweighted pair-group method with arithmetical averages) was carried out based on genetic distances (GD), calculated as $GD_{AB} = 1 - GS_{AB}$, from which a dendrogram representing the relationship between *E. microtheca* populations was obtained.

## Results

The seven primer combinations amplified a total of 223 scorable bands. Between 25 and 43 bands were amplified with each of the seven AFLP primer combinations. Of those bands 78 were polymorphic (Table 2). An example of the AFLP profiles, obtained using primer (in 5' to 3'– direction): GACTGCGTACCAATTCACT (AFLP E38) and GATGAGTCCTGAGTAACCA (AFLP M51) could generate 43 AFLP markers.

**Table 2.** AFLP primer combinations that generated polymorphism products in *EcoRI*/*MseI* enzymes digests

| Primer combination | 3' Selective nucleotides | | Nt[1] | Np[2] | Pp[3] |
|---|---|---|---|---|---|
| | *EcoRI* | *MseI* | | | |
| E37/M47 | ACG | CAA | 26 | 9 | 34.62 |
| E37/M61 | ACG | CTG | 37 | 12 | 32.43 |
| E38/M51 | ACT | CCA | 43 | 17 | 39.53 |
| E38/M55 | ACT | CGA | 32 | 11 | 34.38 |
| E38/M59 | ACT | CTA | 25 | 7 | 28.00 |
| E41/M51 | AGG | CCA | 31 | 12 | 38.71 |
| E41/M60 | AGG | CTC | 29 | 10 | 34.48 |
| Total | | | 223 | 78 | 34.98 |

1) Nt, total number of bands scored for each primer;
2) Np, the number of bands that were polymorphic;
3) Pp, the proportion of polymorphic bands (%).

DNA fingerprints produced by primers were used to calculate similarity coefficients in pairwise comparisons of the populations, as described by Nei and Li (1979). This similarity coefficient was based on the presence of shared amplification products, assuming that fragments with different electrophoretic mobilities were non-allelic and fragments with the same electrophoretic mobility

were allelic. Similarity coefficients ranged from 0.837 to 0.970 among nine populations of *E. microtheca*. In general, similarity coefficients of populations from similar geographic locations were relatively high, with values of 0.937–0.970 for southeastern populations and 0.927–0.963 for northern populations. In the present study, the low level of variation in *E. microtheca* populations was detected by the AFLP analysis.

For genetic distances, a dendrogram displaying genetic relationships among the populations was constructed using the UPGMA method (Figure 1). The resulting dendrogram showed a small differentiation into three clusters. One of the clusters contained populations 12813, 12854 and 12821 (from southeastern Australia), the second cluster contained populations 15073, 15074, 12827 and 15322 (from northern Australia), and the third cluster only contained population 15070 (from western Australia). Population 15430 (from western Australia) lay outside these clusters, and separated from the rest of the populations. Overall, there were only quite small genetic distances among the studied populations.

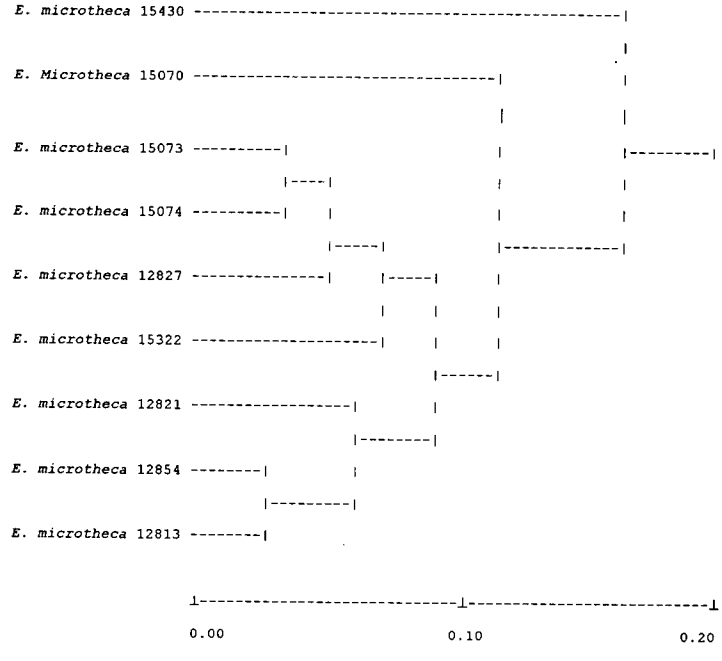


**Figure 1.** Dendrogram of the genetic relationships between the 9 populations of *Eucalyptus microtheca*. Numbers next to species are the population numbers listed in Table 1. The scale indicates the genetic distance.

## Discussion

Molecular marker techniques not only provide an alternative approach for evaluating genetic diversity but are also useful in genetic mapping, plant breeding, and genomic fingerprinting for *Eucalyptus* species (Moran & Bell 1983,

Sale *et al.* 1993, Keil & Griffin 1994, Chen & De Filippis 1996, Hodge *et al.* 1996, Gaiotto *et al.* 1997). Compared with other molecular markers, AFLP procedure detects a large number of polymorphic DNA markers in a relatively short time and is thus an useful technique when high through-put is desired (Vos *et al.* 1995); it is more reliable and reproducible compared with RAPD and RFLP (Sharma *et al.* 1996, Gaiotto *et al.* 1997, Eujayl 1998). The present results indicated that the AFLP marker assay in *E. microtheca* under optimised conditions generated a number of polymorphic markers. Interpretation of data when markers across gels were scored was found to be easier and more reliable with AFLP markers. The conclusion was similar with the first report using AFLP markers for genetic analysis in *Eucalyptus* (Gaiotto *et al.* 1997).

The AFLP methodology revealed the low level of variation in *E. microtheca.* According to genetic distance data, it was possible to construct a phylogenetic tree for the nine *E. microtheca* populations. The resulting dendrogram showed a small differentiation into three clusters. One of the clusters contained populations from southeastern Australia, the second cluster contained populations from northern Australia, and the third cluster contained populations from western Australia. Population 15430 lay outside these clusters, and was separated from the rest of the populations, which was in accordance with the classification of CSIRO where this population was placed in another species, *E. victrix.* However, overall there were only quite small genetic distances among the studied populations. Therefore, apart from a few exceptions, the dendrogram showed overall agreement with the classification of Boland *et al.* (1984).

Diverse environmental conditions in its native habitats, such as seasonal changes in water availability, may have resulted in large intra-specific variation in morphological and physiological traits of *E. microtheca* (Tuomela *et al.* 1993, Tuomela 1997, Li 1998). For example, the most peculiar feature separating the western, northern and eastern populations in *E. microtheca* is the bark type (Tuomela 1997). Despite the marked morphological differentiation occurring between the populations in *E. microtheca,* the genetic variance based on AFLP markers was quite small, indicating high similarity between the populations.

To some extent, the present study was rather elementary. Therefore, more detailed taxonomic studies together with gene analysis should be required in order to accurately define *E. microtheca* populations in the future.

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