

## GENETIC VARIATION IN THE *EPACRIS TASMANICA* COMPLEX (EPACRIDACEAE)

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(with two text-figures)

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Random amplified polymorphic DNA markers (RAPD) were used to delimit species in the *Epacris tasmanica* complex (Epacridaceae) collected from various locations in Tasmania, Australia. The RAPD analysis placed morphologically similar taxa located closely geographically into discrete clusters. However, geographically distant populations of morphometrically similar taxa, such as the southern and northern Tasmanian populations of *E. virgata* and *E. tasmanica*, were less closely related to each other than those taxa classified as different species. The most geographically remote population (*E. glabella* [Serpentine Hill]) shared the least genetic similarities with the other taxa. The genetic information obtained from this study reinforces some previous morphometric data used to delimit species in the *E. tasmanica* complex. Furthermore, the strong geographical structure of the genetic variation is consistent with a model in which gene flow between populations is limited.

**Key Words:** *Epacris tasmanica* complex, genetic variation, RAPD analysis, endangered species.

### INTRODUCTION

The Epacridaceae is one of the five main families that dominate Australian heathlands. The high level of endemism and localised distributions of the Epacridaceae leave many taxa susceptible to extinction. The *Epacris tasmanica* complex comprises seven closely related taxa, all of which are Tasmanian endemics (Crowden & Menadue 1990). Historically, interest has been shown in the delimitation of species of this complex, primarily to clarify the taxonomic relationships between taxa. However, with the listing of six of the seven species of the complex as endangered or vulnerable (Flora Advisory Committee 1994), it is of interest to clearly identify discrete genetic entities to aid in their conservation.

The overlap of vegetative and floral characteristics between taxa in the *E. tasmanica* complex has made separation of distinct species difficult when using identification keys based on plant habit as well as leaf and sepal characteristics (Curtis 1963, Jarman & Crowden 1977), which can be extremely plastic. Other attempts to delimit taxa in the *E. tasmanica* complex have used characters such as pollen surface patterns (Matthews 1965, 1966, Menadue & Crowden 1991) and flavonoid chemistry (Jarman 1975). Crowden & Menadue (1990) used numerical taxonomic methods to revise a key for the *E. tasmanica* complex and suggested there are seven taxa in the complex: *E. tasmanica*, *E. virgata*, *E. apsleyensis*, *E. barbata*, *E. exserta*, *E. stuartii*, and *E. glabella* (described by Jarman & Mihaich 1991 as *E. aff. exserta* [Tunnel Hill]).

Molecular markers are simply inherited characters that distinguish or mark underlying genetic differences between individuals. It is believed that DNA markers are not severely influenced by selection (Avice 1994) and they have been increasingly used to study patterns of relatedness in natural populations (Ritland & Ritland 1996). Randomly amplified polymorphic DNA (RAPD) markers have proved to be very effective in differentiating various degrees of relatedness (Williams *et al.* 1990, Cambecedes *et al.* 1999). They generally have been used within species but are also useful in population studies, when investigating complexes of closely related species; the *E. tasmanica* complex is such a complex. In fact, systematists are increasingly using RAPD

markers and Harris (1995) demonstrated that groupings based on RAPD and restriction fragment length polymorphism (RFLP) analysis were essentially the same in species of *Leucaena* (Leguminosae). In the present study, RAPD markers were used to analyse genetic variation between members of the *E. tasmanica* complex collected from several locations in Tasmania, with a view to clarify the taxonomic relationships between members of this complex.

### METHODS

#### Tissue Collection and DNA Isolation

Tissue samples were collected from 19 Tasmanian populations (fig. 1). New shoots and floral buds were collected from ten representative individuals in each population and individually stored at 4°C in airtight plastic bags, until processed (tissues were stored for a maximum of one week). Young tips were used, as it was expected that this tissue would contain less polysaccharide storage materials and, therefore, provide cleaner DNA compared to mature tissues. In the initial collection (June–July 1996) insufficient new growth was observed on the majority of plants to obtain enough tissue for individual DNA extraction. In some exposed populations, where growth was restricted by local severe environmental conditions (Southport Bluff [*E. stuartii*] and Cape Tourville [*E. barbata*]), it was difficult to obtain fresh plant tissues. In the *E. stuartii* and *E. barbata* populations, tissue was collected from only eight individuals per population. At sites visited later (August–September 1996), when many of the *Epacris* species were beginning to flower, floral buds were also collected to determine their value as a DNA source. Where possible, paler coloured buds were preferentially selected to minimise anthocyanin levels, which might have hindered DNA extraction and analysis.

Preliminary DNA extraction trials showed that 2–5 g of tissue were required to obtain DNA. Due to the lack of available tissue for DNA extraction, tissues from individual plants were pooled in equal proportions to produce a mixture for each population (as discussed in Cambecedes *et al.* 1999). This procedure made it impossible to investigate

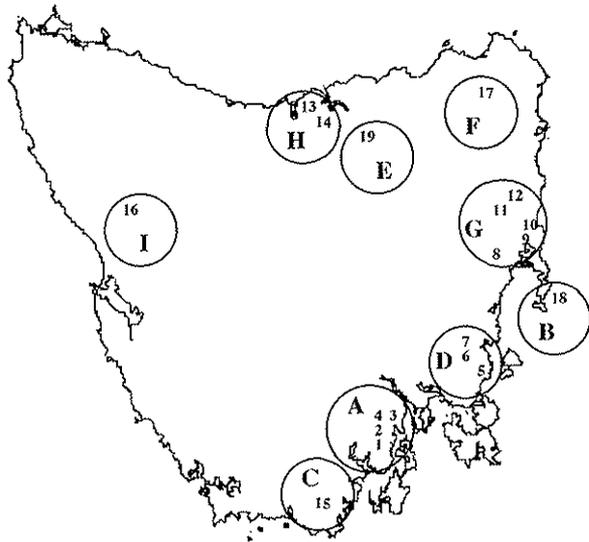


FIG. 1 — Collection sites (numbers) and RAPD groups (letters) for *E. tasmanica* complex populations. *E. virgata* (School Hill [1], Snug "High" [2], Van Morey Rd [3], Southern Outlet [4]); *E. tasmanica* (Hospital Creek [5], Buckland [6], Paradise Gorge [7], Lake Leake Rd [8], "O" Rd [9], Barbers Creek [10], Reynolds Hill [11], Hardings Falls [12]); *E. virgata* (Old Railway [13], Scots Hill [14]); *E. stuartii* (Southport Bluff [15]); *E. glabella* (Serpentine Hill [16]); *E. virgata* (Mt Cameron [17]); *E. barbata* (Cape Tourville [18]); *E. exserta* (Trevallyn Dam [19]). Letters delineate RAPD groups defined in figure 2.

differences within populations; however, it enabled the efficient detection of differences among populations. Tissues were dried in McCartney bottles containing desiccator crystals, or frozen in liquid nitrogen and stored below  $-60^{\circ}\text{C}$  until DNA extraction. Preliminary extractions using floral buds produced high yields of DNA that possessed a similar amount of polysaccharide contamination to that observed in leaves. Consequently, where floral buds were collected, they were used in conjunction with leaf material. DNA was extracted by the CTAB (cetyltrimethyl ammonium bromide) method of Doyle & Doyle (1990), using 5 g of tissue from each population (0.5 g or 0.625 g from each plant when ten or eight samples were collected per population, respectively). For several samples, particularly where leaf material was the only source of DNA, an additional chloroform-isoamyl alcohol extraction was performed prior to DNA precipitation to remove contaminants. To remove further contaminants, the salt concentration of the solubilised DNA was adjusted to 2.5 M with ammonium acetate, and the solution was placed at  $-20^{\circ}\text{C}$  for 5 min. prior to extraction with phenol:chloroform (1:1). For most samples, the ammonium acetate and phenol:chloroform extractions were repeated at least twice. Following the cleaning up procedures, DNA was precipitated in cold ethanol, washed with ethanol, and resuspended in 500  $\mu\text{L}$  of TE buffer to ensure that the different extraction methods did not effect amplification. DNA was quantified using a Hoefer TKO-100 Mini-fluorometer (Hoefer Scientific Instruments, USA) and diluted in sterile water to 3  $\text{ng}\cdot\mu\text{L}^{-1}$ .

## RAPD Analysis

Various primer and magnesium ion concentrations for DNA amplification of *Epacris* species were tested. The best DNA amplification was obtained in polymerase chain reaction (PCR) mixtures (15  $\mu\text{L}$  final volume) containing 1.5  $\mu\text{L}$  Taq polymerase buffer (x10), one unit Taq polymerase (Boehringer, Germany), 100  $\mu\text{M}$  of each dNTP, 1.6 mM  $\text{MgCl}_2$ , 150  $\mu\text{g}\cdot\text{mL}^{-1}$  BSA, 0.25  $\mu\text{M}$  primer and 12 ng genomic DNA (modified from Williams *et al.* 1990). DNA amplification was performed in a Corbett Research Fast Thermal Sequencer (FTS-960) (Corbett Research, Australia) programmed as follows: initial denaturation of 2 min. at  $94^{\circ}\text{C}$ ; 35 cycles of 1 min. at  $92^{\circ}\text{C}$  denaturation, 1 min. at  $35^{\circ}\text{C}$  annealing, 2 min. at  $72^{\circ}\text{C}$  extension; and a final extension of 5 min. at  $72^{\circ}\text{C}$ . A total of 233 10-base primers from UBC (University of British Columbia, Canada) and Operon (Operon Technologies, USA) were screened on the Lake Leake DNA samples (the most abundant sample of DNA). Only 18 of these primers were used in the final analysis, as they produced reproducible products (see Results).

Amplified PCR products were separated using a 1.4% (w/v) agarose gel in TBE buffer containing 0.5  $\mu\text{g}\cdot\text{mL}^{-1}$  ethidium bromide. RAPD bands were visualised on an UV transilluminator and photographed using positive/negative polaroid film (Polaroid 665, USA). All polymorphic bands between 517 and 2645 base pairs were scored, while monomorphic bands were not scored. Each sample was scored manually for band presence (1) or absence (0) and data were recorded in the form of a binary matrix. A similarity matrix was generated using the Simple Matching Coefficient Function of NTSYS-pc (numerical taxonomy system of multivariate statistical programs for PC) (Rohlf 1993). The similarity matrix was used to generate an UPGMA (unweighted pair-group method with arithmetic mean) dendrogram using the SAHN (sequential agglomerative hierarchical non-overlapping) cluster analysis and UPGMA programs in NTSYS-pc (Rohlf 1993).

## RESULTS

Of the 233 arbitrary primers screened, 175 produced amplification of the *Epacris* DNA fragments to varying degrees. However, only 54 of these 175 primers produced RAPD amplification products which were clearly defined and had minimal background smearing. Due to reproducibility problems encountered with some primers, only 18 of the 54 primers were used in the final analysis (UBC 4, 11, 25, 31, 60, 64, 76, 83, 93, 249 and Operon D10, D13, F06, F07, F14, F20, M05, M18). These 18 primers produced 57 scorable polymorphic bands that were used to generate the similarity matrix and UPGMA dendrogram (fig. 2).

The UPGMA dendrogram demonstrates that populations in close geographic proximity formed tight clusters. The groupings do not correspond well to the previous described species, with the two more widespread species split geographically, with all the southeastern Tasmanian populations being grouped together, regardless of species, and geographically distinct populations of the same species from this region becoming more and more distinct. The *E. virgata* populations were divided into three groups, broadly corresponding to geography (figs 1, 2). All populations that were sampled and described by Crowden & Menadue

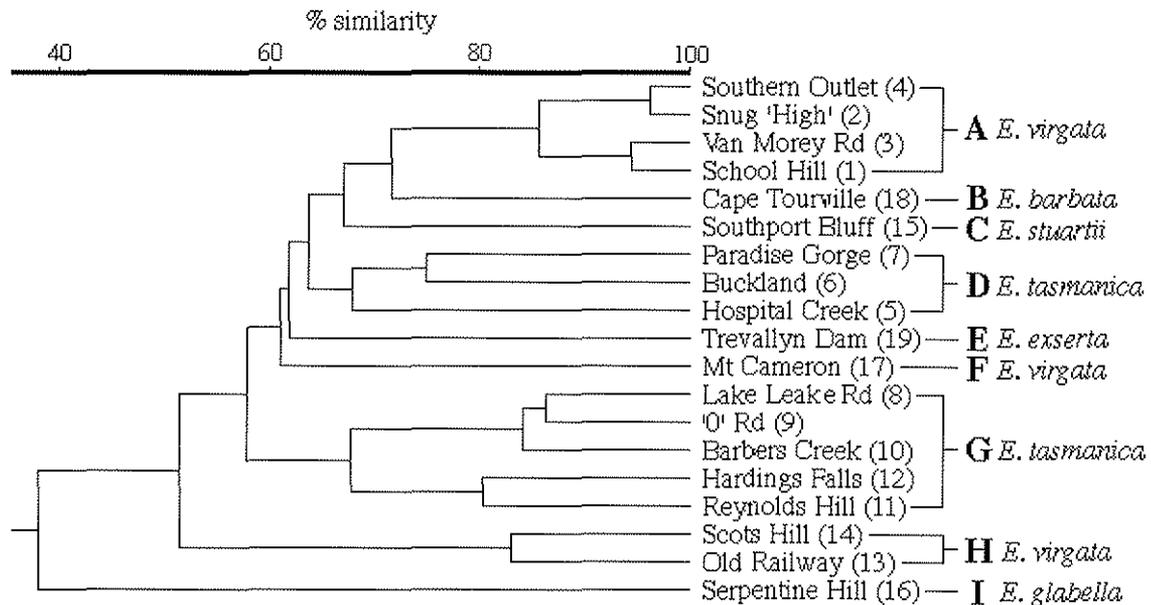


FIG. 2 — UPMGA dendrogram based on RAPD data obtained from populations of the *E. tasmanica* complex. Numbers in parentheses refer to the collection sites (fig. 1), while letters in bold refer to RAPD groups.

(1990) as southern *E. virgata* were tightly clustered, suggesting a close genetic relationship between these populations (Group A). The two northern *E. virgata* populations (Scots Hill and Old Railway) also clustered together (Group H). Populations described as *E. tasmanica* formed two discrete clusters (Groups D and G) separated by *E. exserta* (Trevallyn Dam) (Group E) and a putative disjunct population of *E. virgata* (Mt Cameron) (Group F) (see Discussion). The two *E. tasmanica* clusters do correspond broadly to geography, with populations within each group encompassing approximately 30 km, while approximately 100 km separated the groups.

The dendrogram suggests that the *E. glabella* population (Group I) is an outgroup to the entire *E. tasmanica* complex. However, several primers gave no amplification in the *E. glabella* DNA. Consequently, data were lacking for several reactions, which may have biased the analysis. Interestingly, the positioning of *E. glabella* on the dendrogram does correspond to geography. *E. glabella* occurs in the far northwest of Tasmania, with its closest geographic neighbours being the northern *E. virgata* populations at Scots Hill and Old Railway (Group H).

## DISCUSSION

The genetic data generated by RAPD markers have reinforced some of the taxonomic conclusions generated through the morphometric analyses of the *Epacris tasmanica* complex by Crowden & Menadue (1990). The formation of distinct clusters, representing both the same species and similar geographic locations of the populations, suggests a relationship between the geographical location of the population and genetic distances between populations. The overall small differences in genetic similarities between clusters, suggests a close relationship between all members of the *E. tasmanica* complex.

Interestingly, not all RAPD results correspond to the morphometric data of Crowden & Menadue (1990).

Although morphometrically similar, there is substantial genetic differentiation between the southern (Group A) and northern (Group H) *E. virgata* populations. According to Crowden & Menadue (1990), the northern *E. virgata* population flower measurements are at the smaller end of the *E. virgata* range but are otherwise indistinguishable from the southern *E. virgata* population. The data, therefore, support a model in which gene flow between populations is limited and local differentiation has occurred. Interestingly, the *E. virgata* population at Mt Cameron shows considerable genetic differentiation from both the southern and northern *E. virgata* populations. Crowden & Menadue (1990) tentatively regarded this taxon (originally lodged in the Tasmanian herbarium as *E. aff. exserta* [Mt Cameron]) as an outrider population of *E. virgata*, based on overall habit and appearance. The data in this study add credence to the argument that the *E. virgata* population at Mt Cameron is a well-differentiated population (Crowden & Menadue 1990). Clearly, the *E. virgata* populations at Mt Cameron and at other Tasmanian sites not sampled in this study (e.g. Murchison and Hospital Creek) require further investigation to determine their genetic relationship within the complex.

Crowden & Menadue (1990) suggested that, due to flower type and other shared morphological characteristics, *E. stuartii* and *E. glabella* are more closely related to *E. virgata* (both southern and northern populations) than *E. tasmanica*. However, the RAPD data suggest that *E. glabella* (Group I) and the northern *E. virgata* populations (Group H) are not closely related to the southern *E. virgata* (Group A) and *E. stuartii* (Group C) populations. Furthermore, researchers have suggested that *E. barbata* is more closely related to *E. tasmanica* than *E. virgata* (Menadue & Crowden 1991); however, the RAPD data presented in the present study cannot address this issue. Unfortunately, RAPD data are lacking for the newly described *E. aspleyensis*, so it is not possible to determine its genetic relationship to the other members of the *E. tasmanica* complex.

The association of geographic separation and genetic similarity observed in the dendrogram may, in part, explain these discrepancies. The geographical distance between the southern and northern *E. virgata* and *E. tasmanica* populations would appear to effectively prevent an exchange of genetic material between populations. Over time, drift and different environmental selective pressures at each site would gradually alter the genetic composition of the taxa. Such a scenario would explain the genetic dissimilarity observed between southern and northern *E. virgata* and *E. tasmanica* populations; however, the question of the maintenance of morphological similarities remains unanswered. Additionally, the close proximity of the southern *E. virgata* and southern *E. tasmanica* populations and their genetic similarity could indicate the mixing of genetic material without a noticeable effect on morphology. The premise that geographical isolation is correlated with genetic variation between related taxa is further illustrated by the positioning of other isolated populations (i.e. *E. stuartii* [Southport Bluff], *E. barbata* [Cape Tourville], *E. virgata* [Mt Cameron], *E. virgata* [Scots Hill and Old Railway Line] and *E. glabella* [Serpentine Hill]). Potts & Jordan (1994) reported that populations of *Eucalyptus globulus* from relatively isolated localities often showed larger genetic variation when compared to less isolated populations, due to inbreeding and localised selective pressures. A study of the genetic variances within the populations could clarify whether there is a high degree of inbreeding in these populations.

Subtle differences in leaf morphology, corolla tube length and geographic isolation of members of the *E. tasmanica* complex, are considered sufficient reasons to maintain *E. stuartii* and *E. glabella* as distinct species (Crowden & Menadue 1990). The RAPD data generated in this study supports the maintenance of *E. glabella* as a distinct species; however, this is less clear for *E. stuartii*. Interestingly, Keith (1996) suggested that the distinctiveness of *E. stuartii* from other taxa in the *E. tasmanica* complex could be strengthened by including several other characters, such as leaf thickness and lustre, inflorescence length, width of basal and ultimate branches, and habit.

In conclusion, this RAPD analysis of the *E. tasmanica* complex placed populations of morphologically similar taxa into discrete clusters, which corresponded (in varying degrees) with the geographical location of the populations within the *E. tasmanica* complex. The division of *E. virgata* and *E. tasmanica* into southern and northern groups is consistent with a model in which gene flow between populations is limited and should be investigated further. The fact that not all RAPD results corresponded to the morphometric data of Crowden & Menadue (1990), suggests that it may be simplistic to assume that one methodology is sufficient to address the taxonomic issues in the *E. tasmanica* complex. Further research, therefore, should have a combinatorial approach and include morphological, genetic and breeding systems.

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