Genetic Diversity and Interspecific Relationships in *Banksia* L.f., (Proteaceae).

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Banksia coccinea

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Abstract

Banksias are amongst the best known Australian wild flowers. They are used in ornamental horticulture and last well as fresh cut flowers, or indefinitely as dried arrangements. Breeding and selection of new cultivars for the cut flower industry is currently underway. This thesis aims to increase knowledge essential for conservation biology and for focused and efficient breeding of banksias.

Pollen storage and viability testing are important adjuncts to a plant breeding program. *Banksia menziesii* pollen was stored at 20 ^{0}C, 4 ^{0}C, -20 ^{0}C, -80 ^{0}C and -196 ^{0}C and assessed using a semi solid medium of 1% agar, 15% sucrose, 0.01% boric acid, 0.03% calcium nitrate, 0.02% magnesium sulphate, 0.01 % potassium nitrate, and an incubation temperature of 25 ^{0}C. Germination remained constant at around 70% in all treatments except room temperature 20 ^{0}C, which after six months had only 25% germination. Pollen viability was assessed using fluorescein diacetate (FDA), but the results did not reflect the loss of germinability at 20 ^{0}C. There was no effect of floret position on the inflorescence on germination; but pollen viability varied over the flowering period with maximum germination mid season.

Interspecific hybridisation is assessed as a potential breeding tool, and for the assessment of species relationships within the genus. Pollen tube growth was investigated using controlled hand pollination of the commercially significant species *Banksia coccinea,* to species of *Banksia*, and the related genus, *Dryandra*. Currently, the relationship between *B. coccinea* and the other species groups within *Banksia* is unclear. It has been found previously that success of pollen tube growth in the pistil following interspecific pollination was largely related to taxonomic distance between the species (Sedgley *et al.* 1994). Thus, interspecific hybridisation is a suitable technique to determine the compatibility relationships of the problematic species *B. coccinea*. Some species supported no germination of *B. coccinea* pollen. Others produced pollen tube

abnormalities including thickened walls, bulbous swellings, non-directional growth, burst tubes and branched tubes. Control of pollen tube growth in the pistil was imposed in the pollen presenter, a specialised region of the style for pollen presentation to foraging fauna, and in the upper style. There was no significant reciprocal effect on pollination success in the lower style. The results of pollen tube compatibility in the lower style indicated that *B. coccinea* had a closer affinity to the section *Oncostylis*, than to section *Banksia* where it is currently placed. Given the distinct morphology and close pollen - pistil relationship to section *Oncostylis*, it is proposed to move *B. coccinea* out of section *Banksia* to a new section *Coccinea*, the sister section to *Oncostylis*. Intergeneric crosses of *B. coccinea* with *Dryandra* species resulted in some compatibility, with one cross having low numbers of pollen tubes in the pollen presenter and upper style region. These results indicate a close relationship between *Banksia* and *Dryandra,* which are sister genera in the tribe *Banksiae*, family Proteaceae.

Species relationships within *Banksia* were also assessed using molecular techniques. Random amplified polymorphic DNA (RAPD) markers were assessed for their usefulness at various taxonomic levels within the genus. It was found that RAPDs are informative at the close species level, but not at more distant levels, such as between distantly related series, sections, and subgenera. In addition, species relationships at higher levels were investigated using direct polymerase chain reaction (PCR) sequencing of chloroplast DNA (cpDNA) spacer regions between the *trn*L and *trn*F exons. These regions are thought to be universal for plant species and informative at the intra and interspecific level of plants. Using the region between *trn*L and *trn*F, relationships within *Banksia,* and between *Banksia* and *Dryandra* were investigated. It was found that this region was conservative, with little variation between species. Section *Banksia* formed a group, section *Oncostylis* formed another group, and *B. coccinea* along with two *Dryandra* species was placed between the two sections. Resolution at this node however, was not complete. Subgenus *Isostylis* formed two groups away from the two sections in subgenus *Banksia*, with *B. illicifolia* and *D. formosa* together, while *B. cuneata* was

more distantly related. Based on DNA sequence and RAPD data, it appears that *Banksia* and *Dryandra* may be artificial genera, and that in the presence of each other, they cannot be separated using RAPD or *trn*L DNA sequence data.

Genetic variability within species of *Banksia* was investigated using RAPDs. Levels of genetic diversity were generally high, ranging from 0.59 - 0.90. This agrees with previous work using isozymes, pollen tube and fruit set data, showing that *Banksia* species are predominantly outcrossing. In particular, a detailed study was conducted on a geographically restricted, rare and endangered species, *B. cuneata*. Using RAPDs on all known populations, it was found that levels of genetic diversity were high, ranging from 0.65 - 0.74, and that there was no significant genetic differentiation between populations.

In conclusion, this study contributes to knowledge essential for further improvement and conservation of *Banksia* species, and raises questions regarding the currently accepted taxonomic relationships within *Banksia* and between *Banksia* and *Dryandra*.

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Chapter One

General introduction

The genus Banksia

The genus *Banksia* L.f., which belongs to the family Proteaceae, is named after Joseph Banks who collected the first specimens in 1770. Banksias are amongst the best known Australian wildflowers, with approximately 75 species (George 1987), all of which are native to Australia. The greatest concentration of species is in south west Western Australia where 60 species are found. Fourteen occur in south east and eastern Australia from Eyre Peninsula to Cape York Peninsula, and two of these also occur in Tasmania. One tropical species, *B. dentata*, extends from the Kimberley to Cape York Peninsula, and is also found in Papua New Guinea, Irian Jaya and the Aru Islands. Apart from *B. dentata* no species is common to both east and west Australia, but taxonomically there are strong relationships between the two groups. The geographical range of *Banksia* species varies greatly. Some are widespread and common, such as *B. prionotes*, whereas others are very restricted such as *B. cuneata* (George 1987).

Species relationships within genus *Banksia* are not fully resolved, and some such as *B. coccinea* are problematic and have no known relatives. *B. coccinea* is unusual as it has unique leaves, inflorescences, pollen, infructescences, follicles and seeds (George 1987). A recent cladistic analysis of *Banksia* using morphological characters (Thiele 1993) confirmed the classification of George (1981, 1988) for some species, but not for others. *B. coccinea* was placed *incerte sedis*. Other additional independent data are required to resolve these relationships. Higher order relationships between subgenus *Banksia,* subgenus *Isostylis* and genus *Dryandra* are also unresolved. *Banksia* and *Dryandra* are

sister taxa, in the tribe Banksiae, family Proteaceae, and are thought to show parallel development.

Variability within genus *Banksia* and within species is high, with a wide range of forms, growth habits, morphology, and environmental tolerances. The genus represents a large source of genetic variability, only a portion of which is being utilised in ornamental horticulture. Until recently flowers were harvested from the wild, but now there is commercial planting of banksias for the cut flower industry. Breeding and selection is underway (Sedgley *et al.* 1991, 1994), for improved cultivars for the cut flower industry. Selection of improved varieties from variable populations is important for future breeding efforts, so conservation of natural variation in *Banksia* is essential. This ensures sufficient resources to produce novel hybrids, and improved growth rates, plant form, local adaptation, disease resistance, yield, bloom colours and other important horticultural characters. Effective conservation and management of natural stands depends upon sound knowledge of the biology and population genetics of species. Issues affecting *Banksia* population viability include habitat fragmentation, land clearance, altered fire and recruitment frequencies, disturbance, and introduction of disease and non indigenous plants and animals. Further research into levels and patterns of genetic variation will result in informed decisions, to ensure the viability of *Banksia* populations.

Thesis aims

This project aims to increase knowledge essential to *Banksia* breeding and conservation. Interspecific hybridisation is assessed as a potential tool for the production of novel hybrids. Pollen storage is investigated to increase flexibility of the interspecific breeding program. Species relationships within the genus are investigated; this information is essential for focused breeding efforts. Genetic diversity within species of *Banksia* is investigated for conservation of genetic resources, and patterns of genetic diversity within and between populations of a rare and endangered species *B. cuneata* are investigated. Details of each project aim are given below.

Pollen viability and storage

Pollen storage and viability testing is an important component of plant breeding programs, when the species to be hybridised do not flower at the same time or are geographically isolated. Flexibility of the breeding program will be greatly increased if pollen can be stored successfully for extended periods. There is no published information on pollen collection and storage, or on *in vitro* conditions for successful pollen germination and tube growth of *Banksia* species. The aim of this study is to develop *in vitro* viability testing methods for *Banksia* pollen and to develop practical methods for long term storage.

Species relationships

Species relationships are important for classification and have implications for the evolution of a group. If species relationships are resolved, then breeding and introduction of foreign germplasm for crop improvement can be more focused and efficient. This project investigates species relationships in two ways, through interspecific hybridisation and via molecular techniques. Potential crosses identified using interspecific hybridisation will be investigated further for seed set and new cultivar development. Various molecular methods

can be used to address different questions. Random amplified polymorphic DNA (RAPD) markers are assessed as a potential tool for phylogeny within genus *Banksia*. Another technique, direct polymerase chain reaction (PCR) sequencing of chloroplast DNA, is used to investigate higher order species relationships within genus *Banksia*, between subgenus *Banksia*, and subgenus *Isostylis*, and between genus *Banskia* and genus *Dryandra*. Currently, relationships between these genera and also within genus *Banksia* are not fully resolved.

Genetic diversity in natural populations of Banksia

The breeding system and levels of genetic diversity in *Banksia* species are important in population genetics, conservation and management of endangered or threatened species, and ultimate species survival. To date, there is little information on levels of genetic diversity and breeding systems of *Banksia* species, including many rare or threatened species. This project investigates levels of genetic diversity for a number of species of *Banksia* using random amplified polymorphic DNA (RAPD) markers to estimate genetic diversity in natural populations of *Banksia*.

Patterns of genetic diversity within and between populations of B. cuneata, *a rare and endangered species*

Genetic diversity within and between all known populations of *B. cuneata* is investigated. *B. cuneata* is declared a rare species under the WA Wildlife Conservation Act, and is under threat of extinction due to small population sizes and fragmented distribution over private and public land. Genetic studies including all known remaining populations have not been conducted. Levels and patterns of genetic diversity are investigated using RAPDs, and this information is important for future conservation and management strategies for *B. cuneata*.

Chapter Two

Literature review

Introduction

The biology of *Banksia* breeding systems is important in the context of interspecific pollination, pollen storage, species development and isolation, species relationships, classification, and conservation of natural resources. In addition, related topics such as genetic markers in plant breeding, population genetics and phylogeny make an important contribution to *Banksia* improvement.

Taxonomy

The first species of *Banksia* to be described (*B. serrata, B. integrifolia, B. dentata* and *B. ericifolia)* were collected on the east coast of Australia at Botany Bay and Endeavour Bay by J. Banks and D. Solander in 1770. Carl Linnaeus named *Banksia* based on these four species in 1781. Subsequently, there have been other species described by Brown (1810, 1830), Meissner (1855), Gardner (1928, 1964) and George (1981). Since the monograph of George (1981) only three further taxa have been described. The currently accepted classification of George (1987) is shown in Table 2.1.

Early treatments of *Banksia* were either catalogues of species (Brown), or artificial classifications (Meissner). George attempted a more natural classification, using a variety of leaf, flower, follicle and seed characters to base infrageneric taxa. While George describes "possible lines of evolution" within and between taxa, his classification is difficult to interpret phylogenetically because of a number of taxa with uncertain relationships.

Doust (1983) attempted a cladistic analysis of *Banksia* using 37 characters for 86 taxa, but the trees were poorly resolved. This analysis provided a poor match with the existing taxonomy and failed to suggest a robust phylogenetic scheme. It was not used for a formal classification. More recently Thiele (1993) proposed a classification based on cladistic analysis of morphological characters. Although there is some agreement with the currently accepted classification of George (1987), there are still some uncertainties in the positions of some species and support for some nodes is tenuous.

Relationships within the family Proteaceae have been discussed at length by Venkata Rao (1971), Smith-White (1959), Ramsey (1963) and Johnson and Briggs (1975, 1981). The views of Johnson and Briggs represent the most recent and accepted classification for the family.

The Proteaceae is a large and diverse family of trees and shrubs comprising 75 genera and 1500 species (Johnson and Briggs 1983), found in Australia, South Africa and South America, and is presumably Gondwanan in origin. Few genera are common to South America and Australasia with most genera and species endemic to one continent. Fossil records indicate a very ancient family dating back to the mid to late Cretaceous and Tertiary period (Dettman 1973, Martin 1978). Relationships amongst extant Proteaceae are tentative with *Banksia* placed in the subfamily Grevilleoideae. Within this subfamily there are two ~~subtribes~~ tribes, ~~Bankisieae~~ Banksieae and Musgraveinae, the latter restricted to the rainforests of north eastern Australia. Within subtribe ~~Bankisieae~~ Banksiinae there are two genera *Banksia* and *Dryandra*. All authors agree that *Banksia* and *Dryandra* are each well defined, natural and monophyletic groups, although there are some parallel developments within the two genera.

Banksia comprises two subgenera, *Isostylis* and *Banksia*. The former group is small and the species are similar to *Dryandra*. This ~~lead~~ led George (1981) to propose that *Isostylis* may be closer to *Dryandra* than to subgenus *Banksia*. Nevertheless, he placed it as a

subgenus of *Banksia*, with the comment that a separate genus may be appropriate. This interpretation was based on ~~three~~ four morphological characters. The three species in subgenus *Isostylis* lack the prominent ~~involucal~~ involucral bracts typical of *Dryandra*, but have flower subtending bracts typical of *Banksia*, their follicles are thick, woody and tomentose as in *Banksia* and they have the ovoid inflorescence axis typical of *Banksia*. There is still speculation however, on the relationships of subgenus *Banksia,* subgenus *Isostylis,* and genus *Dryandra*. It is possible that other data such as DNA sequence, or other DNA ~~comparison~~ comparative methods may resolve relationships at this level.

Table 2.1 Classification within *Banksia* (George 1987)

SUBGENUS BANKSIA	
Section Banksia	
Series Salicinae: *B. canei, B. conferta, B. dentata, B. integrifolia, B. marginata, B. oblongfolia, B. paludosa, B. plagiocarpa, B. robur, B. saxicola*	Series Cyrtostylis: *B. ashbyi, B. attenuata, B. audax, B. benthamiana, B. elderiana, B. elegans, B. epica, B. laevigata, B. lindleyana, B. lullfitzii, B. media, B. pilostylis, B. praemorsa.*
Series Grandes: *B. grandis, B. solandri.*	Series Prosratae: *B. blechnifolia, B. chamaephyton, B. gardneri, B. goodii, B. petiolaris, B. repens*
Series Quercinae: *B. oreophila, B. quercifolia*	Series Tetragonae: *B. aculeata, B. caleyi, B. lemanniana*
Series Bauerinae: *B. baueri*	Series Coccineae: *B. coccinea*
Series Banksia: *B. aemula, B. baxteri, B. candolleana, B. menziesii, B. ornata, B. sceptrum, B. serrata, B. speciosa.*	**Section** Oncostylis
	Series *Spicigerae: B. brownii, B. ericifolia, B. littoralis, B. occidentalis, B. seminuda, B. spinulosa, B. tricuspis, B. verticillata*
Series Crocinae: *B. burdettii, B. hookeriana, B. prionotes, B. victoriae.*	Series Dryandroideae: *B. dryandroides.*
Series Cyrtostylis: *B. ashbyi, B. attenuata, B. audax, B. benthamiana, B. elderiana, B. elegans, B. epica, B. laevigata, B. lindleyana, B. lullfitzii, B. media, B. pilostylis, B. praemorsa.*	Series Abietinae: *B. grossa, B. incana, B. lanata, B. laricina, B. leptophylla, B. meisneri, B. micrantha, B. nutans, B. pulchella, B. scabrella, B. sphaerocarpa, B. telmatiaea, B. violacea.*
	SUBGENUS ISOSTYLIS
	B. cuneata, B. ilicifolia, B. oligantha.

Conservation

The reasons put forward for the preservation of species are varied. They range from the belief that all plants and animals have the right to exist, to the view that future generations have the right to expect current resources. Strict preservationism is not the same as conservation. Conservation may allow preservation of species and ecosystems, but also allow their use in a way that is not damaging or wasteful. Preservationism is concerned with preventing destruction of species, ecosystems and wilderness, when they may be regarded as resources by others. To adopt a preservationist view would result in world-wide starvation, and to have uncontrolled consumption would lead to the same through exhaustion of resources. A challenge to the conservationist is to seek middle ground when promoting conservation and controlled exploitation.

Extinction rates for flora and fauna are increasing, through environmental change caused by habitat destruction, introduced weeds and animals, and pollution. Threats to plants include agriculture, urban development, mining, quarrying, chemicals, overgrazing by animals, cultivation, fire, disease and disappearance of pollinators.

Extinction of a species involves a reduction in numbers until it ceases to exist. Extinction is dynamic and occurs when environmental conditions exceed the adaptive capacity of the individuals. Two classes of events lead to extinction, deterministic and stochastic events. Deterministic events are those which involve unalterable change such as deforestation or climate shift. Stochastic events are random and are a result of normal changes or environmental disturbance. Stochastic events reduce population size and density and make them more susceptible to other events. In nature, many extinctions are brought about by deterministic events which reduce numbers and then stochastic events are influential. High environmental variability is the greatest barrier to persistence, rather than demographic and genetic uncertainty.

Stochastic forces fall into three categories. Demographic stochasticity, which is the chance variation of low births and high deaths. The second type is genetic stochasticity, which includes inbreeding depression, chance effects of lethal genes, and the loss of genetic diversity and heterosis. The third type is environmental, and it includes environmental shocks felt by all members of a population. In a dispersed population there is likely to be more habitat variation that there are buffers against change. However, no environment is static and consequently all populations are liable to environmental stochasticity. Deterministic events are readily observed such as storms, fires and feral animals, but stochastic events may go unnoticed until it is too late. This is why monitoring of threatened populations is important for conservation of species.

In each case of potential extinction specific strategies are required to protect the species, based on life history, distribution, habitat, fluctuations in predator relationships and inherent genetic variation. However, in general it can be said that as population size decreases the threat of extinction increases. In the very long term, survival and persistence depend on the adaptability of a population to change its genetic composition in response to long term changes in the environment. This depends on genetic resources available, hence the need for large effective population sizes.

Effective population size is not just the total number of plants. Effective size may be reduced by the presence of non breeding individuals, skewed sex ratios, inbreeding, random variation in progeny, loss of genetic diversity through a bottleneck period of low population size and patterns of pollination. Some populations may be far too small to survive even if they appear to be healthy and viable. Such species may be on the way to extinction unless there is rapid intervention.

Natural populations of *Banksia* are subject to a number of pressures such as land clearing for agriculture, mines, roads, exploitation for cut flowers, burning and disease. In Western Australia (WA) clearing threatens the survival of *B. cuneata* and *B. hookeriana*.

Populations of species that are killed by fire and regenerate from seed, may not survive two fires in quick succession since they would not have enough time to mature, flower and produce more seed. Rare species that would suffer in this respect are *B. aculeata, B. burdettii, B. conferta, B. cuneata, B. dryandroides, B. lanata, B. laricina, B. meisneri, B. pilostylis, B. praemorsa, B. quercifolia, B. scabrella, B. telmatiaea, B. vericillata* and *B. victoriae* (George 1987). Many species are exploited commercially including *B. coccinea, B. hookeriana, B. prionotes, B. menziesii, B. baxteri* and *B. speciosa.* With continued decline in inflorescence number there may be a reduction in the seed bank and subsequent loss of genetic variability. Under the WA Wildlife Conservation Act 1950-1979 there are seven *Banksias* species that are declared rare flora. They are *B. brownii, B. chamaephyton, B. cuneata, B. goodii, B. meisneri, B. sphaerocarpa* and *B. tricuspis.*

It is essential that all species of *Banksia* be conserved in the wild. Banksias are important components of the vegetation in which they occur and are a food source and host for many species of native fauna (George 1987). They are also a propagation source and tourist attraction. Native plants are Australia's greatest horticultural asset and there is a need to safeguard germplasm for the sake of future production and education.

Plant population management

Many aspects of plant population biology must be considered during the development of conservation strategies. These include: breeding systems, life cycle patterns, dispersal modes and their application to management. Species management involves management of particular populations at specific sites, but species conservation cannot be considered to be independent of habitats and ecosystems. The use of land adjacent to the site is relevant to the conservation of particular populations. Social issues are also important, as the particular site or species may be of interest to several groups whose goals may be incompatible with conservation. There are also likely to be budget and staff constraints. Sometimes effective management simply consists of an initial survey, followed by

protection of the site from unwanted human interference, with periodic visits to check the state of the habitat and number of plants. If intervention is necessary, the management choices multiply, and selection of the appropriate method may become difficult. Population management needs to be considered from the following points of view: (1) Ecosystem management depends on maintenance of key species, whose decline could affect the whole ecosystem. (2) Particular species may be threatened because of factors outside the ecosystem, such as harvesting and the consequences of small population size. (3) Some species may require specific habitat manipulation. (4) It cannot be assumed that because a species is present now, that it will still be present in 50 or 100 years time. Strategies need to consider the expected persistence time. (5) People generally find it easier to understand conservation of biodiversity at the species level rather than at the ecosystem and gene levels. Species conservation is best done at the population level. (6) The ultimate test of management success is whether it promotes the persistence of biodiversity into the future. Given adequate protection and assistance in reproduction, many rare and endangered plants can recover in numbers and vitality. The role of conservation biology is to shift the balance in their favour.

Breeding Systems

Which gametes are actually brought together to form zygotes, depends on the breeding system of the plant. There are many floral, physiological and genetic mechanisms that contribute to the mating system of plants. It is essential to have an insight into the breeding system to understand the complex patterns of variation within and between natural populations. There are three basic breeding mechanisms, outbreeding, inbreeding and apomixis. Most higher plants are hermaphrodite and have male and female parts, suggesting self fertilisation. It is interesting however, that many plants are adapted to cross fertilisation, and minimise or prevent self fertilisation. In most cases self incompatibility is involved. Species that are self compatible may be able to flower and fruit in the absence of pollinators, and the progeny are often uniform in appearance. In

apomixis, reproduction is achieved without fertilisation and the sexual process is avoided. Two types of system are found, vegetative apomixis and agamospermy. Vegetative apomixis is the radial growth by rhizomes, stolons and runners, and is characteristic of many perennial species. Plants arising from these propagules are the same genotype as the parent. Agamospermy is when seed is set but no sexual fusion has occurred. Offspring are the same as the plant that produces them.

The breeding system of many banksias is still unclear. Information has come from studies of seed set, pollen tube growth, and more recently enzyme electrophoresis (Fuss and Sedgley 1991, Carthew *et al.* 1988, Coates and Sokolowski 1992, Sampson *et al.* 1994). High levels of outcrossing have been reported in *Banksia*, and are among the highest recorded for plants (Scott 1980, Schemske and Lande 1985, Carthew *et al.* 1988, Coates and Sokolowski 1992). Banksias are predominantly outcrossing, although some studies have shown a mixed mating system (Fuss and Sedgley 1991).

Banksia *flower morphology*

Banksias produce inflorescences composed of numerous hermaphrodite flowers clustered in pairs around a central woody core. Each floret has a single elongated pistil with two ovules in the unilocular ovary. Anthers are attached near the tip of each of the four perianth parts by short filaments. This region of the perianth, prior to anthesis, encloses the distal portion of the style which in many species has an obliquely terminal stigmatic groove. This portion of the style is modified for the special function of pollen presentation. The anthers dehisce prior to anthesis depositing pollen onto the pollen presenter. At anthesis, the style is released from the perianth and pollen is available to foraging insects. The stigmatic groove becomes receptive after the pollen has been removed, when it is ready to receive pollen from another plant. Following fertilisation, both follicles and seeds may develop, taking from a few months to two years to mature.

Floral initiation

Floral initiation and development in relation to the time of flowering has not been widely studied in *Banksia*, although investigations have been conducted on *B. baxteri* and *B. hookeriana* (Rohl *et al.* 1994) and *B. menziesii* and *B. coccinea* (Fuss and Sedgley 1990). Floral initiation occurred in spring in *B. baxteri* and in early summer in *B. hookeriana*. Floral development was rapid in *B. baxteri* taking three months to reach anthesis, with five months in *B. hookeriana,* which flowered in winter. In both species, shoots that flowered were thicker in their first year than those that did not. Floral initiation in *B. menziesii* and *B. coccinea* occurred in late spring, but the rate of development was significantly different.

Flowering times in *Banksia* vary, with some species in flower in all months of the year, with a peak in autumn (Collins and Rebelo 1987). Species vary considerably with regard to the time taken for all flowers on an inflorescence to open, from four days in *B. occidentalis* to 57 days in *B. spinulosa* (Collins and Rebelo 1987). This could be due to factors such as inflorescence size, and period of nectar production required for pollinator attraction. The flowers at the base of the inflorescence open first in most, but not all, species of *Banksia*. The continued elongation of the style in the unopened flower after termination of the growth of the perianth, leads to style curvature, such that the number of flowers opening on an inflorescence is increased by the triggering action due to contact of foraging animals such as birds. Variable amounts of pollen are presented on freshly opened flowers, and counts of 1×10^3 to 3×10^3 have been reported for five species of *Banksia* (Collins and Rebelo 1987).

Within communities, opportunities for hybridisation among species may be minimised by staggered times of flowering, different pollen vectors and possibly by interspecific incompatibility or incongruity. Birds and insects generally forage in such a way as to facilitate both outcrossing and selfing.

Protandry

Protandry occurs when the male reproductive organs are mature before the female, and many genera of the Proteaceae are protandrous. It has been reported in *Banksia* (Carolin 1961, Venkata Rao 1971, Johnson and Briggs 1975) but there have been few estimates of the exact timing. In *B. menziesii* maximum pollen germination and pollen tube growth occurred at 3 days post anthesis (Fuss and Sedgley 1991). The development of maximum stigma receptivity to pollen, as indicated by secretion of esterase and other enzymes by the papillae in the groove, coincides with this change. In *B. prionotes* peak esterase production was recorded at 40 hours after flower opening (Collins and Spice 1986). In most angiosperms, secretion continues for several days after flower opening (Mattson *et al.* 1974).

Pollination

Current knowledge of pollinating fauna associated with the Proteaceae is far from complete. Data suggest that birds such as honeyeaters are common visitors and a variety of small rodents and marsupials are also known to forage on some *Banksia* species. Invertebrate visitors such as bees and wasps have been identified as visitors, and in addition small arthropods have been associated with some species, such as *B. prionotes* (Collins and Rebelo 1987). Vertebrate visitors vary in size and foraging behaviour. For example, honeyeaters often perch on the distal part of the inflorescence and lean forward and downward inserting their bills into recently opened flowers. The forehead and throat of the bird rub against the pollen presenters and stigmas (Collins and Rebelo 1987). The success of particular animals as pollinators depends on their ability to deposit pollen on the stigmas of receptive flowers. Some species of *Banksia* such as *B. baueri* and *B. attenuata* attract a variety of pollinators (Collins and Rebelo 1987). Preferential foraging by honeyeaters has been reported for some species, where partly opened inflorescences maybe preferred regardless of the relative abundance of other inflorescences. The

likelihood of effective pollen transfer is also increased by birds foraging exclusively within 1-2 cm of the boundary between opened and unopened flowers (Collins and Spice 1986).

With hermaphrodite plants, both selfing and outcrossing are theoretically possible. The foraging behaviour of many visitors can facilitate transfer of pollen from freshly opened flowers to older receptive stigmas on the same inflorescence. However, it is likely that birds and mammals promote outcrossing to a greater extent than insects (Collins and Spice 1986), as the former generally can move greater distances.

Consequences of Different Reproductive Modes.

The consequence of self fertilisation is important for our understanding of breeding systems. In the heterozygous diploid, a dominant allele often 'shelters' a recessive allele which may be deleterious in the homozygous state. Self fertilisation quickly results in the accumulation of deleterious recessives. Further selfing results in the production of uniform lines differing in various vegetative and reproductive characters, with weakening and sterility of the plants. If plants from different lines are crossed then hybrid vigour or heterosis may be demonstrated. However, plants that are closely related and arise from the same original parental stock do not give heterotic plants. The long term disadvantages of inbreeding demonstrate the advantages of the outbreeding mode of reproduction, which leads to high levels of variation. The role of incompatibility is very important in considering breeding within populations. While some fruits and seeds may be dispersed long distances, some may fall near the parents and form family groups made up of genetically closely related plants. Crossing between close relatives may lead to inbreeding depression, and incompatibility mechanisms will restrict matings between the closely related individuals.

Generally, obligate outbreeding appears to have advantages, but there may be costs such as uncertain environmental factors influencing cross pollination. Apomixis also has advantages and disadvantages including the propagation of undesirable types. An analysis of the three modes of reproduction shows that each has advantages and disadvantages. It would appear that there are advantages in the short term to produce only well adapted genotypes, but the lineage unable to produce variation is disadvantaged in competition with those capable of change. Lack of variation may not allow a species to withstand selection pressures such as changes in climate and seasonal differences. It is therefore not surprising that flowering plants have several modes of reproduction.

Self incompatibility

Self incompatibility (SI) is the inherited capacity of the flower to reject its own pollen. The degree of self incompatibility in *Banksia* varies among species (Carpenter 1978, Collins and Spice 1986; Salkin 1987). Some show self compatibility following hand pollination, but natural levels of selfed seed set are largely unknown. Biochemical and genetic estimates from isozyme analysis of seeds of two species of *Banksia* showed complete outcrossing (Carthew *et al.* 1988). The high rates imply that there is good mixing of pollen in the populations studied. Pollen gene frequencies were relatively uniform in distribution over plants suggesting that pollen is widely dispersed within the population. Results at different sites were similar despite differences in the level of seed set. In a number of species it has been reported that outcrossing is essential for seed set. Thus, some species have been reported to be self incompatible, for example *B. menziesii* (Ramsey and Vaughton 1991) and *B. prionotes* (Collins and Spice 1986), but some such as *B. spinulosa* (Salkin 1987) have been reported to be self compatible to some extent. Some other members of the Proteaceae show varying levels of self compatibility, for example *Macadamia* (Sedgley 1983). More research is needed however, on breeding systems within the family.

Incompatibility types can be divided into two groups, sporophytic and gametophytic. In sporophytic incompatibility, action of the pollen is determined by the genotype of the plant producing the pollen. In gametophytic incompatibility, the genotype of the individual microspore determines its action.

The system of incompatibility can further be classified into two groups: (1) Polyallelic series at one or two loci (rarely several) in gametophytic and sporophytic systems where the flowers are homomorphic. (2) Two alleles per locus at one or several loci, typically sporophytic and the flowers are heterostylous. The sporophytic system has a dry stigma so the pollen has direct contact with the papillae. In contrast, the gametophytic system has a wet stigma surface on which there is no direct pollen stigma contact, since germination takes place in a fluid medium.

Incompatibility expression can be observed in three sites in the flower: the stigma, style, and the ovary. Depending on the system involved these sites can be used to infer essential points of the incompatibility system in the species.

Stigma.

The stigma reaction is characteristic of species with tri nucleate pollen (Brewbaker 1959), whereas those species that have binucleate pollen are more likely to show inhibition in the style or ovary. Trinucleate pollen is more specific in growth requirements *in vitro* and it is suggested that inhibition in the stigma is due to lack of stimulus on the stigma via the incompatibility reaction. Stigmatic incompatibility is generally restricted to sporophytic systems (Heslop-Harrison *et al.* 1974). It is suggested that incompatibility proteins held in the exine of the pollen grain become immediately available on contact with the stigma. Substances responsible for the stigma reaction are of tapetal origin (Dickinson and Lewis 1973).

Stylar inhibition

Inhibition of the incompatible pollen tube in the style occurs from a few to many hours after germination of the pollen. It is characteristic of self incompatible plants with binucleate pollen. Stylar incompatibility is generally restricted to gametophytic incompatibility and usually leads to swelling and bursting of the pollen tube apex in the upper region of the style. The fact that incompatible and compatible pollen tubes do not influence each other suggests reactions occur on the surface of the pollen tube (Linskens 1965).

Ovary inhibition

In a number of cases the incompatibility reaction does not act until the pollen tubes have reached the ovary and in some cases initiated gametic fusion. In some species where incompatibility occurs in the ovary, the styles are hollow and do not provide pollen tubes with the contact to the stylar tissue necessary for inhibition (Brewbaker 1959).

Evolution of self incompatibility

As outbreeding operates in both gymnosperms and angiosperms, it appears that self incompatibility, which is restricted to the angiosperms, must have coincided with the expansion of the phylum and therefore occurred as a primitive character in the mid-~~cretaceous~~ Cretaceous period (Whitehouse 1950). The rise of a very efficient outbreeding system, such as self incompatibility, is likely to have occurred in conjunction with the evolution of specialised pollinating insects at a very early stage in angiosperm evolution (Grant 1949). Another line of evidence that self incompatibility is a primitive character is that its occurrence is restricted to centres of origin of species (Ernst 1953).

The self incompatibility mechanism

All types of incompatibility appear to proceed as follows: (1) Accumulation of S proteins in the pollen walls and in the inhibition sites in the pistil. (2) Recognition, after incompatible pollination, of identical S proteins in pollen and pistil. (3) Failure of the pollen grain to establish or maintain contact with the stigma and the formation of callose below the pollen within the papillae of the stigma, or in the case of stylar incompatibility the breakdown of the callose-rich inner wall of the tube apex and the accumulation of wall particles in the tube cytoplasm. (4) ~~inhibition~~ Inhibition after this phase or through the process itself of the pollen grains or tubes.

Interspecific Incompatibility

Interspecific incompatibility is defined as any post pollination process preventing formation of hybrid zygotes. This may occur through the absence of pollen germination or via abnormal behaviour of pollen tubes. The phenomenon prevents gene flow between species, in contrast to self incompatibility, which restricts inbreeding, and establishes upper limits to outbreeding and panmixis. Interspecific incompatibility thus contributes to the isolation of populations and favours speciation and the gradual increase of polymorphism within the genus and within the family. It acts as a breeding barrier between sympatric species, but also participates in the rejection of foreign germplasm migrating as pollen grains from allopatric populations, and in the isolation of invaders introduced as seed. Although less studied and far less understood than self incompatibility it displays characteristics in common with the system of self rejection.

Interspecific incompatibility is usually stylar in the genera where SI is expressed in the style and stigmatic in families characterised by stigmatic SI. An important feature of interspecific incompatibility is that it usually occurs unilaterally and often prevents self incompatible species crossing with self compatible species (Harrison and Darby 1955).

Unilateral incompatibility also occurs between self compatible species and also in populations of self incompatible plants.

Interspecific incompatibility occurs throughout the angiosperms in a wide range of families. It is the thesis of Hogenboom (1973, 1975) that interspecific incompatibility, termed incongruity by this author, is a process completely distinct from SI with no association with the S locus. According to Hogenboom the non functioning of the pollen pistil relationship may be due one of two separate mechanisms. One is incompatibility, a mechanism that prevents or disturbs the functioning of the relationship and regulates inbreeding and outbreeding at the intraspecific level. The second is incongruity, the incompleteness of the relationship. In the different species the matching of the genetic systems is not complete. In contrast to incompatibility, which is an evolutionary solution through a precise reaction to the negative effects of inbreeding, incongruity is a by product of evolutionary divergence, which may affect any part of the relationship between pistil and pollen and can be very different in each case. Hogenboom justifies the hypothesis on the basis of anatomical, physiological and genetic studies which suggest basic differences between the two processes of incongruity and incompatibility. With regard to anatomical and physiological differences however, the majority of observations show that SI and interspecific incompatibility affect the same sites. The plant breeder often wishes to break down these incompatibility barriers to transfer germplasm from wild relatives into cultivated species.

Reproductive Ecology

Flowering plants generally produce more seeds than are needed for individual replacement, because of losses during germination and plant establishment. However, low seed set is a common feature of many angiosperms, and may be a result of pollination failure, seed mortality, predatation, limited resources or genetic constraints. In some species of *Banksia,* such as *B. laricina,* resource allocation affects final seed output

(Stock *et al.* 1989). It appears that low seed set in *Banksia* is the result of selection for obligate outcrossing and high seed mineral composition. This maintains genetic diversity of a restricted number of high quality seeds equipped for establishment in the low nutrient soils of their natural habitat (Stock *et al.* 1989). The seeds of *Banksia* carry large reserves of oil, protein and essential mineral nutrients, especially phosphorus, and trace elements (Kuo *et al.* 1982), and seedlings are able to grow rapidly in soils deficient in phosphorus and nitrogen. *Banksia* seeds are unusually rich in proteins which are mostly associated with distinct protein bodies containing globoid inclusions, enriched with mineral elements such as phosphorus, calcium, sulphur and magnesium (Kuo *et al.* 1982). The development of highly nutritious seeds also provides predators a good quality food source in a poor environment. Predators such as birds and insects reduce seed production of *Banksia*.

The valves of the follicles in which the seeds develop are held together by a resinous substance that is destroyed by heat. The structure of valves is quite complex, consisting of three layers of sclereids in various orientations. As the follicles dry, stress increases in the valves, which is relieved when the resin layer melts, causing the valves to open to allow release of the seed (Waldrop 1983). However, heating follicles does not always release the seed. Cowling and Lamont (1986) found that wet-dry cycles increased the rate of seed release. It was suggested that the separator in the follicle is hygroscopic, and with alternate wetting and drying it acts as a lever easing the seeds out.

Fire is also an important component of the life cycle of banksias. *Banksia* species differ in their response to fire. Obligate seeders are killed by fire and regenerate from seedlings, whereas resprouters regrow from epicormic buds beneath the bark, or from underground lignotubers (Zammit and Westoby 1988). While many plants rely on fire to promote germination of seedlings, or regrowth from lignotubers, the frequency of fire is critical to the survival of the species. If fire occurs after too short an interval, plants may be destroyed before they have flowered and set seed. Ultimately, plants which are obligate

seeders may become extinct with too frequent burning. Studies on the behaviour of banksias in fire has been conducted with species such as *B. hookeriana* (Lamont 1985), with a view to understanding regeneration limitations, and to recommend burning intervals. The seed bank at any given time will depend on factors such as growing conditions, predation and the last fire interval. Another factor governing survival after fire is the season in which the fire occurs. Summer/autumn burns give better establishment of seedlings than spring burns, as the weather conditions are more favourable for germination. Seed released in spring will not germinate until the following autumn, and during this time it may be eaten or lose viability. Seed mortality is very high in the first two years after germination with only a few seedlings surviving to adulthood.

Reproductive isolation

The mechanisms of reproductive isolation can be temporal, ecological, or physiological and may exert their effect pre or post zygotically. Differences in flowering time can be an effective breeding barrier and although there may be times when flowering overlaps, the likelihood of producing hybrids is low. Ecological isolating mechanisms such as the absence of common pollinators, and niche conditions select against intermediate hybrid plants. Physiological isolating mechanisms can be no resultant seed set, or reduced viability of seedlings. While the concept of increasing taxonomic distance and increasing genetic disharmony is widely accepted, the amount that this contributes to the extent of hybridisation, in relation to other factors such as incompatibility is not known.

Natural Hybridisation

Presumed natural hybrids occur in wild populations, the most common being *B. robur* x *B. oblongifolia* (George 1987). Other putative natural hybrids are shown in Table 2.2 (Taylor and Hopper 1988). Confirmation of their status relies on a number of criteria including the presence of both parental species in the immediate area, morphological characteristics of the

hybrid intermediate between both parental types, a degree of pollen sterility, and use of techniques such as protein electrophoresis and DNA based methods to establish hybridity. Most *Banksia* hybrids have yet to be confirmed through pollen, protein, and DNA techniques. The hybrid between *B. prionotes* and *B. lindelyana* is significant since the two species are not closely related, and are classified in separate series (George 1981). Only a single tree exists, the fruit is like *B. lindelyana* but the leaves are intermediate between the two parental species. A single hybrid of *B. oblongfolia* and *B. integrifolia* was found to have the leaf shape of *B. integrifolia*, except for a few blunt teeth, and a white undersurface (*B. integrifolia*), but firm texture and rusty hairs on the midrib (*B. oblongfolia*). The flowers had the more silky appearance of *B. integrifolia* rather than *B. oblongfolia.* Another presumed hybrid *B. saxicola* x *B. marginata* has leaves similar to *B. saxicola* but the fruiting cones retained the old flowers and appeared intermediate between the two species. A single old tree presumed to be a *B. hookeriana* and *B. attenuata* hybrid had more than 1000 flower spikes (Taylor and Hopper 1988), but they were small in size and the number of flowers was very low. The flowers were also malformed and never opened properly. The plant was infertile and did not produce seed. Leaves were intermediate and old leaves were retained, as in *B. hookeriana.*

Table 2.2 Recorded hybrid banksias

Female parent		Male parent
B. ericifolia	x	*B. spinulosa*
B. marginata	x	*B. conferta*
B. marginata	x	*B. integrifolia*
B. aemula	x	*B. serrata*
B. paludosa	x	*B. integrifolia*
B. hookeriana	x	*B. prionotes*
B. paludosa	x	*B. marginata*
B. saxicola	x	*B. marginata*
B. integrifolia	x	*B. oblongifolia*
B. ericifolia	x	*B. spinulosa*
B. prionotes	x	*B. lindleyana*
B. prionotes	x	*B. hookeriana*
B. hookeriana	x	*B. attenuata*
B. hookeriana	x	*B. menziesii*

Banksia hybrids are usually intermediate between the parents in appearance and may have appeal for horticulture. A hybrid with large showy spikes 'Giant Candles', occurred spontaneously in Queensland. The parents were thought to be *B. ericifolia* and *B. spinulosa* (George 1987). Other presumed hybrids are rare. Some possible hybrids are being investigated for ornamental appeal as flowering pot plants or cut flowers, including *B. hookeriana* x *B. ~~prinotes~~* prionotes and *B. hookeriana* x *B. menziesii*. (George 1987, Sedgley *et al.* 1991).

Hybrid fitness

In many cases, such as Eucalyptus natural putative hybrids are F1 progeny, with low variability and morphological segregation of parental characters in the seedlings (Ashton and Sandiford 1988). These hybrids may arise due to chance such as following fire. However, their contribution to the gene pool and thus to gene flow between the species will depend on reproductive output and the adaptiveness of their progeny. The three phases of hybridisation described by Drake (1980) are: F1 plant establishment, plant fertility, and evolutionary potential. Some hybrids are reported to be less fertile than average compared to the parents, some are intermediate, and some are better than the parental species and can expand into the parent species' habitats. If the F1 plants are fertile, then the next most important criterion is interaction with the local environment, which determines to what extent the F1 and subsequent generations survive. Hybridisation has also been suggested as a mechanism of species migration (Potts and Jackson 1989, Potts and Reid 1988). In some cases the hybrids may colonise local habitats better than either parent, or provide a link for genetic combinations with a nearby species via long distance pollen dispersal (Potts and Reid 1983).

Patterns of hybridisation

In natural conditions the frequency of hybridisation depends on the co-occurrence of species pairs, synchronous flowering and the presence of pollinating agents. The problems of geographic isolation are overcome in mixed species plantations and flowering times can vary with individuals. Manipulative hybridisation also increases the range of pollinations possible. Another factor determining the success of hybridisation is the extent of reproductive isolation. This can be assessed on the basis of species pair relationships.

Chromosome Studies

Karyological evolution within the Proteaceae has been discussed by Smith-White (1959), Ramsey (1963), Johnson and Briggs (1963) and Venkata Rao (1971). *Banksia* is reported to have 28 chromosomes (n=14) in all eight species tested, *B. asplenifolia, B. ericifolia, B. integrifolia, B. latifolia, B. marginata, B. robur, B. serrata* and *B. spinulosa*. The chromosomes are very small, as is common in perennial tree crops. No natural polyploids or other chromosome abnormalities of *Banksia* have been recorded, and *Dryandra*, the sister taxa to *Banksia*, in the tribe Banksieae, also has 28 (n=14) chromosomes.

Propagation

In natural stands banksias reproduce almost exclusively through sexual reproduction. Some species have lignotubers which regenerate after fire, but this does not lead to the formation of new individuals. The majority of commercial *Banksia* plantations are currently established using seedlings. Seeds germinate readily, with only a few species needing cool conditions to break dormancy (George 1987), and seed remains viable for many years. Once seedlings are established damping-off is a potential problem in the first few months. When selecting particular forms for cultivation, seed propagation can lead to undesirable

variation in the progeny. For this reason, vegetative propagation is a goal for the production of superior plants.

A number of species can be grown from cuttings, but success has been limited with the cut flower species. In general, those with slender glabrous stems such as *B. spinulosa, B. ericifolia,* and *B. integrifolia* will form roots most readily (George 1987). Cuttings usually form roots without hormone treatment, and the plants must be hardened and allowed to develop in the pot before planting out. Grafting of superior plants onto seedling rootstocks is another potential method, although problems with graft incompatibility and low success rates render it difficult. Grafting has been attempted to overcome *Phytophthora* susceptibility by grafting onto a resistant stock such as *B. spinulosa* or *B. ericifolia* (Dixon *et al.* 1985). There has been some success, for example, *B. speciosa* onto *B. integrifolia.*

Australian plants are increasingly propagated by tissue culture, which can increase plant numbers when hard to multiply by other means (Taji and Williams 1990). Tissue culture has also been used to facilitate and supplement conventional breeding programs, for example, by anther culture, embryo rescue and somatic hybridisation. Tissue culture has some advantages over conventional techniques of propagation including rapid multiplication, delivery of plants at any time of year, propagation of plants that are difficult to root by cuttings, or have a low seed set, and disease free plants. Tissue culture is more expensive than seed or conventional vegetative propagation, which limits its application. Australia has an expanding micro-propagation industry due to export markets and increased activity in producing new horticultural and floricultural varieties. Research is currently underway to develop tissue culture techniques for *Banksia.*

Commercial Importance and Improvement of Banksia

Ornamental species of the family Proteaceae are now well established in the international and domestic cut flower industries. Widely accepted genera include *Banksia, Protea,*

Leucospermum and *Leucadendron*. The large showy blooms make them ideal as standard flowers in arrangements, however, this focuses attention on the quality of the bloom. Therefore, the challenge to plant breeders is to improve bloom quality and other desirable characters such as yield, production time, novelty and reliability.

The long production period and location in the southern hemisphere give Australia an opportunity to become a supplier of cut flowers, foliage and potted plants to world markets. Australia, even with a small penetration to those markets could earn significant export income. Exporters and wholesalers buy fresh flowers from growers and air freight to destinations such as the USA, the Netherlands, Japan and Switzerland. Large quantities of flowers and foliage of banksias and other Australian species are exported. The future for *Banksia* cut flowers is good, particularly if methods can be developed to extend the bloom time, from mid October to Christmas, when demand for red and orange flowers is high on northern hemisphere markets. However, most banksias bloom from late summer to spring. *Banksia* flowers last well when cut, and can be dried for use in floral arrangements. The decorative woody fruits can also be used by floral arrangers, while spent flower heads and fruits can be used in craft. Banksias are important for amenity horticulture, as they vary considerably in habit from prostrate bushes to tall trees up to 15 metres. Flower colour covers a wide spectrum including red, orange, brown, yellow, cream, purple and pale mauve. In some species, such as *B. coccinea,* a range of colours from deep red to orange occurs. Banksias are popular for their bird attracting qualities when nectar flow is at its peak, and honeyeaters are frequent visitors. At night pigmy possums feed on the nectar of some species, and insect eating birds also find ample food as the flowers attract insects.

Banksias are grown commercially for cut flowers in South Africa, Israel, USA (Hawaii and California) and Australia. From a genus with over 75 taxa there are approximately eight species, of which *B. coccinea* is the most popular, that are currently exploited. Further species have potential to be used in commercial horticulture. Research is essential to understanding the biology of banksias and to develop strict selection criteria for crop

improvement. Developing selection criteria is one of the most important stages of a breeding program, and for most proteaceous crops the criteria are yield, quality, and *Phytophthora* tolerance. *Phytophthora* is a devastating disease which affects many members of the family Proteaceae and can be a limitation to the cultivation of plants for cut flower production. In addition, this disease can wipe out existing populations and decrease natural resources. Yield is the major factor determining the economic viability of a planting. Presently, plantations are established via seed and show great variation in yield. Through breeding and selection, yield gain can be achieved and vegetative propagation can be used to capture the yield gain, and thus increase efficiency of plantation management. Complete uniformity needs to be balanced against the advantage of mixed crop resistance to environmental pressures, which is especially important in long term crops such as banksias and a mix of improved cultivars is an urgent aim. Quality is very important in marketing and strict quality standards, are being adopted in the industry over a range of crops. Important criteria include: long straight stems, terminal blooms with no interfering foliage, even bloom with minimal aborted florets, attractive floret colour, complementary foliage with attractive colour and shape, and no pest or disease damage.

The South African and American selection and breeding programs for Proteaceae species have resulted in many cultivars that have established the industry (Parvin 1981, Brits *et al.* 1983). These programs have focused on the African genera, and recently there has been a program established for the Australian genus *Banksia* (Sedgley *et al.* 1991). The breeding program focuses on the species *B. coccinea, B. menziesii, B. hookeriana,* and *B. prionotes*, and three cultivars have been registered already. The South African and American programs have used interspecific hybridisation as the basis for cultivar development (Parvin 1981, Brits 1985). The *Banksia* program (Sedgley *et al.* 1991) also aims to use interspecific hybridisation within the genus to produce novel cultivars and combine characters from other species. Hybridisation methods have been developed for *Banksia* based on knowledge of the breeding biology of the genus (Fuss and Sedgley 1991). In addition, research into the structure of the pollen presenter and the stigmatic

groove has shown that there is more than one type of pollen presenter, and the location of the stigmatic groove is different depending on the species (Sedgley *et al.* 1993). This is important in interspecific hybridisation to make sure the target is clearly identified for deposition of interspecific pollen into the stigmatic groove.

The continued interest in Australian species, particularly those from family Proteaceae, for cut flowers provides a challenge to plant breeders to supply new cultivars. Therefore, continued selection and cultivation of new variants and species combinations is essential to expand current markets and maintain high quality products. Importantly, conservation of natural populations of indigenous plants will ensure there is sufficient resource to tap for further crop development and improvement in the future.

Interspecific Hybridisation

As in many other crops, the genetic base in ornamentals can be very narrow. There is clearly scope for broadening the genetic base by interspecific hybridisation, both to introduce novel morphological and physiological characters and to improve specific traits for horticultural performance. There are few commercially improved crops which have not been improved by deliberate interspecific hybridisation (Langton 1987). The advantages of combining two genotypes are many fold. It can transfer genes for disease resistance, plant or fruit quality, or increases in yield. The success of hybridisation can also give information on the relationships of the species. Hybridisation can be effective between strains, between species and sometimes between genera, the success being dependent on the degree of relatedness (Carr *et al.* 1988). Wide hybridisation may be unsuccessful due to the failure to produce F1 progeny or in terms of hybrid weakness or hybrid breakdown.

The technique for artificial hybridisation is very similar to that used in manipulative intraspecific pollination. The *Banksia* flower is hermaphrodite and the pollen must be removed before application of the experimental pollen. Pollen is removed by passing a

looped pipe cleaner over the pollen presenter region of the style (Fuss and Sedgley 1991). It has been shown that banksias are protandrous so pollination cannot proceed until the stigma becomes receptive. Experiments investigating seed set and pistil age and condition have shown that maximum receptivity is related to groove opening and stigmatic secretion and occurs approximately three days after flowering in *B. coccinea* (Fuss and Sedgley 1991). The exact timing depends on the species and the longevity of the flower. In other genera pollinations before the stigma becomes receptive are mostly unsuccessful (Griffin and Hand 1979, Sedgley and Smith 1989). Fresh pollen may be applied by touching the pollen laden presenter region of the species of interest to the stigmatic groove. Flowers are bagged prior to experimentation to avoid pollen contamination.

The use of interspecific pollen presents an additional problem in the availability of fresh pollen. If non-synchronously flowering species are used then the pollen must be stored. *Banksia* pollen has not been tested in storage and *in vitro* methods of germination have not been reported. Viability of fresh *Banksia* pollen has been estimated using the fluorescein diacetate stain (FDA) (Ramsey and Vaughton 1991, Collins and Spice 1986, Ramsey 1986), but attempts to germinate pollen in sucrose and nutrient media were unsuccessful (Prakash 1986). Pollen longevity has been estimated using pollen of different ages on fresh pistils of *B. spinulosa* that have been stained with acetocarmine (Ramsey and Vaughton 1991). It was noted that when flowers opened pollen viability was high, but quickly decreased. Considerable variation between inflorescences was also noted.

Alternative methods of hybridisation

Barriers to effective fertilisation occur between many potential crosses and to overcome these barriers different techniques are used to bypass or reduce the effect. As the site of rejection in many crosses is the stigma or style, many methods facilitate the entry of the pollen into the ovary. Compatible pollen has been used to stimulate the post pollination response. This pollen can be used fresh or killed, or as pollen extract. The function of the

pollen is thought to provide the appropriate recognition substances missing from the foreign species, and hybrid seed has been obtained in ~~*Poplar*~~ Populus using this technique (Knox *et al.* 1972). Another method is the use of solvents on the stigma surface. This is thought to interfere with the maternal recognition system, and hybrids of *Eucalyptus* have been obtained using this method (Pryor and Willing 1974). Temperature can also affect the degree of inhibition of pollen tube growth in styles (Franken *et al.* 1988). Some techniques involve reduction of the distance that the pollen tube has to grow. This is done by using the shortest styled species as the female, or by amputation of the style and pollination of the stump (Raff 1983).

In some interspecific crosses embryos are formed but later abort, due to non function of the endosperm or interaction with the maternal genotype. Embryo rescue is used to overcome this, and can also be used for rapid multiplication of clones and acceleration of germination. Another approach is to bypass sexual reproduction via somatic hybridisation. Protoplasts of two species are fused, screened for hybrid cells, and the plants regenerated *in vitro* (Vasil and Vasil 1980). Bridging species can be used to transfer genes from one to another. This method is not popular due to the long time required and to undesirable characters passing from the bridging species.

Hybrid Verification

The most common method used for the verification of hybrid plants is screening of seedlings for morphological characteristics intermediate between the putative parental species. Hybrids have been identified using cotyledon shape (Kapoor and Sharma 1984), phylotaxy (Brooker 1979), and leaf shape (Ruggeri 1959). Seedling morphology has been used in hybrid verification in other genera, such as *Rhododendron* (Rouse *et al.* 1985). This technique works well when morphological markers are present at the seedling stage. Isozyme analysis is another technique commonly used. Species genotypes can be established and F1 hybrids are identified by their combination of parental

isozymes (Chaparro *et al.* 1987, Parfitt *et al.* 1985). Recently, a technique based on the polymerase chain reaction (PCR), called random amplified polymorphic DNA (RAPD) analysis has been used to verify the hybrid origin of plants (Chong *et al.* 1994).

Pollen Collection and Uses.

When collecting pollen from the field, the quantity available, season in which it can be obtained and the method of handling and storage depends on the plant species. In insect pollinated plants, the insects that visit the flowers do so primarily to collect the nectar at the base of the style, or the pollen from the dehisced anthers. Pollen in such flowers is readily located. In contrast, pollen from wind pollinated species needs to be collected before anther dehiscence which can be more difficult. In collecting pollen it is essential that the pollen be free of contamination and that the genetic purity of the pollen be assured.

Pollen may be used in hybrid production, when specific progeny are required, and known parental sources of pollen are used in the breeding program. In some species, pollen from plants of early varieties may need to be stored to facilitate pollination of late maturing varieties, or pollen from species with different flowering times may need to be stored to allow the production of interspecific hybrids.

Factors Controlling Pollen Availability

Genetic controls

The most important factors determining the time of pollen development and anther dehiscence are inherited. Genotypic variation and blocks to hybridisation are often established when pollen dispersal does not coincide with stigma receptivity of plants within pollinating distance (Stanley and Kirby 1973). Time of pollen shedding can be

related to geographical conditions and anther dehiscence depends in part, on meteorological conditions which can change day to day. In most plants the pollen quality as measured by pollen germination remains about the same throughout the period of dehiscence.

External controls

Temperature and moisture are the primary elements that affect pollen development in mature plants. If anther dehiscence is early in one location then it is possible to find similar genotypes at a higher elevation or colder location where the pollen has not yet been shed. Variations in temperature and moisture can also shorten the interval of pollen dispersion from a plant. Low or high temperatures during pollen development can adversely affect the quantity and quality of pollen, and moisture and nutrient status of the plant can affect pollen viability and abundance.

Collecting times can affect the quality of pollen, as pollen collected in the morning is often more viable than that collected in the afternoon. Radiation can have a negative influence on development, as exposure of pollen to ultra violet light can injure some pollens, so pollen is best dried in darkness or diffuse light. Chemicals applied to plants for insect control or defoliation can adversely affect pollen quality, and plants exposed to fungicides, herbicides, or pesticides applied during flower development will often yield damaged pollen. Awareness of the factors that influence pollen germination from field grown plants can help the plant breeder select plants and recognise sources of deviation in pollen behaviour.

Pollen Storage

The earliest reports of storage and transport of pollen ~~was~~ were about 1000 B.C. when date pollen was transported by hand to pollinate female flowers (Wodehouse 1935). The

maintenance of pollen germination capacity depends on the conditions of storage. Critical factors include: relative humidity, temperature and the atmosphere surrounding the pollen.

Relative humidity (R.H.)

The humidity of the air during storage affects longevity. Most species retain their viability best at low relative humidity. Various reports indicate that pollen longevity, in general, increases with reduction in RH to about 6% during storage (Stanley and Linskens 1974, Akihama and Omura 1986). Many species lose viability at very high or very low RH. However, low moisture levels must be avoided in the storage of certain pollen, such as *Tulipa* where the water content cannot be brought below a critical level of 40% (Stanley and Linskens 1974). Frequent fluctuation of RH during storage causes quick loss in viability, as pollen apparently cannot withstand variations which induce alternate phases of low and high metabolic activity. For many pollens pre drying is essential before storage.

Temperature

Another important factor that affects pollen viability during storage is temperature, and many pollens can be stored successfully at temperatures below zero (Sedgley and Griffin 1989). It is assumed that in pollen stored at extremely low temperatures, such as liquid nitrogen -196 ^{0}C, the metabolic activity is zero with potential storage for an unlimited time (Sedgley and Griffin 1989).

Gas atmosphere and oxygen pressure

Correct atmosphere, such as increased levels of carbon dioxide when pollen is stored over dry ice, can prolong viability, however, storage in pure oxygen can shorten viability

(Stanley and Linskens 1974). Reduction of the partial pressure of oxygen can prolong the viability of some pollen. When pre dried under reduced pressure pollen can retain high germination. In comparison some pollens die when placed under reduced pressure (Stanley and Linskens 1974).

Causes of decreased viability during storage

Little is known about the influence of change during storage on the genetic and physiological capacity of pollen. The primary reason for decreased pollen viability in storage is probably related to enzyme activity, which decreases respiration substrates, as the mechanism by which pollen retains its viability during storage is related to intracellular rates of respiration. This is confirmed by the requirement of stored pollen for higher concentrations of sugar for normal germination than fresh pollen, and as the respiration rate decreases with age, so the boron sensitivity increases (Stanley and Linskens 1974). Activity of enzymes and changes in endogenous hormones can also decrease viability. Reduction of germination capacity under certain storage conditions can therefore be attributed to inactivation of enzymes and metabolic substrates essential for germination. Other factors may affect pollen survival in storage, such as accumulation of toxic cell compounds, oil deposits in the exine, mineral nutrition of the plant during pollen development, bacterial or viral contamination, and chemical additives from bee bodies.

Viability tests

After pollen is collected or removed from storage, assessment of the capacity to germinate and grow normally is important. In an *in vivo* assay a long time can elapse between pollination and seed set, and such a test may not be valid, since incompatibility reactions may inhibit pollen growth in the style even though the pollen can germinate normally.

Viability tests which can determine growth potential have been developed. These include *in vitro* assays and non germination tests.

In vitro *assays*

Most pollen viability tests germinate a small sample of pollen and observe under the microscope the percent pollen grains producing tubes after a given time. Such tests assume that optimum conditions have been established, so that germination approximates that on the plant. However, most pollen tubes stop growth before they reach the length they normally obtain in the style, and the rate of pollen tube growth is often not as rapid as *in vivo*. Another assumption is that the sample of pollen tested is typical of the source. This assumption is permissible if care is taken to ensure that the pollen is mixed before drawing the sample. Plant to plant variation must be guarded against in drawing a sample from bulk pollen for viability testing.

Non germination assays

Tests which stain pollen grains with a coloured dye are often used as indices of viability. In such tests the chemicals are absorbed into specific cell constituents present in the mature pollen grain. Examples of such stains include acetocarmine, aniline blue, and potassium iodide. Most stains are not sufficiently accurate when compared to germination tests, and can give only crude estimates of viability. Viable pollen grains contain functioning enzymes and certain dyes on reduction change from a colourless compound to a coloured form. Active enzymes in the pollen grain are usually necessary to catalyse the colour change of the dye. Examples of these dyes are TTC (2,3,5-triphenyl tetrazolium chloride), or a fluorescent reaction with fluorescein diacetate (FDA).

Recording data

A false germination count can result under certain conditions. In certain germination conditions a minimum concentration of pollen grains must be placed in a given volume of solution if maximum germination is to be attained. Conversely, high pollen concentrations can inhibit germination. Care must be taken in the viewing and counting of the pollen grains. Pollen spread over agar or a slide can give slight distortion of viability counts, if the grains are not evenly dispersed. Growth inhibiting or stimulation chemicals must also be recognised as potential sources of error. Inhibition products such as organic acids can diffuse out of the pollen grains and accumulate on the medium. Water source, pH and nutrient factors can also affect the results. In addition, the rehydration of stored pollen before viability testing is important for maximum germination.

Comparison of viability tests

It should be noted that fair to good seed formation may occur even though the results of a *in vitro* pollen germination assay indicate low viability. The same problem holds true for results of non germination assays. If the test indicates 40% or better the pollen may be quite satisfactory for use in field pollination (Stanley and Linskens 1974). Inert materials are often used to dilute pollen to lower the percent viable grains in the sample. The capacity to form seed and seedlings is the ultimate test of pollen viability, and thus different pollen viability methods must be judged for their relative merits. The ability of pollen to grow or respond to *in vivo* or *in vitro* assay is dependent on the inherent chemistry of the pollen, which can provide an understanding of the metabolic factors facilitating growth and seed formation.

Molecular Techniques to Study Relationships in Plants

There are a number of molecular techniques available to the plant biologist and it is important to select the one most suitable for the problem. One of the most important factors is the degree of relationship among the organisms being studied. If the organisms are closely related, a technique which detects highly variable regions of the genome is most useful. When working with distantly related organisms more slowly evolving regions of the genome are needed. Different molecular markers provide varying amounts of information about the actual variation at the nucleotide level and can have varying levels of usefulness. Some techniques provide small numbers of highly informative characters, while others have large numbers of less informative characters. The breeding system of the plant is also important in the choice of the marker, since this affects the levels of variation expected. Types of markers used to study plant relationships include restriction fragment length polymorphisms (RFLP) (Byrne *et al.* 1994), random amplified polymorphic DNA (RAPD) (Huff *et al.* 1993, Chalmers *et al.* 1992, Russell *et al.* 1993), DNA sequencing (Phillips *et al.* 1994, Sang *et al.* 1995) and techniques to study repetitive DNA (Rieseberg *et al.* 1990). There are many other techniques available, but the following are amongst the most commonly used for studies of plant species relationships.

The polymerase chain reaction (PCR) technique

The PCR technique rests on the use of synthetic primers to prime the synthesis of a complementary strand of DNA using the thermostable polymerase enzyme obtained from a thermophilic bacterium (*Taq* I polymerase). Primers are used for the two complementary strands at the opposite ends of the region to be amplified. After one round of amplification, the reaction mix is denatured through heating and allowed to reanneal with excess primer, and the polymerase reaction is carried out a second time. Repeated

cycling of these conditions results in a geometric increase of the sequence determined by the initial primers.

Random amplified polymorphic DNA (RAPD)

RAPDs were recently introduced by Welsh and McClelland (1990) concurrently with Williams *et al.* (1990). They are a PCR based technique and as such require only a small amount of DNA and tissue. This is an important consideration in non destructive sampling of rare and endangered plants, or when experimental material is scarce. The technique differs from conventional PCR in that the primers are short arbitrary sequences, in contrast to primers that are complementary to a known region. The resultant bands correspond to regions in the genome where primers anneal to form a product able to be amplified with the amplification enzyme *Taq* polymerase. The distance between the primer sites determines the size of the product.

Although reaction conditions can alter the number of bands produced, it is usual to optimise the reaction conditions for a given species or genus. A primer is assessed as suitable if it will amplify a scorable number of bands. Some primers amplify too few bands, and some amplify too many, which may be uninformative. Due to the high levels of variability detected using RAPDs they are very useful for population studies (Huff *et al.* 1993, Chalmers *et al.* 1992, Russell *et al.* 1993, Dawson *et al.* 1993, Yu and Pauls 1993), in the identification of cultivars (Dunemann *et al.* 1994, Mailer, *et al.* 1994, Schnell, *et al.* 1995) and hybrids (Chong *et al.* 1994), in mapping genomes (Carlson *et al.* 1991, Cai *et al.* 1992, Lodhi *et al.* 1992, Grattapagalia *et al.* 1992), parentage determination (Welsh *et al.* 1991) and estimation of gene flow (Arnold *et al.* 1991). RAPDs are generally considered suitable below the species level. However, they have been used in taxonomic studies and have confirmed existing classifications based on more traditional methods, such as morphology, cytology and enzyme electrophoresis (Demeke *et al.* 1992, Howell *et al.* 1994).

RAPDs are dominant markers, and as such cannot distinguish between a homozygote and a heterozygote; however the large number of polymorphic markers produced offsets this disadvantage. A major advantage of RAPDs is that no prior sequence information is required, and the technique can be applied to any species where DNA of reasonable quality can be obtained. RAPD techniques are quick, relatively cheap and can process large sample sizes. In addition, RAPDs do not require radioactive labelling, a potential hazard in some other methods, and automated machines are available.

Each RAPD fragment produced is separated using gel electrophoresis, and two bands which co-migrate are generally assumed to be homologous. However, it is possible that pairs of priming sites will occur in the same orientation separated by approximately the same distance more than once in the genome. Hence bands that co-migrate may not be homologous (Bachmann 1994, Lynch and Milligan 1993, Stammers *et al.* 1995). To check this, southern blot analysis of RAPD bands have produced varied results. In some cases the co-migrating bands are all homologous (Wilikie *et al.* 1993), and in others some are homologous, and some are repetitive DNA (Williams *et al.* 1990). Ideally each data set needs to be tested to confirm that co-migrating bands are homologous, but this is usually not feasible given the scope of many projects.

Because PCR is such a sensitive technique, some RAPD bands may arise due to contamination. In general, this can be avoided by using optimised methods and careful technique. Many workers have shown that reproducible results can be obtained if specific conditions are used and care is taken to avoid any alteration of the conditions (Virk *et al.* 1995).

Debate continues as to which is the best method of analysis for RAPDs. Analysis for systematic or classification purposes can be done using similarity coefficients or methods such as parsimony using PAUP (Phylogenetic Analysis Using Parisomony) (Swofford 1990). Choices of coefficients and their implications for subsequent analysis should be

carefully considered (Jackson *et al.* 1989, Swofford and Olsen 1990, Wier 1991), as should earlier choices regarding sampling (Baverstock and Moritz 1991). Each RAPD data set should ideally be tested with different methods of analysis to determine their suitability, and compared to independent information.

DNA sequencing

Direct PCR sequencing enables rapid and precise determination of sequence identity and variation, which is useful in most aspects of molecular biology and genetics. PCR improves the ease and capacity of DNA sequencing activities by simplifying the screening, preparation and manipulation of the DNA templates. The technique is so powerful that it is one of the most utilised molecular techniques in phylogenetic studies (Hillis *et al.* 1990). The sequence chosen is of the utmost importance as it must contain the appropriate level of variation across the taxa to be studied. The target sequence must also be present in all the organisms studied and be capable of alignment. Sequencing can be used to produce phylogenies of species (Taberlet *et al.* 1991), genera (Phillips *et al.* 1994, Sang *et al.* 1995), orders (Frye and Hedges 1995), subfamilies (Hsiao *et al.* 1995), families (Olmstead and Sweere 1994) and to investigate relationships at higher taxonomic levels (McCourt 1995, Raff *et al.* 1994).

Molecular phylogenetic analysis has become easier because of PCR. A general strategy that can be used is to apply a general set of primers to amplify and then sequence the region directly using *Taq* polymerase (Ruanto and Kidd 1991). Usually the primers flank a hypervariable region and hybridise to conserved flanking regions such that comparisons can be made against a wide variety of genotypes. Introns are particularly good for this purpose, because they generally evolve faster than exons (Wolfe *et al.* 1989) and tend to be short in plants (Hanley and Schuler 1988, Hawkins 1988).

In order to use PCR amplification of the target sequence, the individuals need to be homozygous for that sequence (Hillis *et al.* 1990). If the sequences to be analysed are homogeneous a single sequence results, but if a mix of similar sequences is present, a direct sequence analysis will reveal ambiguous signals at the mixed positions. Cloning PCR products allows sequence variants to be separated before sequencing. Generating direct sequences from PCR products is technically more difficult than analysis of cloned single stranded DNA templates, as the PCRs generate complementary strands that can compete with a sequencing primer to bind the template, and contain salt and other reagents that can affect the sequencing chemistry. These contaminants can lead to artefacts and completely failed reaction 'stops' in every lane. Sequence reactions are complex mixtures and it is often difficult to trace the precise source of a sequencing problem.

PCR products can be sequenced by conventional radioactive methods, or by fluorescent automated DNA sequencing (Smith *et al.* 1988, Voss *et al.* 1989). The fluorescent methods are well suited for relatively high throughput studies, with direct computer entry of the final data. Manual methods are expensive and time consuming. The radioactive methods are generally more successful when templates of poor quality are used, while the fluorescent methods require more stringent variation of the reagents. This is particularly true of the methods that mix four different colours in a single lane, as the chemistry of each sequence reaction must be carefully balanced. The current fluorescent DNA sequencing strategies work most favourably with oligonucleotides with dyes attached at the 5' terminus. PCR provides a simple alternative and fragments can be amplified by primers that have a universal sequencing recognition site at the 5' terminus. This site is incorporated into the fragment via one PCR primer and then commercially available fluorescent primers are used (McBride *et al.* 1989). An advantage of the fluorescent assay is that the detection of the primary signal offers a linear response to the amount of material that is present over a wider range than radioactive methods. As a result fluorescent DNA sequencing via PCR is becoming the preferred method. Combinations

of PCR and DNA sequencing are now standard items in the molecular biology tool kit and the range of applications in molecular biology is likely to continue to increase.

Molecular Approaches to Plant Systematics

Traditionally plant systematists have sought to determine relationships based on a wide range of criteria, including morphological similarities at the gross, anatomical and ultrastructural levels, and similarities with respect to secondary metabolites, isozymes and other protein systems. Recently DNA analysis has been used as a basis for biosystematic study (Palmer 1987, Ritland and Clegg 1987). Genetic relationships between plants can be estimated from the pattern of DNA sequence change.

The investigator is faced with various choices regarding the level of genetic resolution appropriate for the materials under study. Common choices in plants include the chloroplast genome, (cp DNA) or components of the chloroplast genome, the nuclear ribosomal RNA genes (r RNA), or nuclear encoded, single copy genes. Another consideration is the method employed to provide direct or indirect measures of sequence divergence. Genetic divergence can be determined using restriction site changes. Restriction site analysis requires some knowledge of the physical map of the genome. Empirical studies have shown that restriction site analysis provides good resolution at or below the family level. A second method is DNA sequencing. Until recently DNA sequencing required molecular cloning of the gene or DNA fragment under study, which was subjected to a 'sequencing strategy' for the production of overlapping sequencing runs. Two related technological advances have overcome these necessities. It is now possible to synthesise oligonucleotides that can prime dideoxy sequencing in the gene of interest, based on previously determined sequence information. The second major advance is PCR. PCR allows direct amplification of the DNA fragments from heterogenous DNA samples, and thereby circumvents the cloning procedure. Application

of PCR to chloroplast genes, together with direct primer sequencing, provides an excellent method to obtain large data sets.

Another interesting area of PCR application is in the amplification of DNA fragments from fossil plant and animal tissues. Fossil sequences have been obtained from Miocene-aged leaf compression fossils (17-20 million years old). The *rbcL* gene has been amplified and compared to other related plants (Clegg and Durbin 1990), confirming its identity. The study of ancient DNA opens up a whole new area of palaeobotanical research. In the past it was necessary to infer relationships of plants; now the extant plants can be analysed directly, eliminating some of the problems that complicate the study of plant phylogeny.

Higher plant cells contain three genetic compartments which separately transcribe DNA and translate messenger (m) RNA into polypeptides. Most plant proteins are derived from the nuclear DNA, with fewer genes in the mitochondrial or plastid DNA. The cytoplasmic genomes are much smaller than the nucleus and are present in multiple copies in each organelle. As there are many chloroplasts and mitochondria in the cells these genomes can contribute a large proportion of the total cellular DNA. Chlorpolast DNA in particular has been extensively used to study plant phylogeny.

Chloroplast DNA

Chloroplast DNA (cp DNA) continues to be used as a phylogenetic tool for many plant species (Hoot *et al.* 1994, Mummenhoff and Koch 1994) and a number of reviews have been published detailing its suitability for such studies (Clegg and Durbin 1990). Chloroplast DNA occurs as a circular molecule with multiple copies per organelle, and is relatively easy to isolate (Clegg and Durbin 1990). Most angiosperms transmit chloroplasts through the maternal lineage and progeny usually receive the entire chloroplast genome from one parent (Sears 1980). Thus the use of chloroplast DNA

ensures that the DNA examined originates from a single parental lineage, rather than a mixture of recombined DNA as in the case of nuclear DNA. In addition, foreign DNA is not incorporated into the chloroplast genome, in contrast to nuclear and mitochondrial genomes (Palmer 1987).

It has been suggested that the chloroplast genome evolves at a slow rate and is ideal for analysis of plant evolution. The slow rate of evolution may also be disadvantageous for some purposes, but should be appropriate for the intergeneric and subgeneric levels (Palmer *et al.* 1988).

PCR analysis of chloroplast DNA

PCR amplification of variable regions of the chloroplast genome, such as introns or intergeneric spacers, displays high levels of variation, while conserved coding regions allow comparison over a wide range of taxa. Polymorphism of the PCR products is detected by direct sequencing of the product, or restriction endonuclease digestion of larger products. To amplify across a large number of taxa the region of interest must be free of structural rearrangement, although some insertions or deletions may alter the size of the product and this may be useful as a marker itself. Compared to traditional sequencing methods, PCR amplification allows the use of small amounts of tissue and heterogenous DNA samples and is also quicker and cheaper than other cloning methods. The primers used can be chosen for the level of variation required. Slowly evolving sequences can be used for divergent taxa, and rapidly evolving sequences can be used for intraspecific studies. Sequencing of intergeneric spacers of cp DNA can be used for phylogenetic study of closely related species or as intraspecific markers.

Taberlet *et al.* (1991) designed primers to amplify regions between the *trnT* and *trnF* genes in the large single copy region which included an ~~intergeneric~~ intergenic spacer region between the *trnT* and *trnL*, the *trnL* intron and another intergeneric spacer between the

trnL 3' exon and *trnF*. These were studied by sequencing the PCR products. The primers were tested across a wide range of taxa including green algae, bryophytes, pteridophytes, gymnosperms and angiosperms. The region is thought to be universal for plant taxonomy studies.

Analysis of DNA sequence data

Many techniques exist for the analysis of DNA sequence data. The simplest algorithm is the unweighted pair group method (UPGMA), which clusters based on similarity. A drawback of the analysis is that the statistical error associated with the topology of the tree is not evaluated, because it is not based on a probabilistic model. Pairs of sequences that have been separated for the same length of time may have different numbers of substitutions because the occurrence of a mutation is a random event. It is therefore desirable to estimate the statistical error associated with the process of nucleotide substitution. ~~Felenstein~~ Felsenstein (1981) developed a maximum likelihood algorithm for the estimation of phylogenetic topologies that is based in a probabilistic model of the substitution model and allows calculation of confidence intervals of the branch lengths. A major drawback of this method is that it is computationally difficult and a large amount of computer time is required for a moderate number of taxa and site differences. Resampling methods such as the bootstrap method are being used to quickly estimate the statistical error of phylogenetic topologies (~~Felenstein~~ Felsenstein 1985). Sequence analysis can also be performed using parsimony methods such as Phylogenetic Analysis Using Parsimony (PAUP) (Swofford 1990).

Conclusions

In summary, there is little doubt that the introduction of PCR has resulted in a large increase in genetic knowledge of plant species, and that this will continue to grow. The ultimate DNA marker is DNA sequencing, and when this becomes cheap to obtain and

analyse large amounts of sequence data will be produced and some of the older methods will be superseded. In the mean time it seems that it will be possible to make genetic maps and screen thousands of genetic loci quickly. This will directly affect new crops that have not received much research attention. It will also allow ecological and population geneticists to address novel questions because of the high marker output and the capacity to analyse large numbers of individuals through automation. The range of molecular techniques now available for studying relationships in plants allows a technique to be chosen which is appropriate for the genetic question under investigation.

Chapter Three

Viability testing of *Banksia menziesii* pollen after storage at different temperatures

Abstract

Germination after storage
~~Storage~~ of *Banksia menziesii* pollen was assessed at 20, 4, -20, -80 and -196 ^{0}C using a semi solid medium of 1% agar, 15% sucrose, 0.01% boric acid, 0.03% calcium nitrate, 0.02% magnesium sulphate, 0.01 % potassium nitrate, and an incubation temperature of 25 ^{0}C. Germination remained constant in all treatments except room temperature, 20 ^{0}C, which after six months had only 25% germination. Pollen viability was assessed using fluorescein diacetate (FDA), but the results did not reflect the loss of germinability at 20 ^{0}C. There was no effect of floret position on the inflorescence on germination, but pollen viability varied over the flowering peroid with maximum germination mid season.

Introduction

Pollen storage is an important component of plant breeding programs, when the species or individuals to be hybridised do not flower at the same time, or are geographically isolated. In the genus *Banksia,* there is no published information on pollen collection and storage, or on *in vitro* conditions for successful pollen germination and tube growth. Storage conditions vary for plant species, and in most woody species low humidity and low temperature storage is optimal (Stanley and Linskens 1974, Akihama and Omura 1986). Dehydration of pollen to 3-5% moisture increases pollen longevity at low temperature, but the pollen must be re-hydrated before it will germinate (Yates and Sparks 1989). Pollen viability testing methods include staining with materials such as fluorescein diacetate (FDA), or acetocarmine, and *in vitro* pollen germination (Sedgley

and Griffin 1989). Staining methods rely on the activity of enzymes in the pollen, which may persist after the ability of the pollen grain to effect seed set has declined. Similarly, *in vitro* pollen germination does not always reflect the true viability and lack of reproducibility of results has been demonstrated in some genera including *Acacia* (Sedgley *et al.*, 1992). The ability of pollen to effect seed set is the most accurate test of viability, but time constraints dictate that indirect methods are widely used.

There is considerable interest in cultivation of *Banksia* species for cut flower and potted plant industries. Recent work by Fuss and Sedgley (1991) on the development of hybridisation techniques for *Banksia* has stimulated the use of interspecific hybridisation in banksia breeding (Sedgley *et al.*, 1994), and flexibility of the breeding program would be greatly increased if pollen could be stored successfully. The objective of this study, was to develop pollen viability testing methods for *Banksia*, and to develop practical methods for long term storage.

Materials and methods

Plant material

Plants of *B. menziesii* were located in collections at Happy Valley and Nangkita, South Australia (latitude 35°10' S, longitude 138°34'E). Climate and soils data are presented for the two sites (Figs 3.1, 3.2; Table 3.1). All experimental plants were grown from seed, and ranged in age from 3 to 15 years. Experiments were conducted over the flowering season of *B. menziesii* during the period of 1993 to 1995.

Pollen collection

Banksia menziesii inflorescences comprise hundreds of florets, and in preliminary experiments pollen was collected from the upper, middle and lower thirds of four

inflorescences from the same plant. Pollen was also collected from the middle third of three inflorescences from three plants at monthly intervals during the flowering period.

In subsequent experiments, pollen was collected in the middle of the season from the middle third of up to 15 inflorescences, from three plants. Pollen from all inflorescences of each plant were mixed together to give a pooled sample.

Pollen storage

Florets with dehisced anthers were removed and placed in 1.5 mL eppendorf tubes in a desiccator over silica gel (pre-equilibrated for 1 month) for 24 hours at 4 ^{0}C. They were placed in containers with silica gel at 20, 4, -20, -80 or -196 ^{0}C (liquid nitrogen). The liquid nitrogen treatment had no silica gel since the vapour pressure is negligible. Pollen was stored for up to 6 months and tested following a period of hydration at 100% humidity for one hour. Killed pollen, which was kept at 60 ^{0}C for 5 days, was used as a control.

Fluorescein diacetate (FDA) test

Pollen was placed on a slide with two drops of FDA (1mg FDA in 1 mL acetone; added dropwise to 50 mL 10% sucrose, until mixture appears milky) (Heslop Harrison *et al.* 1984). The pollen was allowed to sit for 30 mins to allow penetration of the FDA into the pollen grain before observation with a fluorescence microscope. At least 250 grains were counted in different areas of the slide, and scored for the presence of fluorescing and non-fluorescing grains. Grains which were bright and consistent in fluorescence were scored as viable, and those which were non-fluorescing, or had weak and patchy fluorescence as inviable.

In vitro *germination*

The basal semi solid medium comprised 1% agar, 0.01% boric acid, 0.03% calcium nitrate, 0.02% magnesium sulphate, and 0.01% potassium nitrate in 7 cm diameter petri dishes. Three sugar sources, sucrose, lactose and fructose were tested at a concentration of 10%. The best sugar source was tested at 5, 10, 15 and 20%. Incubation temperatures tested for the best medium were 15, 20, 25, 30, and 35 ^{0}C. Pollen germination was scored using a dissecting microscope after incubation for 18 hours on the medium. At least 250 randomly selected pollen grains in different fields of view were scored for germination, the criteron for which was a tube longer than the grain diameter. Pollen tube length was measured for 10 grains per replicate.

Statistical analysis

All experiments were randomised complete block designs, using plants as blocks with three replicates per treatment. Results were analysed using analysis of variance (ANOVA) with the statistical package Genstat 5 (Payne 1987). Correlation coefficients were calculated by plotting a line of best fit.

Results

In vitro *germination*

For pollen collected from the middle third of the inflorescence in the middle of the flowering season, sucrose gave the highest germination, with glucose and lactose supporting poor germination (Table 3.2). There was no significant difference between 10, 15 and 20% sucrose in germination or tube length, but measurements were lower with 5% sucrose. A concentration of 15% sucrose was used in further experiments. Pollen germination was highest at 15-25 ^{0}C with no significant difference between these

temperatures. There was a drop in germination at 30 ^{0}C and almost none at 35 ^{0}C. Temperature also had a significant effect on pollen tube length which decreased with increasing temperature. An incubation temperature of 25 ^{0}C was used in further experiments.

Effect of position on the inflorescence and time during the flowering season

In vitro tests showed no significant effect of position on the inflorescence on germination percentage or on pollen tube length (Table 3.3). There was a significant effect of time of season on FDA staining and pollen germination, with pollen at the start of the season showing lower viability than mid and late season (Table 3.3). The correlation between FDA staining and *in vitro* germination was good with $r^2 = 0.8$.

Pollen storage

There was a significant effect of storage temperature, time and an interaction between temperature and time on pollen germination. All treatments retained germinability, with the exception of 20 ^{0}C (Table 3.4). The effect of storage temperature on pollen tube length was not significant, although time in storage was. Pollen viability as measured by FDA staining showed no significant effect of temperature, with all treatments showing similar results (Table 3.4). There was a significant effect of time only. The correlation coefficients for the germination and FDA tests were low, in the case of 20 ^{0}C and -80 ^{0}C and high for 4 ^{0}C, -20 ^{0}C and -196 ^{0}C (Table 3.5). Killed pollen showed zero germination and FDA staining indicated 3% viability.

Table 3.1 Soil profile of Happy Valley and Nangkita field sites. Soil classification according to Northcote (1979).

Site	Subsite	Soil class	Position	Depth (cm)	Texture	pH	Cond-uctivity (µs/cm)	Structure
Happy Valley	A	Uc4	Crest of dune	0-60	Loamy sand	6.0	2.8	Loose grain
				60-70	Loamy sand	6.0	4.7	Loose grain
				70-80	Loamy sand	6.0	3.8	Loose grain
	B	Dy5	Dune swale	0-25	Loamy sand	6.0	2.8	Loose grain
				25-55	Medium clay	5.5	15.2	Massive
Nangkita		Uc2	Plain (<5%) slope	0-15	Loamy sand	6.0	3.2	Loose grain
				15-55	Sand	6.5	2.9	Loose grain
				55-70	Loamy sand	6.0	3.8	Loose grain

Table 3.2 Optimisation of in *vitro* pollen germination medium for *B. menziesii*

Variable	Pollen germination (%)	Pollen tube length (μm)
Sugar source (10%)	Incubated at 25 ^{0}C	
Sucrose	61.4	97.5
Lactose	12.0	101.3
Glucose	20.3	100.0
Probability	<0.05	ns
Sucrose concentration (%)	Incubated at 25 ^{0}C	
5	18.1	67.5
10	50.6	96.3
15	68.9	100.0
20	60.1	121.3
Probability	<0.01	<0.001
Incubation temperature (^{0}C)	10% sucrose medium	
15	57.9	137.5
20	56.4	116.3
25	57.0	100.0
30	13.6	77.5
35	0.5	28.8
Probability	<0.001	<0.05

Table 3.3 Effect of position on the inflorescence and time during the flowering season on viability of pollen of *B. menziesii.*

Variable	Pollen germination (%)	Pollen tube length (µm)	Fluorescence (%)
Position on inflorescence			
Upper third	72.2	88.8	-
Middle third	77.6	91.3	-
Lower third	71.8	96.3	-
Probability	ns	ns	
Time during season			
June	63.4	115.0	52.2
July	75.8	96.3	82.9
August	70.2	90.0	83.1
Probability	<0.001	<0.05	<0.001

\- Not tested

Table 3.4 Effect of storage temperature and time on viability of pollen of *B. menziesii*.

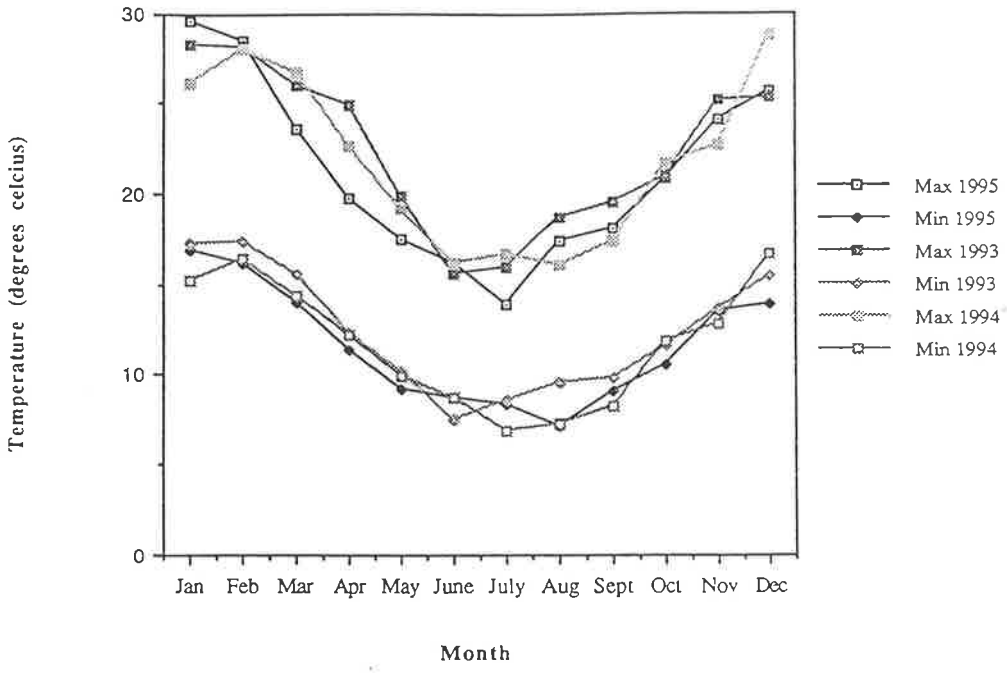
Storage conditions	Pollen germination (%)	Pollen tube length (μm)	Fluorescence (%)
20 ^{0}C			
0 months	84.6	100.0	85.4
3 months	53.1	87.5	62.0
6 months	26.3	63.8	59.7
4 ^{0}C			
0 months	84.6	100.0	85.2
3 months	72.1	85.0	70.0
6 months	65.2	63.8	66.2
-20 ^{0}C			
0 months	84.6	100.0	85.4
3 months	71.3	90.0	74.1
6 months	72.6	73.8	65.5
-80 ^{0}C			
0 months	84.6	100.0	85.4
3 months	66.6	92.5	53.8
6 months	68.5	77.5	53.6
-196 ^{0}C			
0 months	84.6	100.0	85.4
3 months	67.6	90.0	58.0
6 months	72.7	76.3	67.9
Probability			
Temperature	<0.001	ns	ns
Time	<0.001	<0.001	<0.001
Interaction	<0.05	ns	ns

Table 3.5 Correlation coefficient between FDA staining results and *in vitro* germination percentage for pollen storage over six months.

Storage Temperature (^{0}C)	Correlation coefficient (r^2)
20	0.536
4	0.902
-20	0.875
-80	0.462
-196	0.805

Figure 3.1 Temperature (A) and rainfall (B) data recorded at the nearest weather station to the Happy Valley experimental site for 1993, 1994 and 1995.

A Mean maximum and minimum temperature at Happy Valley in 1993, 1994 and 1995



B Total rainfall (mm) at Happy Valley in 1993, 1994 and 1995

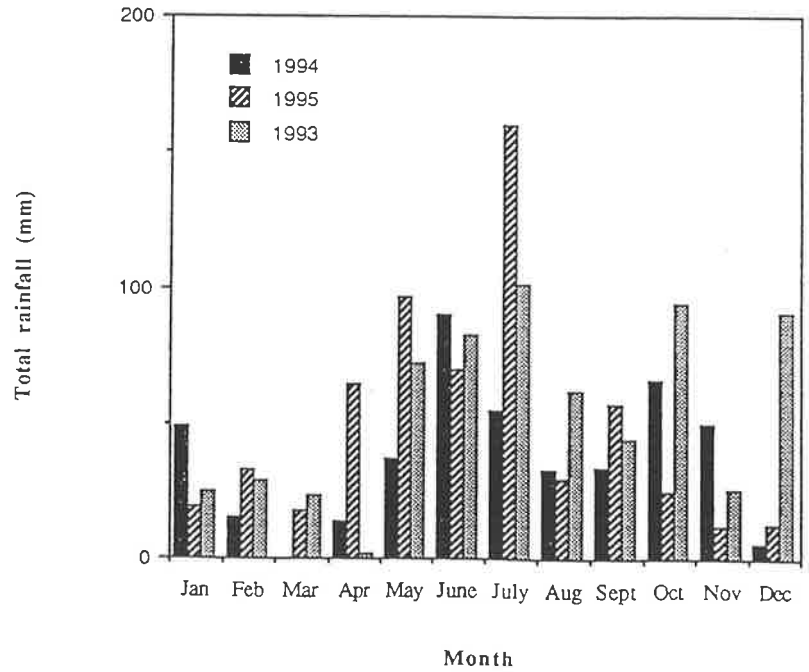
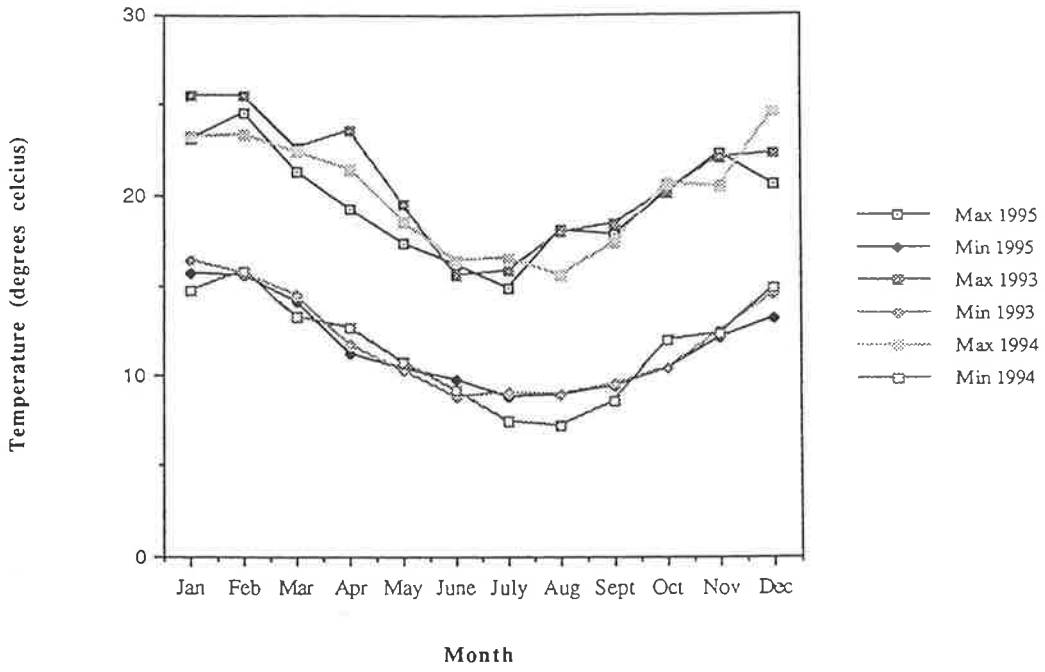


Figure 3.2 Temperature (A) and rainfall (B) data recorded at the nearest weather station to the Nangkita experimental site for 1993, 1994 and 1995.

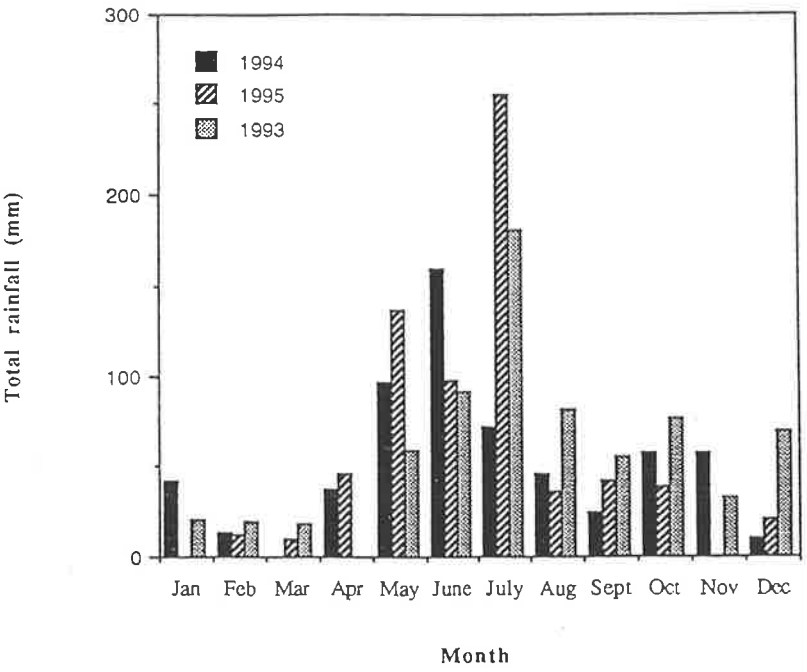
A

Mean maximum and minimum temperature at Nangkita in 1993, 1994 and 1995



B

Total rainfall (mm) at Nangkita in 1993, 1994 and 1995



Discussion

Banksia pollen stored for six months in a ~~fridge~~ refrigerator (4 ^{0}C), freezer (-20 ^{0}C), ultra freeze (-80 ^{0}C) or liquid nitrogen (-196 ^{0}C) retained a germinability of around 70%. For crossing purposes, a standard ~~fridge~~ refrigerator or freezer is the most practicable method to store pollen. Storage above zero degrees is not feasible, and extended pollen storage leads to a decrease in the length of the pollen tubes obtained *in vitro*. This may influence the ability of a pollen to grow a tube down the style, and effect fertilisation. Theoretically, pollen in liquid nitrogen retains its viability indefinitely provided precautions are taken to reduce the moisture content before storage (Stanley and Linskens 1974, Sedgley 1981). The level of liquid nitrogen must be maintained, otherwise freezing, thawing and refreezing may kill the pollen.

Media for *in vitro* germination of pollen have been developed for a number of genera, but there were no published reports of an optimal medium for *Banksia*. Germination was not significantly affected between temperatures of 15-25 degrees for *B. menziesii*, perhaps reflecting cool natural conditions in the field during the flowering season. The species *B. coccinea* and *B. integrifolia* were also tested, and germinated well on the *in vitro* medium.

The FDA test appeared to be less reliable than *in vitro* germination. In particular, the pollen stored at 20 ^{0}C fluoresced well, but had low *in vitro* germinability. Correlations between the media and FDA results were good in some cases, and poor in others, indicating differences in sensitivity between the two techniques. Poor correlation of FDA and *in vitro* germination has been shown in *Elocharis, Lonicera* (Shivanna and Heslop-Harrison 1981) and *Acacia* (Sedgley and Harbard 1993). FDA tests two properties of the pollen grain, the integrity of the plasmalemma of the vegetative cell and the presence of esterase capable of cleaving the fluorogenic ester fluorescein diacetate (Heslop Harrison *et al.*, 1984). Thus, FDA tends to overestimate the germinability of the pollen. It is

however, considered to be more accurate than other forms of viability testing such as lactophenol, fuschin, acetocarmine and benzidine, as tested on *Solanum* pollen (Heslop-Harrison and Heslop-Harrison 1970).

Floret position on the inflorescence had no significant effect on pollen viability, so pollen can be collected from any part of the inflorescence, as long as the pollen is fresh. The optimal time for pollen collection was mid to late season, perhaps reflecting a climatic influence of temperature as the season progresses from winter to spring.

The results of this study indicate that *Banksia* pollen can be stored relatively cheaply in an ordinary household ~~fridge~~ refrigerator or freezer for long enough to suit breeding requirements, after drying and storage over silica gel.

Chapter Four

Interspecific and intergeneric pollination with *Banksia coccinea* R.Br. (Proteaceae)

Abstract

Interspecific and intergeneric pollen tube growth were investigated using controlled hand pollination of the commercially significant species *Banksia coccinea,* to species of *Banksia*, and the related genus *Dryandra.* Currently, the relationship between *B. coccinea* and the other species groups within the *Banksia* genus is unclear. It has been found previously that success of pollen tube growth in the pistil following interspecific pollination was largely related to taxonomic distance between the species (Sedgley *et al.* 1994). Thus, interspecific hybridisation is a suitable technique to determine the compatibility relationships of the problematic species *B. coccinea* . Some species supported no germination of *B. coccinea* pollen. Some supported normal pollen tube growth and others produced pollen tube abnormalities including thickened walls, bulbous swellings, non-directional growth, burst tubes and branched tubes. Control of pollen tube growth in the pistil was imposed in the pollen presenter, a specialised region of the style for pollen presentation to foraging fauna, and in the upper style. There was no significant reciprocal effect on pollination success in the lower style. The results of pollen tube compatability in the lower style indicated that *B. coccinea* had a closer affinity to the section *Oncostylis*, than to the section *Banksia* where it is currently placed. It is suggested that a new section *Coccinea* be erected. Intergeneric crosses of *B. coccinea* with *Dryandra* species resulted in some compatibility, with one cross having low numbers of pollen tubes in the pollen presenter and upper style region. These results indicate a close relationship between *Banksia* and *Dryandra,* which are sister genera in the tribe *Banksiae*, family Proteaceae.

Introduction

~~The genus *Banksia* L.f., with over 70 species, is a well-known member of the family Proteaceae (George 1981, 1988).~~ Banksias are cultivated for the amenity, cut flower and potted plant industries. ~~They have attractive blooms and last well as cut flowers or indefinitely as dried arrangements.~~ Breeding is currently underway to select new cultivars for the cut flower industry, and interspecific hybridisation has recently been assessed as a potential breeding tool (Sedgley *et al.* 1991, 1994, 1996).

Banksias produce inflorescences composed of numerous hermaphrodite flowers clustered in pairs around a central woody core. Each floret has a single elongated pistil with two ovules in a unilocular ovary. Anthers are attached near the tip of each of the four perianth parts by short filaments. This region of the perianth, prior to anthesis, encloses the distal portion of the style, which in many species has an obliquely terminal stigmatic groove (Sedgley *et al.* 1993). This portion of the style is modified for the special function of pollen presentation. Anthers dehisce prior to anthesis, depositing pollen onto the pollen presenter, and at anthesis the style is released from the perianth and pollen is available to foraging fauna. The stigmatic groove becomes receptive after the pollen has been removed, when it is ready to receive pollen from another plant (Fuss and Sedgley 1991). Following fertilisation, follicles and seeds may develop, taking from a few months to two years to mature.

To obtain interspecific hybrids, the taxonomic relationships of the species must be known in order to increase the likelihood of a successful cross. It is assumed that closely related species will hybridise, while distantly related species will not. Taxonomic relationships within genus *Banksia* however, are still unresolved. In the current classification *Banksia* is divided into two subgenera, *Banksia* (72 spp.) and *Isostylis*. (3 spp.) (George 1981, 1988). Subgenus *Banksia* is divided into two sections, *Banksia* and *Oncostylis*, based on flower form. Section *Banksia* contains those species with straight styles (50), while

section *Oncostylis* contains species with hooked styles (22). Within the larger section *Banksia* there are ten series, and section *Oncostylis* has three series. *Banksia* and *Dryandra*, are sister taxa in the tribe ~~*Banksiae*~~ Banksieae, of the family Proteaceae.

Self incompatibility is common in the genus (Ramsey and Vaughton 1991), and inhibition of pollen tube growth in the pollen presenter has been shown in *B. coccinea* (Fuss and Sedgley 1991), *B. prionotes* and *B. menziesii* (Sedgley *et al.* 1994). Mechanisms of both intraspecific and interspecific incompatibility remain obscure for *Banksia,* although pollen tube inhibition in the style indicates a gametophytic system (Fuss and Sedgley 1991). Further evidence for gametophytic incompatibility arises from the binucleate pollen grains seen in *Banksia* and other genera in the Proteaceae (Brewbaker 1959). Binucleate pollen grains commonly characterise incompatibility systems of the gametophytic type in which the inhibition occurs during pollen tube growth in the pistil.

Interspecific hybridisation is of interest from economic, ecological and taxonomic viewpoints. Economically, the potential of hybrids may exceed that of the parental species for novel or improved varieties in ornamental horticulture. Hybrids occurring in the wild may have a competitive advantage over both parental species in a disturbed habitat (Potts and Reid 1985). Hybridisation generally occurs only between related taxa and can therefore have systematic and evolutionary implications for a group (Erickson *et al.* 1983). Some presumed natural interspecific hybrids have been reported in *Banksia* (Taylor and Hopper 1988) but little information is available on the limits to interspecific hybridisation. Crossing studies of *Banksia* have encouraged speculation on the relationships of species within the genus. Lewis and Bell (1981) crossed four *Banksia* species and found that species of the same style morphology crossed. Examples are *B. menziesii* and *B. attenuata* in section *Banksia*, which have straight styles and *B. littoralis* and *B. telmatiaea* in section *Oncostylis*, which have hooked styles. There appeared to be a barrier between the two style morphology groups, with species having straight styles

unable to cross with hooked style species. However, pollen germination was observed only in the stigma, and this is only an initial step towards obtaining a successful hybrid. In a study by Sedgley *et al.* (1994), crosses were conducted involving two Western Australian species, *B. prionotes* and *B. menziesii*. It was found that success of pollen tube growth was largely related to taxonomic distance between the species, and the results showed a close affinity between the series *Banksia* and *Crocinae*. Pollen tube growth appeared to be controlled in the pollen presenter and upper style regions.

Banksia coccinea R. Br. (scarlet banksia) is currently the only species in *Banksia,* subgenus *Banksia,* section *Banksia*, series *Coccineae*. It is placed in subgenus *Banksia* as its styles are straight at anthesis. However, prior to anthesis the styles are hooked as in section *Oncostylis*, series *Spicigerae*. Placement of *B. coccinea* within the genus is unclear as it has no obvious close relatives having unique seedling leaves, adult leaves, inflorescences, pollen, infructescences, follicles and seeds. A recent cladistic analysis of *Banksia* based on morphological characters was undertaken by Thiele (1993); he noted however, that his analysis should be treated with caution, as support for some nodes was tenuous. In this analysis *B. coccinea* and other taxa were problematic and were placed *incerte sedis*. Additional independent data are needed to resolve these relationships.

This study investigated interspecific and intergeneric compatibility in *Banksia*, by studying pollen tube growth using fluorescence microscopy. In particular, the study focused on the relationship of the commercially significant species *Banksia coccinea,* with other species in the genus *Banksia*, for the production of potential commercial hybrids.

Materials and methods

Plant material

Species of *Banksia* and *Dryandra* were located in collections at Happy Valley and Nangkita, South Australia. (latitude 35°10' S, longitude 138°34'E). Climate and soils data are presented for the two sites in chapter three. All experimental plants were grown from seed, and ranged in age from 3 to 15 years. Selected species represented two genera, two subgenera, two sections, and twelve series (Table 4.4). Species were chosen on the basis of taxonomic position, flowering time, and commercial potential. Classification follows that of George (1981, 1988).

Pollinations for assessment of pollen tube growth

Inflorescences were prepared for experimentation (Plate 4.1) by removing the open florets and isolating the inflorescences with glassine bags secured with a twist tie (Fuss and Sedgley 1991b). One day later, self pollen was removed from open florets by passing a looped synthetic pipe-cleaner over the pollen presenter region of the style, after which the inflorescence was rebagged. Approximately 100 florets were pollinated 3 days after anthesis at peak stigma receptivity (Fuss and Sedgley 1991a) with fresh pollen, on at least two inflorescences per species. *B. coccinea* was the male parent in all interspecific and intergeneric crosses as the unique pollen grain morphology allowed easy identification of cross pollen from chance self pollination (Sedgley *et al.* 1993). Reciprocal crosses were selected to study possible effects of unilateral incompatibility on pollination success. Pistils were fixed in Carnoy's solution at 6 days after pollination. They were transferred to 90% ethanol for storage, before processing for squash preparations for fluorescence microscopy (Fuss and Sedgley 1991a) (Plate 4.2). Counts were made of the number of pollen grains in the stigmatic groove, pollen tubes in the pollen presenter, and in the upper and lower regions of the style. Pollen tube data were

analysis of deviance based on generalised linear models
analysed by ~~ANOVA~~ using the statistical package, Genstat 5 (Payne 1987). Experimental interspecific pollinations were conducted throughout the 1993 and 1994 seasons, and intergeneric pollinations were conducted in 1995.

Results

Effect on pollen tube growth of site, month and year

A subgroup of the interspecific crosses, tested at both Happy Valley and Nangkita, showed a significant site effect (Table 4.1). Pollinations conducted at Happy Valley were more successful than those at Nangkita for all species except *B. hookeriana.* There was a significant effect of time of pollination during the season on intraspecific *B. coccinea* and *B. menziesii* crosses, but all showed successful lower style penetration (Table 4.2). There were differences in the extent of pollen tube penetration during the 1993 and 1994 flowering season for the species *B. ericifolia, B. menziesii* and *B. blechnifolia,* but not for *B. hookeriana, B. coccinea* or *B. brownii* (Table 4.4). Analysis of the interspecific cross data was adjusted for the site effect using generalised linear models.

Reciprocal crosses

Reciprocal crosses were conducted between *B. coccinea* and the species *B. gardneri, B. praemorsa, B. speciosa, B. ericifolia* and *B. menziesii* (Table 4.3). There were no significant reciprocal effects with *B. gardneri* and *B. praemorsa.* Significant reciprocal effects with *B. speciosa, B. ericifolia* and *B. menziesii* were observed in the stigmatic groove and pollen presenter, but not the upper and lower style.

Interspecific and intergeneric pollination with B. coccinea *as male parent*

Numbers of pollen grains per stigmatic groove, and pollen tubes per pistil were low (Table 4.4), but pollen tubes of *B. coccinea* were observed in the lower style of the species *B. coccinea, B. ericifolia, B. micrantha* and *B. sphaerocarpa.* A Poisson model was fitted to the number of pollen grains per cross, and analysis of deviance showed a significant effect of female parent ($P < 0.001$). Pollen tubes of *B. coccinea* were observed in low numbers in the pollen presenter and upper style regions of the species *Dryandra squarossa* (Table 4.4). To account for the low pollen grain and pollen tube numbers observed, even in intraspecific crosses, predicted ~~probabilities~~ proportions of pollen tube growth were calculated based on binomial models, relating the number of pollen tubes in the pollen presenter to the number of pollen grains on the stigma, the number of pollen tubes in the upper style to the number in the pollen presenter, and the number in the lower style to the number in the upper style. Analysis of deviance showed a significant interaction between the female parents and *B. coccinea* pollen in the pollen presenter ($P < 0.001$), upper style ($P < 0.05$) and the lower style ($P < 0.001$). The ~~probabilities~~ proportions based on the binomial models predicted that, in addition to species in which lower style penetration was observed, success could be expected with the species *B. menziesii, B. prionotes, B. ashbyi, B. attenuata, B. leavigata, B. blechnifolia, B. petiolaris, B. lemanniana, B. tricuspis, B. nutans, B. telmatiaea* and *B. cuneata* (Table 4.5). Results from observed and predicted pollen grain and tube numbers were averaged across the taxonomic series (Tables 4.6, 4.7). Pollen tubes of *B. coccinea* (series *Coccineae)* were observed in the upper style of interseries crosses to *Cyrtostylis, Prostratae, Tetragonae, Spicigerae, Abietinae,* and of the intersubgeneric cross to *Isostylis*. Pollen tubes were present in the lower style of interseries crosses to *Spicigerae* and *Abietinae*. Predicted ~~probabilities~~ proportions indicated that additional lower style success could be expected with the series *Crocinae, Cyrtostylis* and *Tetragonae* and with the subgenus *Isostylis*.

Pollen tube abnormalities

Some interspecific crosses produced apparently normal pollen tubes, but others showed abnormalities. Pollen grain and pollen tube abnormalities were observed on the stigmatic surface, but most pollen tube abnormalities were observed in the pollen presenter and upper style region. There were no pollen tube abnormalities observed in the lower style (Table 4.8). The type of abnormalities included directionless and branched pollen tubes, those with thickened walls, bulbous swellings, and burst tips (Plate 4.3).

Table 4.1 Site effect on mean pollen grain and tube numbers in interspecific crosses of *Banksia* with *B. coccinea* as the male parent.

Species	Site	Pollen grains	Pollen presenter	Pollen tubes in Upper style	Pollen tubes in Lower style
Series *Bauerinae*					
B. baueri	Happy Valley	2.60	0.00	0.00	0.00
	Nangkita	1.03	0.00	0.00	0.00
Series *Banksia*					
B. menziesii	Happy Valley	0.36	0.00	0.00	0.00
	Nangkita	0.29	0.02	0.02	0.00
Series *Crocinae*					
B. hookeriana	Happy Valley	0.09	0.00	0.00	0.00
	Nangkita	0.55	0.00	0.00	0.00
Series *Cyrtostylis*					
B. praemorsa	Happy Valley	0.43	0.02	0.00	0.00
	Nangkita	0.12	0.00	0.00	0.00
Series *Abietinae*					
B. telmatiaea	Happy Valley	2.30	0.48	0.11	0.00
	Nangkita	2.31	0.15	0.05	0.00
Mean	Happy Valley	1.32	0.09	0.00	0.00
	Nangkita	0.72	0.03	0.00	0.00
Probability		<0.05	<0.05	ns	ns

Table 4.2 Effect of time of pollination during the flowering season on mean pollen grain and tube numbers in intraspecific crosses of *Banksia coccinea* and *B. menziesii*

Time of pollination	Pollen grains	Pollen presenter	Pollen tubes in Upper style	Pollen tubes in Lower style
B. coccinea x B. coccinea				
10 August	1.75	0.97	0.49	0.14
27 September	3.79	0.99	0.48	0.25
Probability	ns	<0.05	ns	<0.05
B. menziesii x B. menziesii				
16 July	0.93	0.74	0.55	0.11
10 August	0.55	0.16	0.10	0.02
Probability	ns	<0.05	ns	ns

Table 4.3 Mean pollen grain and tube numbers in interspecific crosses of *Banksia* with *B. coccinea* as the male parent.

Cross	Pollen grains	Pollen presenter	Pollen tubes in Upper style	Pollen tubes in Lower style
B. gardneri x B. coccinea	7.90	0.00	0.00	0.00
B. coccinea x B. gardneri	0.02	0.00	0.00	0.00
Probability	ns	ns	ns	ns
B. praemorsa x B. coccinea	0.28	0.01	0.00	0.00
B. coccinea x B. praemorsa	0.10	0.00	0.00	0.00
Probability	ns	ns	ns	ns
B. speciosa x B. coccinea	0.03	0.00	0.00	0.00
B. coccinea x B. speciosa	2.21	0.00	0.00	0.00
Probability	<0.01	ns	ns	ns
B. ericifolia x B. coccinea	1.85	0.33	0.19	0.04
B. coccinea x B. ericifolia	3.39	0.19	0.02	0.00
Probability	<0.01	<0.01	ns	ns
B. menziesii x B. coccinea	0.45	0.00	0.00	0.00
B. coccinea x B. menziesii	2.98	1.54	0.17	0.00
Probability	<0.01	<0.01	ns	ns

Table 4.4 Mean pollen grain and tube numbers in intraspecific, interspecific, and intergeneric crosses pollinated with *B. coccinea* pollen. Taxonomy follows George (1981, 1988).

Taxon	Pollen grains	Pollen tubes in: Pollen presenter	Upper style	Lower style	Year
Genus *Banksia*					
Subgenus *Banksia*					
Section *Banksia*					
Series *Salicinae*					
B. integrifolia L.f.	0.04	0.01	0.00	0.00	1994
B. marginata Cav.	0.45	0.03	0.00	0.00	1993
B. paludosa R. Br.	0.69	0.00	0.00	0.00	1993
Series *Grandes*					
B. grandis Willd.	0.72	0.00	0.00	0.00	1994
Series *Quercinae*					
B. quercifolia R. Br.	0.08	0.00	0.00	0.00	1993
Series *Bauerinae*					
B. baueri R. Br.	1.80	0.00	0.00	0.00	1993
Series *Banksia*					
B. candolleana Meissn.	1.22	0.00	0.00	0.00	1993
B. menziesii R.Br.	0.33	0.01	0.01	0.00	1993
	0.45	0.00	0.00	0.00	1994
B. ornata F. Muell. ex Meissn.	1.07	0.02	0.00	0.00	1993
B. speciosa R. Br.	0.03	0.00	0.00	0.00	1994
Series *Crocinae*					
B. burdettii E.G. Baker	0.19	0.00	0.00	0.00	1993
B. hookeriana Meisnn.	0.37	0.00	0.00	0.00	1993
	0.33	0.00	0.00	0.00	1994
B. prionotes Lindley	0.61	0.11	0.01	0.00	1993
Series *Cyrtostylis*					
B. ashbyi E.G. Baker	1.62	0.27	0.04	0.00	1993
B. attenuata R. Br.	1.18	0.07	0.02	0.00	1994
B. laevigata Meisnn.	0.56	0.21	0.13	0.00	1994
B. media R. Br.	0.34	0.00	0.00	0.00	1993
B. praemorsa Andrews	0.28	0.01	0.00	0.00	1993
Series *Prostratae*					
B. blechnifolia F. Muell.	0.89	0.01	0.00	0.00	1993
	0.92	0.14	0.07	0.00	1994
B. gardneri A.S. George	0.10	0.00	0.00	0.00	1993
B. petiolaris F. Muell.	1.55	0.71	0.05	0.00	1994
B. repens Labill.	0.04	0.00	0.00	0.00	1993
Series *Tetragonae*					
B. lemanniana Meisnn.	7.35	0.81	0.22	0.00	1994
Series *Coccineae*					
B. coccinea R. Br.	3.20	0.98	0.48	0.22	1993
	6.18	3.81	1.10	0.34	1994
Section *Oncostylis*					
Series *Spicigerae*					
B. brownii Baxter ex R. Br.	0.24	0.00	0.00	0.00	1993
	0.17	0.00	0.00	0.00	1994
B. ericifolia L. f.	1.01	0.14	0.11	0.00	1993
	1.85	0.33	0.19	0.04	1994
B. occidentalis R. Br.	0.33	0.01	0.00	0.00	1993
B. tricuspis Meisnn.	1.47	0.21	0.13	0.00	1993
Series *Abietinae*					
B. laricina C. Gardner	1.01	0.07	0.00	0.00	1993
B. micrantha A. S. George	2.34	0.57	0.26	0.13	1993
B. nutans R. Br.	5.17	0.13	0.02	0.00	1994
B. pulchella R. Br.	0.31	0.00	0.00	0.00	1993
B. sphaerocarpa R. Br.	3.56	0.34	0.09	0.06	1994
B. telmatiaea A. S. George	2.30	0.32	0.08	0.00	1993
Subgenus *Isostylis*					
B. cuneata A. S. George	3.77	0.12	0.04	0.00	1993
Probability	<0.001	<0.001	<0.05	<0.001	
Genus *Dryandra*					
D. praemorsa	0.81	0.00	0.00	0.00	1995
D. sessilis	0.27	0.00	0.00	0.00	1995
D. squarossa	0.40	0.08	0.02	0.00	1995
Probability	<0.05	ns	ns	ns	

Table 4.5 Predicted ~~probability~~ proportions of pollen tube growth in intraspecific, interspecific and intergeneric crosses pollinated with *B.coccinea* pollen. Taxonomy follows George (1981, 1988).

Taxon	Pollen grains	Pollen presenter	Pollen tubes in Upper style	Pollen tubes in Lower style	Year
Genus *Banksia*					
Subgenus *Banksia*					
Section *Banksia*					
Series *Salicinae*					
B. integrifolia L.f.	0.0400	0.2500	0.0038	0.0000	1994
B. marginata Cav.	0.4500	0.0667	0.0004	0.0000	1993
B. paludosa R. Br.	0.6970	0.0001	0.0000	0.0000	1993
Series *Grandes*					
B. grandis Willd.	0.7200	0.0001	0.0000	0.0000	1994
Series *Quercinae*					
B. quercifolia R. Br.	0.0864	0.0002	0.0000	0.0000	1993
Series *Bauerinae*					
B. baueri R. Br.	1.6865	0.0001	0.0000	0.0000	1993
Series *Banksia*					
B. candolleana Meissn.	1.2233	0.0001	0.0000	0.0000	1993
B. menziesii R.Br.	0.3020	0.0264	0.9981	0.0001	1993
	0.4624	0.0002	0.0000	0.0000	1994
B. ornata F. Muell. ex Meissn.	1.0515	0.0196	0.0005	0.0000	1993
B. speciosa R. Br.	0.0304	0.0002	0.0000	0.0000	1994
Series *Crocinae*					
B. burdettii E.G. Baker	0.1915	0.0002	0.0000	0.0000	1993
B. hookeriana Meisnn.	0.4396	0.0002	0.0000	0.0000	1993
	0.3267	0.0001	0.0000	0.0000	1994
B. prionotes Lindley	0.7294	0.2097	0.0769	0.0002	1993
Series *Cyrtostylis*					
B. ashbyi E.G. Baker	1.6563	0.1698	0.1482	0.0002	1993
B. attenuata R. Br.	1.1800	0.0593	0.2857	0.0001	1994
B. laevigata Meisnn.	0.5700	0.3684	0.6667	0.0001	1994
B. media R. Br.	0.3409	0.0002	0.0000	0.0000	1993
B. praemorsa Andrews	0.2546	0.0266	0.0004	0.0000	1993
Series *Prostratae*					
B. blechnifolia F. Muell.	0.9038	0.0106	0.0005	0.0000	1993
	0.9216	0.1489	0.5000	0.0001	1994
B. gardneri A.S. George	0.0962	0.0002	0.0000	0.0000	1993
B. petiolaris F. Muell.	1.5657	0.4581	0.0704	0.0001	1994
B. repens Labill.	0.0374	0.0002	0.0000	0.0000	1993
Series *Tetragonae*					
B. lemanniana Meisnn.	7.5977	0.1029	0.2794	0.0001	1994
Series *Coccineae*					
B. coccinea R. Br.	2.9562	0.3272	0.4906	0.4231	1993
	6.1800	0.6165	0.3176	0.2810	1994
Section *Oncostylis*					
Series *Spicigerae*					
B. brownii Baxter ex R. Br.	0.2427	0.0002	0.0000	0.0000	1993
					1994
B. ericifolia L. f.	0.9811	0.1442	0.8000	0.0002	1993
	1.8824	0.1771	0.5882	0.2000	1994
B. occidentalis R. Br.	0.3301	0.0294	0.0005	0.0000	1993
B. tricuspis Meisnn.	1.5775	0.1346	0.6191	0.0002	1993
Series *Abietinae*					
B. laricina C. Gardner	1.0316	0.0714	0.0005	0.0000	1993
B. micrantha A. S. George	2.3301	0.2417	0.4655	0.4815	1993
B. nutans R. Br.	5.1765	0.0246	0.1538	0.0001	1994
B. pulchella R. Br.	0.2772	0.0001	0.0000	0.0000	1993
B. sphaerocarpa R. Br.	3.5600	0.0955	0.2467	0.6667	1994
B. telmatiaea A. S. George	1.9634	0.1265	0.2154	0.0002	1993
Subgenus *Isostylis*					
B. cuneata A. S. George	3.7250	0.0468	0.2632	0.0002	1993
Probability	<0.001	<0.001	<0.05	<0.001	
Genus *Dryandra*					
D. praemorsa	0.8100	0.00001	0.0000	0.0000	1995
D. sessilis	0.2700	0.00001	0.0000	0.0000	1995
D. squarossa	0.4000	0.19512	0.0000	0.0000	1995
Probability	<0.05	ns	ns	ns	

Table 4.6 Mean pollen grain and tube numbers in intraspecific, interspecific and intergeneric crosses pollinated with *B. coccinea* pollen averaged over taxonomic groups. Taxonomy follows George (1981, 1988).

Taxonomic group	Pollen grains	Pollen presenter	Pollen tubes in Upper style	Pollen tubes in Lower style
Genus *Banksia*				
Subgenus *Banksia*				
Section *Banksia*				
Series *Salicinae*	0.39	0.01	0.00	0.00
Series *Grandes*	0.72	0.00	0.00	0.00
Series *Quercinae*	0.08	0.00	0.00	0.00
Series *Bauerinae*	1.80	0.00	0.00	0.00
Series *Banksia*	0.62	0.01	0.00	0.00
Series *Crocinae*	0.38	0.03	0.00	0.00
Series *Cyrtostylis*	0.80	0.11	0.04	0.00
Series *Prostratae*	0.70	0.17	0.02	0.00
Series *Tetragonae*	7.35	0.81	0.22	0.00
Series *Coccineae*	4.69	2.40	0.79	0.28
Section *Oncostylis*				
Series *Spicigerae*	0.85	0.12	0.07	0.01
Series *Abietinae*	2.45	0.24	0.08	0.03
Subgenus *Isostylis*	3.77	0.12	0.04	0.00
Genus *Dryandra*				
D. praemorsa	0.81	0.00	0.00	0.00
D. sessilis	0.27	0.00	0.00	0.00
D. squarossa	0.40	0.08	0.02	0.00

Table 4.7 Predicted ~~probability~~ proportions of pollen tube growth in intraspecific, interspecific and intergeneric crosses pollinated with *B. coccinea* pollen averaged over taxonomic groups. Taxonomy follows George (1981, 1988).

Taxonomic group	Pollen grains	Pollen presenter	Pollen tubes in Upper style	Pollen tubes in Lower style
Genus *Banksia*				
Subgenus *Banksia*				
Section *Banksia*				
Series *Salicinae*	0.3957	0.1056	0.0014	0.0000
Series *Grandes*	0.7200	0.0001	0.0000	0.0000
Series *Quercinae*	0.0864	0.0002	0.0000	0.0000
Series *Bauerinae*	1.6865	0.0001	0.0000	0.0000
Series *Banksia*	0.6139	0.0093	0.1997	0.0000
Series *Crocinae*	0.4218	0.0526	0.0192	0.0001
Series *Cyrtostylis*	0.8004	0.1249	0.2202	0.0001
Series *Prostratae*	0.7049	0.1236	0.1142	0.0000
Series *Tetragonae*	7.5977	0.1029	0.2794	0.0001
Series *Coccineae*	4.5681	0.4719	0.4041	0.3521
Section *Oncostylis*				
Series *Spicigerae*	1.0028	0.0971	0.4016	0.0401
Series *Abietinae*	2.3898	0.0933	0.1803	0.1914
Subgenus *Isostylis*	3.7250	0.0468	0.2632	0.0002
Genus *Dryandra*				
D. praemorsa	0.8100	0.00001	0.0000	0.0000
D. sessilis	0.2700	0.00001	0.0000	0.0000
D. squarossa	0.4000	0.19512	0.0000	0.0000

Table 4.8 Pollen tube abnormalities observed in interspecific crosses with *B. coccinea* pollen.

Pollen tube abnormaility	Percentage of species with abnormality in:			
	Stigma	Pollen presenter	Upper style	Lower style
Thickened walls	5.7	25.7	0.0	0.0
Bulbous swellings	11.4	11.4	20.0	0.0
Directionless growth	11.4	20.0	2.9	0.0
Burst tip	5.7	0.0	2.9	0.0
Branched pollen tube	0.0	14.3	2.9	0.0

Plate 4.1 Interspecific hybridisation technique for *Banksia* crosses.

Figure 1 Removal of all opened florets on a *B. menziesii* inflorescence prior to bagging.

Figure 2 Wire cage placed on inflorescence.

Figure 3 Isolation of inflorescence with a glassine bag secured with a twist tie.

Figure 4 After one day all unopened florets are removed.

Figure 5 After removing all unopened florets, a ring of florets one day old are left for pollen removal.

Figure 6 Pollen removal using a synthetic pipe cleaner, the inflorescence is then re-bagged.

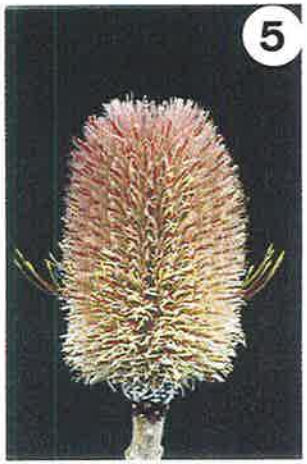
Figure 7 Pollination of florets after 3 days, at maximum stigma receptivity, with fresh *Banksia coccinea* pollen, the inflorescence is then re-bagged. After 6 days florets are harvested for fluorescence microscopy.

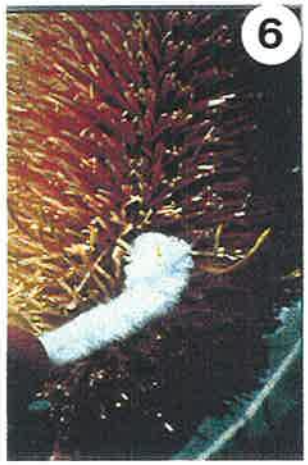

1


2


3


4


5


6

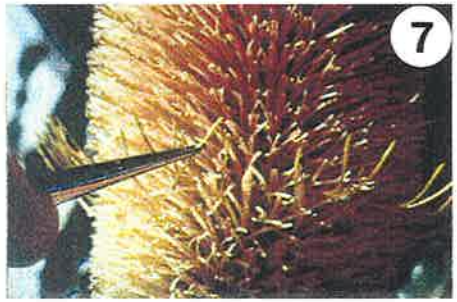

7

Plate 4.2 Scanning and fluorescence micrographs of *Banksia*.

Figure 1 Scanning micrograph of a *B. prionotes* pistil showing the stigmatic groove at the tip of the pistil (arrow), the ridged pollen presenter region (arrow), and the upper style below the constriction of the the pollen presenter (arrow). Scale bar represents 500 µM.

Figure 2 Scanning micrograph of a *B. sceptrum* stigmatic groove showing papilla cells (arrow) inside the groove. Scale bar represents 50 µM.

Figure 3 Fluorescence micrograph of a disected *B. prionotes* pistil stained with aniline blue. Arrows show the stigmatic groove at the tip of the pistil, and the transmitting tissue in the centre of the pistil. Scale bar represents 500 µM.

Figure 4 Fluorescence micrograph of a disected *B. pulchella* ovary stained with aniline blue, showing two ovules. Scale bar represents 150 µM.

Figures 5, 6 Fluorescence micrograph of normal pollen tube growth of *B. coccinea* through the pollen presenter region (Fig 5), and the upper style of *B. coccinea* (Fig 6). Scale bar represents 150 µM.

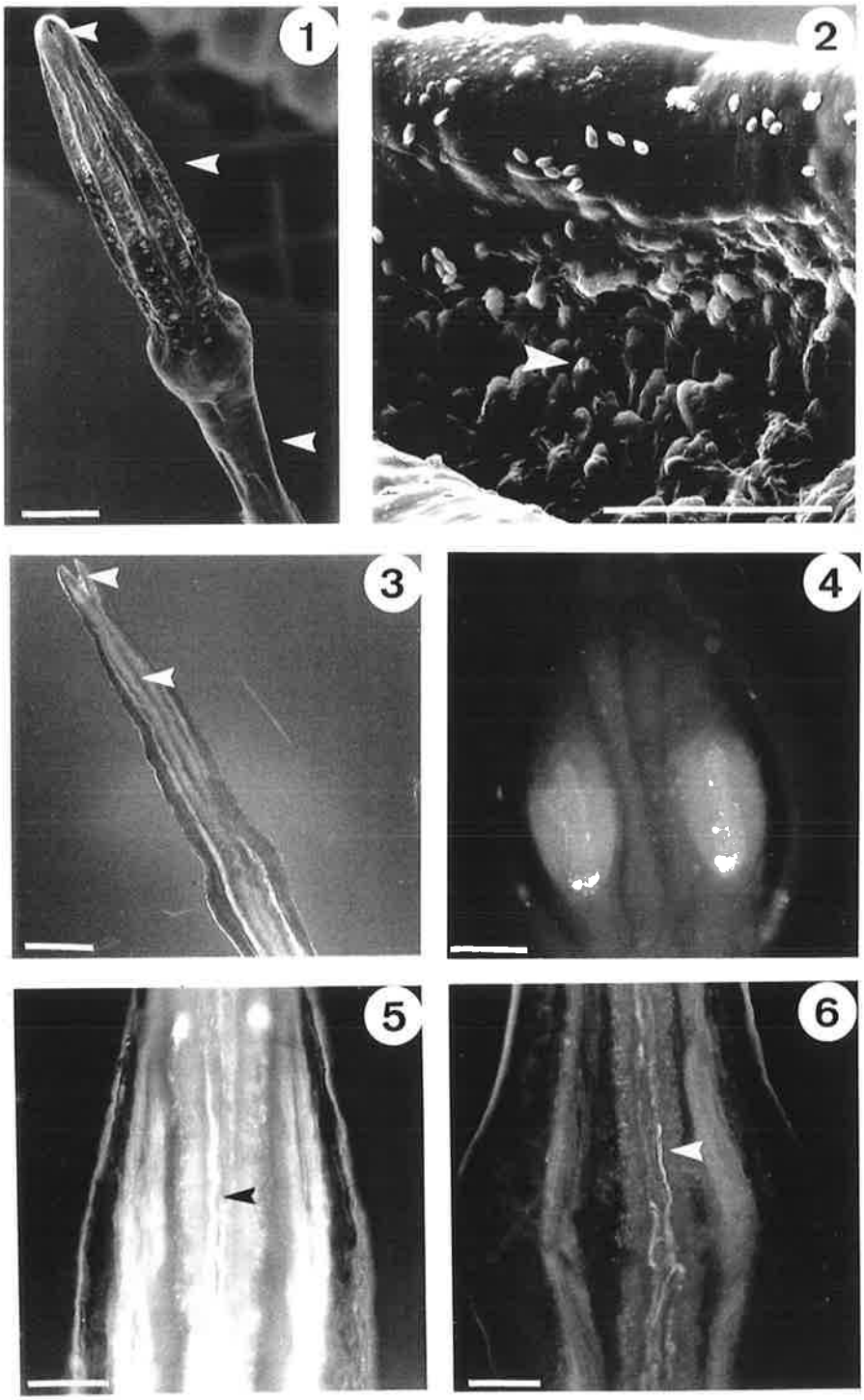

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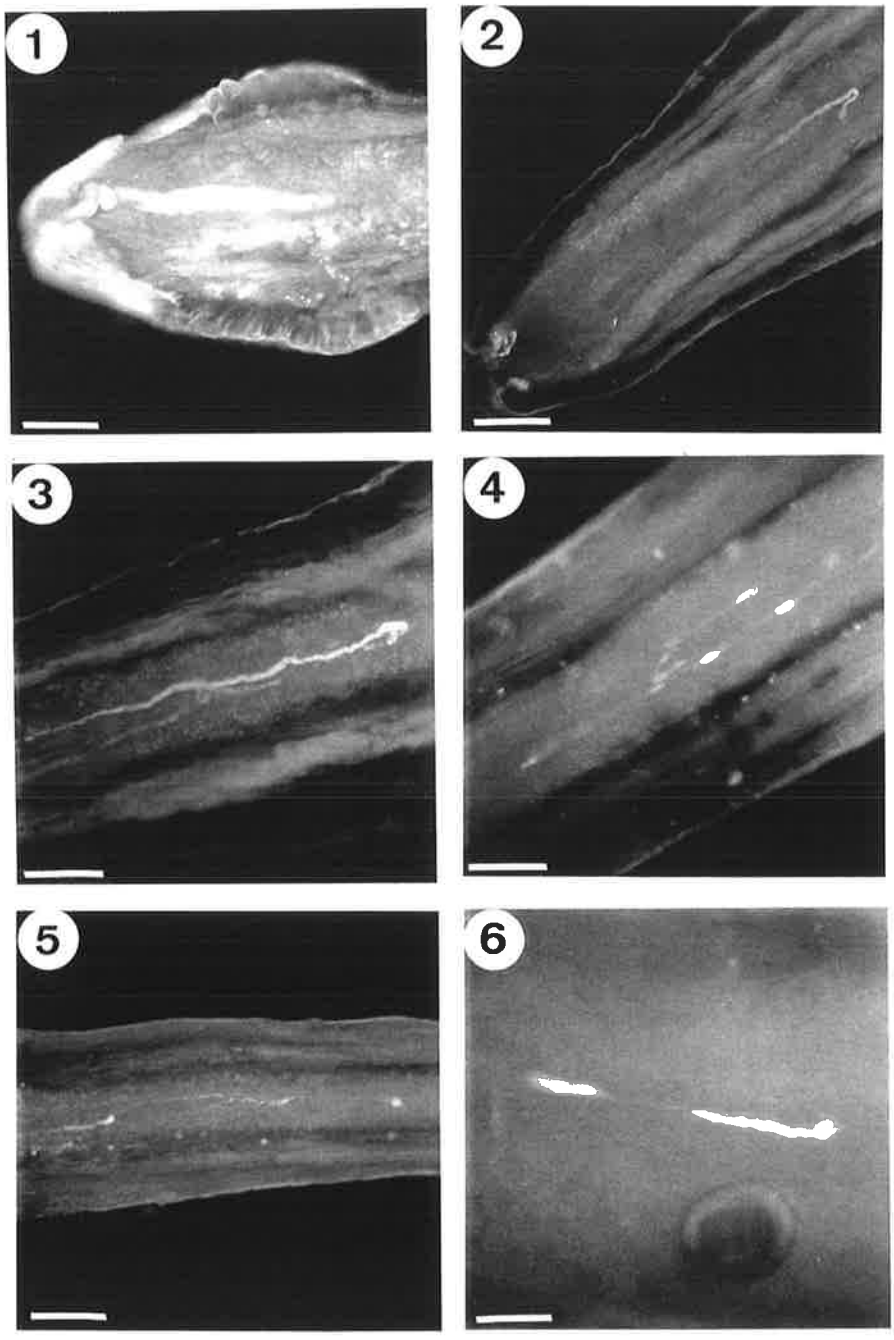
Plate 4.3 Pollen tube abnormalities observed in *Banksia coccinea* interspecific crosses using fluorescence microscopy.

Figure 1 Thickened pollen tube in the pollen presenter of *B. laricina.* Scale bar represents 150 μM.

Figure 2 Directionless pollen tube in the pollen presenter of *B. candolleana.* Scale bar represents 150 μM.

Figures 3, 4 Branched pollen tubes in the upper style of *B. telmatiaea* (Fig 3), and *B. micanthra* (Fig 4). Scale bars represent 75 μM.

Figures 5, 6 Pollen tubes with bulbous tips in the upper style of *B. prionotes.* Scale bar Fig 5 represents 150 μM, Fig 6, 75 μM.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Discussion

There was observed or predicted sexual compatibilty between *B. coccinea* and species in subgenus *Banksia*, section *Banksia* series *Cyrtostylis*, *Prostratae*, and *Tetragonae*, section *Oncostylis* series *Spicigerae*, and *Abietinae* and subgenus *Isostylis*. There was no interspecific pollen tube growth with species from subgenus *Banksia* section *Banksia* series *Grandes, Quercinae* or *Bauerinae*. This generally agrees with the hierarchical classification of George (1981, 1988). However, the particular success of interspecific pollination of *B. coccinea* with *B. ericifolia* (section *Oncostylis*, series Spicigerae), *B. micrantha* and *B. sphaerocarpa* (section *Oncostylis*, series *Abietinae*), as measured by pollen tube growth into the lower style, suggests a closer relationship of *B. coccinea* to section *Oncostylis* than to section *Banksia,* where *B. coccinea* is currently placed. The relationship of *B. coccinea* to section *Oncostylis* has previously been suggested by George (1981) and Thiele (1993), and the results of this study provide further evidence that there is a strong affinity to this section.

These results indicate that the current classification of *B. coccinea* in section *Banksia* may be inappropriate, as compatibility within the section was variable, with no cross observed with pollen tube growth to the base of the style. The strong affinity of *B. coccinea* to section *Oncostylis* is supported by morphological evidence, as *B. coccinea* has hooked styles prior to anthesis, as do the members of series *Spicigerae* (George 1981). Despite the strong pistil-pollen compatibility, it is not appropriate to place *B. coccinea* in section *Oncostylis* as all its members have hooked styles at anthesis, whereas *B. coccinea* does not, and this is a primary character determining the section classification (George 1981). In addition, follicles of species in this section have no lateral beak after opening. Moreover, inflorescence development in section *Oncostylis* is basipetal (with the exception of *B. nutans*) in contrast to that of *B. coccinea.* Placement of *B. coccinea* in subgenus *Isostylis* is also inappropriate on morphological grounds, due to *Isostylis* characters such as inflorescence shape which is short and conical, ovoid receptacles,

loosely packed perianths, straight slender pistils and obliquely ovoid follicles. In view of the distinct morphology and lack of pistil-pollen compatibility between *B. coccinea* and section *Banksia,* it is proposed to erect a new section, *Coccinea*, containing only one species, *B. coccinea* (Maguire *et al.* 1996 in preparation). Chapter 5

This study also highlights unusual aspects of the breeding systems operating in *Banksia.* Generally low pollen tube numbers are observed in *Banksia* compared to other genera. This may be partly due to the small size of the stigmatic groove, which restricts pollen grain numbers (Sedgley *et al.* 1993), and the morphology of the pistil, which in *B. menziesii* supports the growth of no more than one or two pollen tubes to the ovary (Clifford and Sedgley 1994). The *Banksia* inflorescence characteristically shows low seed set in relation to the numbers of flowers produced (Collins and Rebelo 1987, Walker and Whelan 1991). Many hypotheses have been proposed to explain this phenomenon, including breeding system controls (Lamont and Barrett 1988, Goldingay *et al.* 1991), pollinator behaviour (Collins and Rebelo 1987), resource control (Stock *et al.* 1989), seed predation (Witkowski *et al.* 1991) and spatial limitation on the infructescence (Collins and Rebelo 1987, Fuss and Sedgley 1991a).

The results of this study are in agreement with intraspecific and interspecific pollen tube data previously obtained (Fuss and Sedgley 1991a, Sedgley *et al.* 1994, 1996), showing that the pollen presenter and upper style are control points in pollen tube growth. The pollen presenter has a different morphology from the rest of the style, with an abundance of transfer cells surrounding the transmitting tissue (Clifford and Sedgley 1994). This indicates a region of high flux, which may influence inhibition of pollen tubes. The upper style is the next controlling region, with lower numbers of pollen tubes inhibited than in the pollen presenter. It appears that once pollen tubes pass this region they are uninhibited to the base of the style. Seed set and seedling growth are the ultimate tests of success of interspecific crosses.

Pollen tube growth in most interspecific crosses of *Banksia* appeared to be normal, as seen in intraspecific crosses, but some combinations showed abnormalities. Pollen grain and pollen tube abnormalities were observed on the stigmatic surface, but most were observed in the pollen presenter and upper style regions. Perhaps this is due to higher numbers of pollen tubes and more pollen tube selection in these regions. No pollen tube abnormalities were observed in the lower style. Similar observations of pollen tube abnormalities have been reported in interspecific crosses of *Eucalyptus* (Ellis *et al.* 1991), *Solanum* (Fritz and Hanneman 1989), *Lycopersicon* (deNettancourt *et al.* 1973) and *Rhododendron* (Williams *et al.* 1982). Dumas and Knox (1983) reported that pollen tube morphology and callose formation are strongly influenced by the incompatibility reaction. Pollen tubes of compatible pollinations have long thin tubes with defined callose plugs, and those that are incompatible are often thick, ending in tapered, swollen or burst tips. It is possible that the pollen tube behaviour observed on the stigma in *Banksia* interspecific crosses may be a more immediate and severe display of the interspecific incompatibility mechanism.

The mechanism of interspecific incompatibility has long been a subject of debate. Interspecific incompatibility is defined as any post pollination process preventing the formation of hybrid zygotes through absence of pollen germination or abnormal pollen tube behaviour. This phenomenon prevents gene flow between species, in contrast to self incompatibility which restricts inbreeding and establishes upper limits to outbreeding. Interspecific incompatibility acts as a breeding barrier between sympatric species, allowing gradual speciation within populations, but prevents foreign germplasm migration via pollen from allopatric populations and isolates invaders introduced as seed.

Current scientific thought on the mechanism of interspecific incompatibility is divided into two main hypotheses: the incongruity hypothesis of Hogenboom (1975, 1984) and the protein - glycoprotein mediated response related to the self incompatibility (SI) mechanism (Nasrallah *et al.* 1985, Anderson *et al.* 1986). The incongruity hypothesis

maintains that pollen tube growth ceases in the style due to the inability of the pollen tube genotype to recognise and utilise the stylar cell secretions. The glycoprotein - protein mediated response has been more widely accepted, and recent molecular data also support it (Ai *et al.* 1990, Gray *et al.* 1991, Jahnen *et al.* 1989). In other genera, interspecific incompatibility parallels gametophytic SI, in which a stylar glycoprotein response has been found in *Prunus* (Williams *et al.* 1982, Mau *et al.* 1982), *Nicotiana* (Anderson *et al.* 1984, 1986) and *Petunia* (Kamboj and Jackson 1986).

Commonly, unilateral interspecific incompatibility is observed in interspecific crosses. Unilateral incompatibility prevents pollen from SC species to grow down the styles of SI species (SI x SC), whereas the reverse (SC x SI) is allowed. Unilateral incompatibility appeared to be absent in the *Banksia* interspecific crosses in this study, with no significant effect of cross direction on pollen tube numbers in the lower style. The apparent absence of unilateral incompatibility in this study may be attributable to the fact that most *Banksia* species, from both taxonomic sections of *Banksia*, are predominantly outcrossing and SI (Scott 1980, Ramsey and Vaughton 1991, Carthew *et al.* 1988, Carthew 1993, Collins and Spice 1986, Goldingay *et al.* 1991, Fuss and Sedgley 1991a). Alternatively, the crosses may be too far distant to be successful, and cross direction may be irrelevant, with some other factor(s) determining cross success. Lewis and Bell (1981) crossed four *Banksia* species and found pollen germination in species of the same style morphology: for example, *B. menziesii* and *B. attenuata* which have straight styles, in section *Banksia*, and *B. littoralis* and *B. telmatiaea* with hooked styles, in section *Oncostylis*. However, there appeared to be a barrier between the two style morphology groups, with species with straight styles unable to cross with hooked style species. The results of the present study show that this is not however, a general phenomenon. *B. coccinea*, which has straight styles, crosses more readily with hooked style species, than species with straight styles. It is suggested that this is because *B. coccinea* is more related to the hooked styled species, rather than to other straight styled species. Other factors besides interspecific incompatibility may also isolate *Banksia*

species. Factors that isolate species include geographical separation, and environmental and ecological and genetic factors. It should be noted, that most crosses are not possible in the wild, with many species geographically isolated, with differing habitat requirements and adaptations for specific pollinator attraction. These factors in combination with genetic incompatibility may allow some crosses to hybridise while others will not, and evidence of this is the low numbers of natural hybrids that occur. Further work is required on the mechanisms isolating species, including those that are geographically isolated, and those which co-occur, yet remain sexually isolated from one another.

This study demonstrated the close relationship of *Banksia* to *Dryandra,* with some pollen tube growth in the pollen presenter and upper style regions in the cross between *D. squarrosa* and *B. coccinea*. *Banksia* and *Dryandra* are sister genera in the tribe Banksiae, family Proteaceae. Intergeneric compatibility is not unknown in other crops for example intergeneric hybrids have been reported between *Litchi* and *Dimocarpus* (McConchie *et al.* 1994). Breeding barriers between genera within tribe ~~*Banksiae*~~ Banksieae have not been previously investigated, and the taxonomic relationship between genus *Dryandra*, genus *Banksia*, subgenus *Banksia*, and subgenus *Isostylis* is currently unresolved. This study confirms the close relationship of the genera; but it does not resolve relationships. Further studies such as DNA sequencing are needed to address these questions.

In conclusion, the species relationships highlighted by this study contribute to determining the taxonomic position of the problematic species, *B. coccinea*. In addition, species combinations are suggested that may be successful in the production of novel hybrids, which may have potential for the cut flower and potted plant industries.

Chapter Five

Banksia sect. *Coccinea* (A.S. George) T. Maguire *et al.*, (Proteaceae). A new section

Abstract

A new section of *Banksia,* sect. *Coccinea* (A.S. George) T. Maguire *et al.*, is described to include the single, but phylogenetically isolated species, *Banksia coccinea* R. Br. Sectional classification is based on the distinct morphological features of *B. coccinea* and its pollen - pistil compatibility to other species of *Banksia.* Interspecific pollination shows greatest compatibility with species of sect. *Oncostylis*, rather than sect. *Banksia*, where *B. coccinea* is currently placed. Due to the lack of lower style compatibility with sect. *Banksia* and *B. coccinea's* distinctive morphology, it is proposed to erect a new monotypic section as sister to sect. *Oncostylis.*

Introduction

The genus *Banksia* is endemic in Australia, with the exception of *B. dentata* which extends to Papua New Guinea, Irian Jaya and the Aru Islands. In the current classification, *Banksia* is divided into two subgenera: *Banksia* (72 spp.) and *Isostylis* (3 spp.) (George 1981, 1988). Subgenus *Banksia* is further divided into two sections, *Banksia* and *Oncostylis*, based on flower form. Section *Banksia* contains 50 species with straight styles, and section *Oncostylis* contains 22 species with hooked styles. There are 10 series within sect. *Banksia* and three in sect. *Oncostylis*. *Banksia coccinea* R. Br. (Scarlet banksia) is currently the only species in *Banksia* subg. *Banksia* sect. *Banksia* ser. *Coccineae*. Although it is placed in subg. *Banksia*, as its styles are straight at anthesis, the styles are hooked prior to anthesis as in sect. *Oncostylis* ser. *Spicigerae*.

The placement of *B. coccinea* within the genus has been problematic. It has no obvious close relatives and has a unique combination of characters comprising cotyledons, seedling leaves, adult leaves, inflorescences, pollen, infructescences, follicles and seeds. *B. coccinea* cotyledons are cuneate-obovate, with auricles (George 1981). *Banksia* seedling leaves are usually different from the adult leaves, and in *B. coccinea* the first seedling leaves are small, broadly spathulate with narrowly attenuate bases, deeply lobed margins, with a single obtuse lobe on each side and rounded sinuses (Thiele 1993). The broadly oblong-obcordate adult leaves are also distinctive in the combination of size, shape and marginal dentation (Thiele 1993). The inflorescences of *B. coccinea* have looped styles before anthesis, resulting from unequal growth of the pistil and perianth. The flowers are spirally arranged, but once the perianths are exserted they assume a vertical alignment. In most other species of subg. *Banksia* the spiral arrangement is either evident in the exserted perianths through to anthesis, or becomes obscured and unpatterned. The exceptions are ser. *Quercinae* and ser. *Spicigerae* in which there is a superficial vertical alignment. In *B. coccinea* the style twists through c. 90 ^{0} so that the upper part (when hooked prior to anthesis) achieves a more-or-less horizontal position. In each pair of flowers the styles twist in opposite directions so that one overlaps with a flower of the adjacent pair. This is also the pattern adopted in ser. *Spicigerae*. *B. coccinea* pollen is unique in the genus in having an elongate cylindrical shape rather than an oblong or crescent shape (Sedgley *et al.* 1993). The infructescence of *B. coccinea* is relatively small and the follicles are the smallest in the genus. At dehiscence the valves of the follicle split at the insertion point of the style to leave a lateral beak. The seeds have unique features including the small size, cuneate shape, unevenly rugose inner face, unevenly wrinkled outer face and a slightly oblique, obovate, wrinkled wing.

A recent cladistic analysis of *Banksia* based on morphological characters was undertaken by Thiele (1993). In this analysis, *B. coccinea* and some other taxa were problematic and were placed *incertae sedis*. The analysis should be treated with caution, as it was noted that support for some nodes was tenuous. Because additional independent data may help

to resolve these relationships, the pistil-pollen compatibility of *B. coccinea* was studied through interspecific pollination to other species groups within the genus.

Materials and methods

Banksia *flower morphology*

Banksias produce inflorescences composed of numerous hermaphrodite flowers clustered in pairs around a central woody core. Each floret has a single elongate pistil with two ovules in the unilocular ovary. Anthers are attached by short filaments near the tip of each of the four perianth parts. This region of the perianth, prior to anthesis, encloses the distal portion of the style which in most species has an obliquely terminal stigmatic groove. This portion of the style is modified for the function of pollen presentation. Prior to anthesis the anthers dehisce, depositing pollen onto the pollen presenter. At anthesis the style is released from the perianth and pollen is available to foraging fauna. *Banksia* flowers are protandrous, so the pollen is mature before the stigmatic groove becomes receptive. The stigmatic groove becomes receptive after 3 days, when it is ready to receive pollen from another plant (Fuss and Sedgley 1991). Following fertilisation, follicles and seeds may develop, taking from a few months to 2 years to mature.

Interspecific pollination

The technique for interspecific pollination was that used in manipulative intraspecific pollination (Fuss and Sedgley 1991). Inflorescences from one species of subg. *Isostylis* and 34 species of subg. *Banksia* (Table 5.1) were prepared for experimentation by removing opened florets and isolating the inflorescences inside glassine bags secured with twist ties. Between one and six species per series were selected as female parents. Self pollen was removed from open florets by passing a looped pipe cleaner over the pollen presenter region of the style, after which the inflorescence was rebagged.

Approximately 100 florets were pollinated three days after anthesis, the time of peak stigma receptivity (Fuss and Sedgley 1991), with fresh pollen from *B. coccinea*, applied to at least 2 inflorescences per species. *B. coccinea* was used as the male parent in all crosses as the unique pollen grain shape clearly distinguished interspecific pollination from any chance self pollination. In addition, pollen grains of *B. coccinea* were able to physically fit in the stigmatic grooves of all other *Banksia* species (Sedgley *et al.* 1993). Insufficient *B. coccinea* inflorescences to use as female parents for the large number of pollinations required was a further important factor. Control intraspecific pollinations were carried out on 2 species (*B. coccinea* and *B. menziesii*) for pollen removal success, and pollination success to confirm validity of the technique.

Pistils were fixed at 6 days after pollination in Carnoy's solution (6 : 3 : 1 ethanol : chloroform : acetic acid) for one week. They were transferred to 90% ethanol for storage before processing for fluorescence microscopy. Pistils were hydrated in 70% ethanol (30 mins), 30% ethanol (30 mins) followed by two washes in reverse osmosis (RO) water (30 mins each). Pistils were softened in 0.8 M NaOH at 60^0C (30-45 mins) and stained in decolourised aniline blue (0.1% W.S. aniline blue in 0.1 M $Na_2K_3PO_4$) overnight. Aniline blue stains callose in the pollen tube walls. Pistils were dissected longitudinally under the microscope to facilitate observation of pollen tubes, mounted in 80% glycerol on microscope slides and covered with a coverslip. Observations were made of the number of *B. coccinea* pollen grains and pollen tubes in the style.

Results

Most interspecific crosses had apparently normal pollen tube growth down the style. Some interspecific combinations showed abnormal pollen tube growth, however, the frequency of abnormal pollen tube behaviour was low. The control intraspecific cross of *B. coccinea x B. coccinea* showed pollen tube growth to the base of the style (Table 5.1). Some interspecific combinations also had pollen tube growth to the base of the style.

Those species with lower style penetration by *B. coccinea* pollen tubes were from series *Spicigerae* and *Abietinae* (sect. *Oncostylis*, subg. *Banksia*). Pollen tube growth in the upper style, but not the lower style, was observed in species from the series *Salicinae, Banksia, Crocinae, Cyrtostylis, Prostratae* and *Tetragonae* of sect. *Banksia* and with subg. *Isostylis*. There was no interspecific pollen tube growth with species from series *Grandes, Quercinae* or *Bauerinae* of sect. *Banksia* (Table 5.1).

Table 5.1 Pollen tube penetration of interspecific and intraspecific pollen in *Banksia* pistils pollinated with *B. coccinea* pollen (pp: pollen presenter, us: upper style, ls: lower style, * : pollen tubes present, blank cell: pollen tubes absent). Taxonomy follows George (1981, 1988).

Taxon	Pollen tubes in: pp	us	ls
Genus *Banksia*			
Subgenus *Banksia*			
Section *Banksia*			
Series *Salicinae*			
B. integrifolia L.f.	*		
B. marginata Cav.	*		
B. paludosa R. Br.			
Series *Grandes*			
B. grandis Willd.			
Series *Quercinae*			
B. quercifolia R. Br.			
Series *Bauerinae*			
B. baueri R. Br.			
Series *Banksia*			
B. candolleana Meissn.			
B. menziesii R.Br.	*	*	
B. ornata F. Muell. ex Meissn.	*		
B. speciosa R. Br.			
Series *Crocinae*			
B. burdettii E.G. Baker			
B. hookeriana Meisnn.			
B. prionotes Lindley	*	*	
Series *Cyrtostylis*			
B. ashbyi E.G. Baker	*	*	
B. attenuata R. Br.	*	*	
B. laevigata Meisnn.	*	*	
B. media R. Br.			
B. praemorsa Andrews	*		
Series *Prostratae*			
B. blechnifolia F. Muell.	*	*	
B. gardneri A.S. George			
B. petiolaris F. Muell.	*	*	
B. repens Labill.			
Series *Tetragonae*			
B. lemanniana Meisnn.	*	*	
Series *Coccineae*			
B. coccinea R. Br.	*	*	*
Section *Oncostylis*			
Series *Spicigerae*			
B. brownii Baxter ex R. Br.			
B. ericifolia L. f.	*	*	*
B. occidentalis R. Br.	*		
B. tricuspis Meisnn.	*	*	
Series *Abietinae*			
B. laricina C. Gardner	*		
B. micrantha A. S. George	*	*	*
B. nutans R. Br.	*	*	
B. pulchella R. Br.			
B. sphaerocarpa R. Br.	*	*	*
B. telmatiaea A. S. George	*	*	
Subgenus *Isostylis*	*	*	
B. cuneata A.S. George			

Discussion

Interspecific hybridisation is of interest from economic, ecological and taxonomic viewpoints. Economically, the potential of hybrids may exceed that of the parental species for novel or improved varieties in ornamental horticulture. Ecologically, hybrids occurring in the wild may have a competitive advantage over both parental species in a disturbed habitat (Potts and Reid 1985). Hybridisation generally only occurs between related taxa and can therefore have systematic and evolutionary implications for a group (Erickson *et al.* 1983). Some presumed natural interspecific hybrids have been reported in *Banksia* (Taylor and Hopper 1988, Clifford 1993), but little information is available on interspecific hybridisation. Interspecific pollination within the genus *Banksia* has been previously reported with *B. prionotes* and *B. menziesii* (Sedgley *et al.* 1994). It was found that success of pollen tube growth was largely related to taxonomic distance between the species, and the results showed a close affinity between the series *Banksia* and *Crocinae*. Pollen tube growth appeared to be controlled in the pollen presenter and the upper style regions. Crossing studies with *Banksia* have encouraged speculation on the relationships of species within the genus. Lewis and Bell (1981) crossed four *Banksia* species, observing pollen germination on the stigma, and found that species of the same style morphology crossed, viz *B. menziesii* and *B. attenuata* which have straight styles and belong in section *Banksia*, and *B. littoralis* and *B. telmatiaea*, which have hooked styles and belong in section *Oncostylis*. They suggested a barrier between the two style morphology groups, with species having straight styles unable to cross with hooked style species.

The current study was conducted on the basis that pollen tube compatibility in *Banksia* is largely related to taxonomic distance, as shown previously by Sedgley *et al.* (1994). This study shows that *B. coccinea* has the strongest interspecific pistil-pollen compatibility with species in subg. *Banksia* sect. *Oncostylis* as demonstrated by successful pollen tube growth to the base of the style. This suggests that the current classification of *B. coccinea*

in sect. *Banksia* is inappropriate, as although compatibility within the section was variable, there were with no crosses having pollen tube growth to the base of the style. The strong affinity to sect. *Oncostylis* is further supported by morphological evidence, as *B. coccinea* has hooked styles prior to anthesis, as do the members of series *Spicigerae* and *Abietinae* (George 1981). However despite the strong pistil-pollen compatibility, it is not appropriate to place *B. coccinea* in sect. *Oncostylis* as all its members have hooked styles at anthesis, whereas *B. coccinea* does not, and this is a primary character determining the sectional classification (George 1981). In addition, follicles of the species in this section have no lateral beak after opening, and inflorescence development in sect. *Oncostylis* is basipetal with the exception of *B. nutans*, in contrast to that of *B. coccinea* where it is acropetal. Placement within subgenus *Isostylis* is also inappropriate on morphological grounds due to characters such as inflorescence shape, which is short and conical, ovoid receptacles, loosely packed perianths, straight slender pistils and obliquely ovoid follicles. In view of the distinct morphology and lack of lower style pistil-pollen compatibility between *B. coccinea* and sect. *Banksia,* it is proposed to erect a new section *Coccinea* containing only *B. coccinea.* It is further suggested that section *Coccinea* is the sister section to *Oncostylis*, based on lower style compatibility with *B. coccinea.*

Taxonomy

Banksia subg. *Banksia* sect. *Coccinea* (A.S. George) T. Maguire *et al,*. sect., stat. nov.
Banksia sect. *Banksia* series *Coccineae* in A. S. George *Nuytsia* 3:389 (1981).
Type species: *Banksia coccinea* R.Br.
Derivation of name. From the Latin *coccineus* (scarlet), in reference to the styles of the type (and only) species.
Stems erect. *Leaves* broadly oblong to obcordate. *Common* and *floral bracts* linear-subulate. *Flowers* at anthesis vertically seriate. *Pistil* strongly curved laterally before anthesis, afterwards straight; pollen presenter conical. *Follicles* very small, after dehiscence with a lateral beak. *Cotyledons* cuneate-obovate.

This section contains only one species, *Banksia coccinea* R. Br., which warrants its own section in the distinguishing characters described above.

Chapter Six

Seed set following interspecific pollination with *B. coccinea* R. Br

Abstract

Following analysis of pollen - pistil compatibility of *B. coccinea* to other *Banksia* species, species combinations with successful pollen tube growth were selected for seed set experiments. Of the five species combinations tried, two crosses initiated follicle set. In the cross *B. micrantha x B. coccinea* all seed were aborted, indicating post fertilisation selection. The cross *B. ericifolia x B. coccinea* produced seed. These seed showed low viability, with only two seedlings produced, one of which died. The remaining seedling, based on early seedling morphological characters, cannot be distinguished from the female parent *B. ericifolia.*

Introduction

Banksia breeders aim to produce novel hybrids which are faster growing, high quality, and give increased yields for the cut flower market. Heterosis, through interspecific hybridisation, has been reported in other crops (Meskimen and Franklin 1984) but some studies show no increase in heterosis with hybrid growth rates being intermediate or equal to those of the parents (Potts *et al.* 1987). Moreover, some crosses give rise to inferior offspring, as seen in some eucalypts (Potts *et al.* 1987). Breeding of novel banksias depends on production of advanced generation hybrids, which is determined by interspecifc hybrid fertility and fitness of the subsequent offspring.

To produce interspecific hybrids in *Banksia*, species relationships need to be assessed. The accepted classification of *Banksia* (George 1981, 1988) includes some species whose positions are uncertian. One of these is *Banksia coccinea,* a major commercial species, which has showy red blooms on long straight stems, with foliage that does not obstruct the bloom, making it ideal as a cut flower. In a previous chapter interspecific pollination was conducted with *B. coccinea* to other *Banksia* species to assess sexual compatibility. Following interspecific pollination, some combinations were found to have compatible pollen-pistil interactions, or a predicted probability of compatibility.

Some self incompatibility and interspecific isolation systems have been shown to operate at the post fertilisation stage (Franken *et al.* 1988, Seavey and Bawa 1986), and maternal resource allocation can also influence which seeds develop (Stephenson and Bertin 1983 Stock *et al.* 1989). The extent to which interspecific isolation occurs post zygotically in *Banksia* can be assessed by seed set, seed germination and seedling growth. The aim of this study is to determine the probable success of interspecific seed set following pollination with *B. coccinea* to species that were identified as compatible, or potentially compatible in the pistil - pollen interaction.

Materials and methods

Plant material

Following analysis of pollen-pistil interactions, a further series of interspecific pollinations ~~were~~ was conducted for seed set on species combinations that were identified as compatible, or predicted to be compatible. Parental species and locations were the same as those previously described.

Interspecific seed set

Fresh *B. coccinea* pollen was applied to pistils at peak stigma receptivity as described previously. Two thirds of ~~each of four - eight~~ the flowers on four to eight inflorescences per cross were pollinated. Female species included *B. menziesii* (section *Banksia*), *B. tricuspis, B. ericifolia, B. micrantha* (section *Oncostylis*), and *B. cuneata* (subgenus *Isostylis*). A control pollination with *B. coccinea* was included. After pollination, bags were left in place until follicle maturity. Cones with follicles were collected after 12 months, at maturity. Measurements of follicle set, and length, width and height of follicles were taken. Follicles were opened by burning over a bunsen burner in a fume hood; seed and the woody ~~separator~~ separators were removed using tweezers. Seeds were classed as full seed, or as aborted seed which were thin and papery, containing no embryo tissue. Full seeds were measured for seed weight, length, width and wing width. Differences in follicle and seed characters were tested between species using analysis of variance (ANOVA) with the statistical package Genstat 5.

Germination trials

Seed viability was tested in sterile conditions. Seed were surface sterilised in 70% ethanol for 2 min, Milton's antibacterial solution for 5 min, followed by 3 washes in reverse osmosis (RO) water. Seeds were transferred to sterile petri dishes with moist filter paper, sealed and kept at 15 °C in an incubator and scored weekly for germination. Seeds were scored as germinated when the radicle emerged from the seed and was about 0.5 mm long. After 3 months any ungerminated seed was squashed to test for the presence of a white embryo, confirming full seed development. Differences in seed germination time and viability were tested between species using ANOVA. After germination, seeds were transferred to potting soil (State Flora mix), placed in a glasshouse (maximum 25 °C, minimum 15 °C) and fertilised after 2 months with slow release fertiliser (Osmocote).

Hybrid verification

Cotyledon morphology of newly germinated parental and hybrid seedlings were recorded. Morphological measurements were taken from 4 seedlings of parental and hybrid seedlings (with the exception of hybrids as there was only one surviving individual). Measurements were taken from 1 leaf from the first and fifth pairs of leaves. When leaves were alternate measurements were taken from the uppermost leaf. Variables measured included: cotyledon length, width, length/width, number of leaf lobes, leaf length, width, length/width, number of teeth, length of teeth, number of nodes, distance from the first to the fifth node, hairs on upper surface, lower surface of the leaves (0 = absent, 1 = present), stem hairiness (0 = absent, 1 = present), leaf arrangement, opposite, spiral (0 = absent, 1 = present). To verify seed parentage from controlled pollinations data from the two parental species and putative hybrid were analysed using the statistical package PATN (Belbin 1991).

Results

Interspecific seed set

Successful interspecific crosses were *B. ericifolia x B. coccinea* and *B. micrantha x B. coccinea* (Table 6.1). The control cross also had follicle set. Interspecific pollination of *B. micrantha* with *B. coccinea* showed a significant difference from intraspecific pollination for follicle length (<0.001), width (<0.001), height (<0.001), and number of seed per follicle (Table 6.2). Pollination of *B. ericifolia* with *B. coccinea* showed a significant difference from intraspecific pollination for follicle length (<0.001), width (<0.001), height (<0.001), seed length (<0.001), width (<0.001), and wing width (<0.001) (Table 6.3, Fig 6.1). There was no significant difference between species in number of seed per follicle, or seed weight.

Seed germination of B. coccinea, B. ericifolia *and* B. coccinea *x* B. ericifolia

There was a significant effect (<0.001) of species combination in germination percentage, under the conditions of the experiment, and a marginally significant effect (<0.07) of time to germination (Table 6.4). There was no significant interaction of species and germination time.

Seedling survival and vigour

The cross *B. ericifolia* x *B. coccinea* gave germination and growth of two seedlings. One seedling died within 1 month of germination. Healthy cotyledons emerged from the seed, and when planted out the seedling emerged from the soil and stopped growth. After a period of no further growth, the growing tip senesced before production of the first leaves and the seedling died. The second seedling continued to grow and appeared to be healthy (Fig 6.2).

Hybrid verification and early seedling characteristics

Cotyledon shape is characteristic of taxonomic groups within *Banksia*. The presumed hybrid had a cotyledon shape similar to the female parent *B. ericifolia* (Fig 6.3). Leaf morphology in many *Banksia* species changes from seedling to adult, so leaf measurements were taken at the first and fifth leaves to standardise leaf measurements. A matrix of variables measured for each species (Table 6.5) was used in distance calculations (Gower association measure) and subjected to group average (UPGMA) clustering using the program PATN (Belbin 1991). The ~~dendogram~~ dendrogram (Fig 6.4) shows relationships between the parental species and the presumed hybrid, which clusters with the female parent *B. ericifolia*. The data matrix was subjected to multidimensional scaling, with a minimum spanning tree, using PATN. The data were clearly separated into two dimensions (Fig 6.5) with a stress value of 0.05. A two dimensional plot of the

data showed spatial relationships between individuals of each species and the presumed hybrid. The hybrid grouped with the female parent *B. ericifolia*, indicating that it may be a self. Group statistics were calculated by PATN for each of the morphological characters (Table 6.6). The relative contribution of each character was evaluated for separating the data into two groups (*B. ericifolia, B. coccinea*). A probability value calculated using the Kruskal - Wallis test showed the level of significance for each character. Characters 4, 5, 7, 8, 9, 10, 11, 12, 13, 14, 15, 17, 18, and 19 were significant.

Table 6.1 Number of fertile cones and mean number of follicles/cone following interspecific pollination with *B. coccinea* pollen (SE = standard error).

Interspecific cross	No fertile cones	No barren cones	Total	Follicles/ cone (SE)
B. menziesii	0	4	4	0
B. tricuspis	0	6	6	0
B. ericifolia	2	2	4	12 (2.0)
B. micrantha	3	1	4	16 (1.0)
B. cuneata	0	8	8	0
B. coccinea	4	0	4	22 (0.6)

Table 6.2 Mean follicle and seed set data for parental species and putative interspecific hybrid *B. micrantha* x *B. coccinea* .

Character	*B. micrantha* open pollinated	*B. micrantha* x *B. coccinea*	*B. coccinea* x *B. coccinea*	Probability
Follicle length (mm)	22.60	16.10	8.67	<0.001
Follicle width (mm)	17.73	13.13	3.83	<0.001
Follicle height (mm)	11.93	8.17	3.40	<0.001
Seed/follicle	1.00	0.00	1.00	ns

Table 6.3 Mean follicle and seed set data for parental species and putative interspecific hybrid *B. ericifolia* x *B. coccinea* .

Character	*B. ericifolia* open pollinated	*B. ericifolia* x *B. coccinea*	*B. coccinea* x *B. coccinea*	Probability
Follicle length (mm)	22.70	19.45	8.25	<0.001
Follicle width (mm)	10.25	9.95	3.65	<0.001
Follicle height (mm)	8.90	9.00	3.10	<0.001
Seed/follicle	1.00	1.00	1.00	ns
Seed length (mm)	8.55	8.70	6.20	<0.001
Seed width (mm)	5.65	6.50	4.35	<0.001
Wing width (mm)	11.70	10.65	6.40	<0.001
Seed weight (g)	0.025	0.025	0.020	ns

Table 6.4 Seed germination percentage and germination time for parental and putative hybrid seed.

Cross	*B. ericifolia* open pollinated	*B. ericifolia* x *B. coccinea*	*B. coccinea* x *B. coccinea*	Probability
Germination %	30	7	52	<0.001
Time to max germination (weeks)	3	5	5	<0.070

Table 6.5 Seedling morphology data for 3 month old seedlings of *B. coccinea, B. ericifolia*, and putative *B. ericifolia x B. coccinea* hybrid. Characters measured are: cotyledon length (CL), cotyledon width (CW), cotyledon length/width (CL/W), characters from the first leaf pair, number of leaf lobes (1LLo), leaf length (1LL), leaf width (1LW), leaf length/width (1LL/LW), number of teeth (1T), length of teeth (1TL), number of nodes (NN), distance from node one to node five (ND), stem hairiness (SH), leaf arrangement, oppsite (O), spiral (S), leaf hairs on the upper (U), or lower (L) surface, characters from the fifth leaf pair, number of leaf lobes (5LLo), leaf length (5LL), leaf width (5LW), leaf length/width (5LL/LW), number of teeth (5T), length of teeth (5TL).

Char (mm)	CL	CW	CL/W	1LLo	1LL	1LW	1LL/LW	1T	1TL	NN	ND	SH	O	S	U	L	5LLo	5LL	5LW	5LL/LW	5T	5TL
Eric 1	11	8	1.4	0	18	3	6	8	1	7	40	0	1	0	0	1	0	27	4	6.75	8	1
Eric 2	12	7	1.7	0	20	2	10	6	1	8	40	0	1	0	0	1	0	26	3	8.7	6	1
Eric 3	9	8	1.1	0	19	3	6.3	8	1	7	40	0	1	0	0	1	0	24	4	6	5	1
Eric4	11	7	1.6	0	14	3	4.7	8	1	8	40	0	1	0	0	1	0	24	3	8	8	1
Cocc 1	9	8	1.1	3	14	10	1.4	0	0	5	30	1	0	1	0	1	8	31	11	2.8	0	0
Cocc 2	9	7	1.3	3	9	3	3	0	0	5	20	1	0	1	0	1	13	38	14	2.7	0	0
Cocc 3	8	6	1.3	3	10	5	2	0	0	5	20	1	0	1	0	1	13	35	14	2.5	0	0
Cocc 4	10	9	1.1	3	9	3	3	0	0	5	17	1	0	1	0	1	8	19	10	1.9	0	0
E x C	10	9	1.1	0	19	2	9.5	6	1	9	30	0	1	0	0	1	0	24	3	8	6	1

Table 6.6 Morphological character evaluation. Group statistics given two groups, *B. ericifolia* (1) and *B. coccinea* (2). Character mean, standard deviation and probability using Kruskal - Wallis test, calculated by the program PATN (Belbin 1991).

Character	Group	Mean	St. Dev.	Prob.
1	1	10.60	1.02	
	2	9.00	0.71	0.0595
2	1	7.80	0.74	
	2	7.50	1.12	0.7024
3	1	1.38	0.24	
	2	1.20	0.10	0.3039
4	1	0.00	0.00	
	2	3.00	0.00	0.0047
5	1	18.00	2.09	
	2	10.50	2.06	0.0184
6	1	2.60	0.49	
	2	5.25	2.86	0.0591
7	1	7.30	2.08	
	2	2.35	0.68	0.0139
8	1	7.20	0.97	
	2	0.00	0.00	0.0088
9	1	1.00	0.00	
	2	0.00	0.00	0.0047
10	1	7.80	0.75	
	2	5.00	0.00	0.0098
11	1	38.00	4.00	
	2	21.75	4.92	0.0142
12	1	0.00	0.00	
	2	1.00	0.00	0.0047
13	1	1.00	0.00	
	2	0.00	0.00	0.0047
14	1	0.00	0.00	
	2	1.00	0.00	0.0047
15	1	0.00	0.00	
	2	10.50	2.50	0.0067
16	1	25.00	1.27	
	2	30.75	7.22	0.2129
17	1	3.40	0.49	
	2	12.25	1.79	0.0120
18	1	7.49	0.97	
	2	2.48	0.35	0.0139
19	1	6.60	1.20	
	2	0.00	0.00	0.0098

Figure 6.1 Schematic drawing of parental and putative interspecific hybrid seed. Seed are drawn to actual size. (a) *B. ericifolia*, (b) *B. ericifolia x B. coccinea*, (c) *B. coccinea*, (d) *B. micrantha*, (e) *B. micrantha x B. coccinea*, (f) *B. coccinea*.

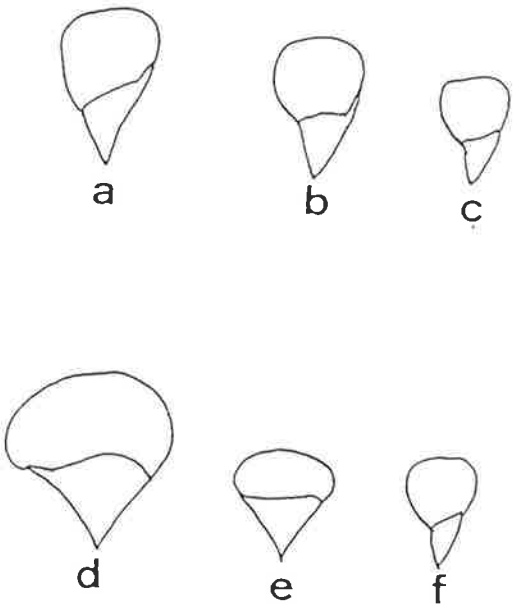

a
b
c
d
e
f

Figure 6.2 Putative interspecific hybrid seedling between *B. ericifolia* and *B. coccinea* at 3 months.


B. ericifolia
Putative
B. ericifolia x B. coccinea
B. coccinea

Figure 6.3 Schematic drawing of parental and putative interspecific hybrid cotyledons. Cotyledons are drawn to actual size. (a) *B. ericifolia*, (b) *B. ericifolia x B. coccinea*, (c) *B. coccinea.*

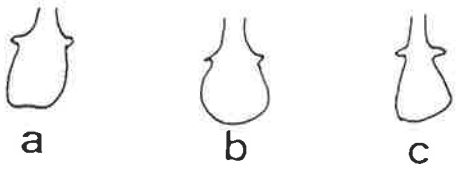

a
b
c

Figure 6.4 Dendogram showing relationships between *B. coccinea*, *B. ericifolia* and putative interspecific hybrid. Distance matrix calculated using the Gower association matrix, followed by group average (UPGMA) clustering.

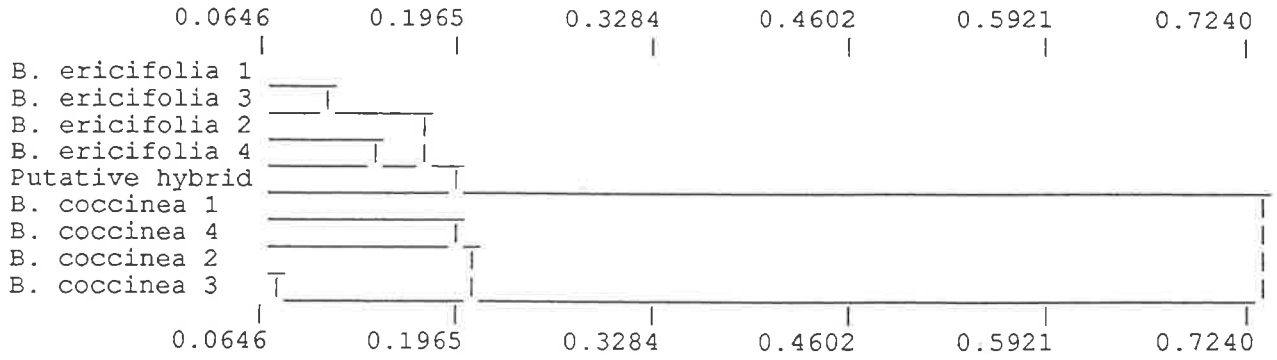
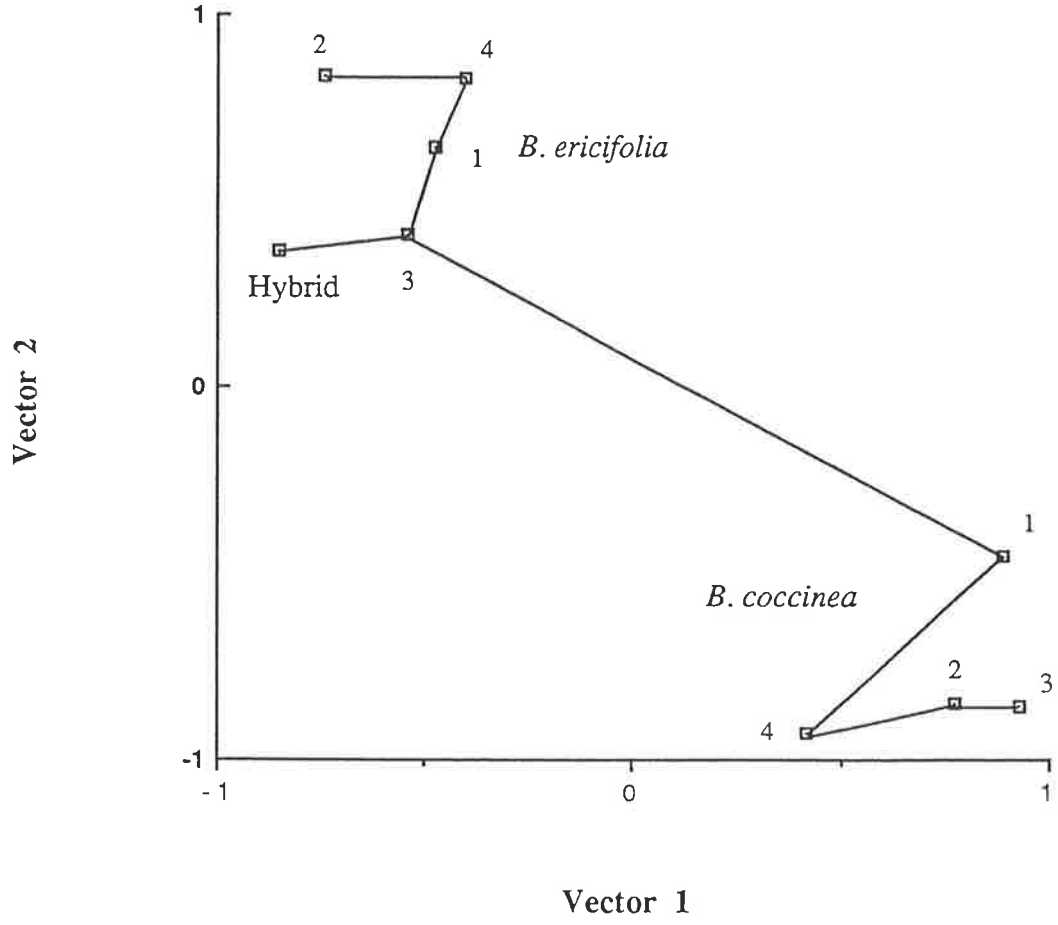

0.0646
0.1965
0.3284
0.4602
0.5921
0.7240
B. ericifolia 1
B. ericifolia 3
B. ericifolia 2
B. ericifolia 4
Putative hybrid
B. coccinea 1
B. coccinea 4
B. coccinea 2
B. coccinea 3
0.0646
0.1965
0.3284
0.4602
0.5921
0.7240

Figure 6.5 Multidimensional scaling of data matrix into two dimensions (stress value 0.05). The plot shows spatial relationships between individulas of *B. ericifolia, B. coccinea* and the putative interspecific hybrid.


1
0
-1
- 1
0
1
Vector 2
Vector 1
2
4
1
B. ericifolia
Hybrid
3
1
B. coccinea
2
3
4

Discussion

The species combinations tested in this study resulted in only one cross (*B. ericifolia x B. coccinea*) with full seed formation. Of these seed only two seedlings were produced, one of which died possibly due to hybrid breakdown, and one survived. The surviving plant, based on early seedling morphological characters, appears to be a self of *B. ericifolia.* This does not necessarily mean it is a self, as hybrids may display dominant and recessive characters of each parent, but based on early seedling characters it cannot be distinguished from the female parent. As the plant reaches maturity, it is possible that it may display more intermediate characters.

The cross *B. micrantha x B. coccinea* initiated follicle set, but all seed were aborted showing post fertilisation selection. Seed formation with *B. ericifolia,* and partial success with *B. micrantha,* both in section *Oncostylis,* demonstrates the close compatibility of *B. coccinea* to section *Oncostylis*, as indicated previously using pollen - pistil data. Therefore, pollen - pistil interactions can predict possible hybrid combinations and species relationships. Interspecific hybridisation has previously been conducted with *Banksia* species to investigate species relationships Lewis and Bell (1981). crossed four *Banksia* species, observed pollen tube growth on the stigma and found that species of the same style morphology crossed. *B. menzeisii* and *B. attenuata,* which have straight styles in section *Banksia* crossed, and *B. littoralis* and *B. telmatiaea,* which have hooked styles in section *Oncostylis* crossed. There appeared to be a barrier between the two style morphology groups, with species having straight styles unable to cross with hooked style species. However, this does not appear to be the case in general. The current study shows *B. coccinea* (straight style) crosses with *B. ericifolia* and partially with *B. micrantha* (hooked style). Another study by Sedgley *et al.* (1994) involving *B. prionotes* and *B. menziesii* found that success of pollen tube growth in the style was largely related to taxonomic distance between the species, and the results showed a close affinity

between the series *Banksia* and *Crocinae*. Thus, interspecific hybridisation is a suitable technique to determine compatibility relationships between *Banksia* species.

An important consideration in hybrid production for plant breeding is the presence of pre and post fertilisation barriers. This study demonstrates that both pre and post fertilisation barriers occur in *Banksia*. The crosses with *B. menziesii*, *B. tricuspis*, and *B. cuneata* (predicted by lower style pollen tube penetration) failed to produce follicles, suggesting selection at the ovary. The cross *B. micrantha* x *B. coccinea* set follicles, but all seed were aborted after follicle initiation. In *Banksia* there is no evidence of parthenocarpy, and there may be a number of reasons why seeds may abort, including space limitation, or resource allocation (Stock *et al.* 1989). Embryo selection may occur, with the maternal parent selecting the best genotype combinations, ensuring survival of the fittest individuals, hence species purity and survival. Reports of hybrid banksias in the wild are few (Taylor and Hopper 1988). Reported hybrids are usually between taxonomically close species, appear to be intermediate between the parental species, and occur in the same area. Thus, hybrid formation may be beneficial in extending species range, species adaptation, or increasing pollinator attraction. The low occurrence of natural hybrids supports the hypothesis of selection of the fittest individuals and maintenance of genetic purity of the species.

In this study the cross *B. ericifolia* x *B. coccinea* set both follicles and seed, however seed viability was low with only one seedling surviving which appeared to be a self. Embryo rescue has been used in other crops to recover potential hybrid zygotes before abortion and subsequent breakdown. It is possible that many of the hybrid seed formed in the *B. ericifolia x B. coccinea* cross resulted in embryo breakdown and were therefore inviable. In *Banksia,* little information is available on interspecific hybridisation and the mechanisms controlling pre and post fertilisation selection.

Chapter Seven

DNA isolation methods for *Banksia* and other members of the Proteaceae

Abstract

The application of current nucleic acid technologies to crop improvement require the development of efficient DNA extraction techniques which yield DNA of adequate purity. Three different DNA extraction methods are described, suitable for genera in the family Proteaceae, which are both rapid and efficient. Due to the different types of plant tissue available, methods were developed for mature leaves, seedling leaves and resting buds, and seed material. These procedures combine and modify previously published techniques. The DNA is suitable for restriction digestion and PCR amplification.

Introduction

The applications of current nucleic acid technologies to crop improvement include gene mapping, genetic fingerprinting, population studies, and phylogenetic analyses. These techniques are appropriate for research into banksias, proteas and other plants for cut flower production. The development of efficient DNA extraction techniques, which yield DNA of a purity adequate for restriction enzyme digest and PCR, has proven difficult from plant materials in the Proteaceae. The problems associated with DNA extraction from plants high in polysaccharides and phenolics, such as occurs in *Banksia*, have been recognised previously (Couch and Fritz 1990, John 1992, Do and Adams 1991).

Plants contain three types of DNA, in the nucleus (genomic), mitochondria (mDNA) and chloroplasts (cpDNA). Methods of extraction for each DNA type are available for many

plant genera, although most experiments require preparation of only genomic DNA. Extraction procedures include removal of the cell wall and nuclear membrane; separation of DNA from the other cellular components, such as cell walls, proteins, lipids or RNA; with maintenance of DNA integrity, and protection from nucleases and mechanical shearing. Commonly, cells are broken by grinding in liquid nitrogen, when the low temperature prevents degradation by nucleases. The extraction buffer usually contains compounds such as sarkosyl, to dissolve membranes and denature proteins, and EDTA which complexes Mg^{2+} ions, an essential co-factor for nucleases. After phenol/chloroform extraction the aqueous phase contains the DNA and RNA, while polysaccharides and some proteins and lipids are in the organic phase. Cell debris and protein aggregates occur in the interphase, between the organic and aqueous phases. Iso-propanol precipitation of the aqueous phase concentrates the DNA, and after a series of washes the DNA is ready to use.

An optimal DNA extraction method needs to be quick and uncomplicated, allowing maximum number of extractions per day, and minimising time and cost. Developing DNA extraction methods that are applicable to a broad range of species is an important goal. The aim of this research is to develop efficient procedures for the extraction of DNA from *Banksia* and other genera in the family Proteaceae.

Materials and methods

Plant material from *Banksia, Dryandra, Leucandendron, Isopogon, Protea, Macadamia, Leucospermum, Grevillea, Hakea* and *Serruria* (Proteaceae) was obtained from mature leaves, seedling leaves, buds and seeds. Material can be stored for a few months at -20 ^{0}C and -80 ^{0}C indefinitely. Two methods for mature and seedling leaf, and bud material adapted from Doyle and Doyle (1988) using CTAB (Maguire *et al.* 1994) were developed, and a third "miniprep" extraction method applied to seed material, is a slightly modified method of Weining and Langridge (1991). DNA extracted using the seedling

leaves method was restricted using Hae III or Dra I endonucleases, or subjected to random amplified polymorphic DNA (RAPD) techniques (Hu and Quiros 1991, Klein-Lankhorst *et al.* 1991).

Results

Protocol 1: DNA extraction from mature leaves

1 Extraction buffer (5 to 7.5 mL) [2% (w/v) CTAB, 1.4 M NaCl, 0.2% (v/v) 2-mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl, (pH 8.0)] was pre-heated to 60°C in a water bath in a 10 ml plastic centrifuge tube.

2 Fresh tissue [0.5 to 1 g] was ground to a powder in liquid nitrogen in a pre-chilled mortar and pestle.

3 The powder was scraped directly into pre-heated buffer in a pre-warmed mortar and mixed thoroughly with a spatula. The sample was poured into a centrifuge tube and inverted gently several times.

4 The sample was incubated at 60°C for 30 min with occasional gentle inversions.

5 The solution was extracted once with chloroform-isoamyl alcohol (24:1, v/v), by gentle but thorough mixing for 5 min.

6 The phases were separated by centrifugation in a swinging bucket rotor-type centrifuge at 4000 rpm at room temperature for 3 min.

7 The aqueous layer was removed with a wide-bore pipette and transferred to a clean plastic centrifuge tube. Two to 3 volumes of ice-cold isopropanol was added.

8 The nucleic acids were recovered by centrifugation at 4000 rpm for 3 to 5 min. As much supernatant as possible was removed without disturbing the precipitate. 70% ethanol was added directly to the pellet and swirled gently. If the precipitate was not obvious the solution was recentrifuged (this may cause some difficulty in washing and requires lifting the pellet from the bottom with a glass rod to allow thorough washing).

9 The pellet was briefly vortexed and allowed to stand for 20 min in 70% ethanol and then centrifuged for 15 min at 4000 rpm.

10 The supernatant was carefully removed and the pellet allowed to air dry at room temperature for approx 5 to 10 min or until there were no visible drops of liquid in the tube.

11 The pellet was resuspended in 400 µl TE buffer [10 mM Tris-HCl (pH 7.4), 1 mM EDTA].

For more difficult plant material, such as bud material, this procedure was modified by incubating for 45 mins in CTAB and allowing the DNA to precipitate in isopropanol at -20°C for approx 30 mins.

Protocol 2: DNA extraction from seedling leaves and other material high in phenolics and polysaccharides

1 Extraction buffer (5 to 7.5 mL) [3% (w/v) CTAB, 1.4 M NaCl, 0.4% (v/v) 2-mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl, (pH 8.0), 2% PVP] was pre-heated to 60^{o}C in a water bath in a 10 ml plastic centrifuge tube.

2 Fresh leaf tissue [0.5 g] was ground to a powder in liquid nitrogen in a pre-chilled mortar and pestle.

3 The powder was scraped directly into pre-heated buffer in a pre-warmed mortar and mixed thoroughly with a spatula. The sample was poured into a centrifuge tube and inverted gently several times.

4 The sample was incubated at 60^{o}C for 45 min with occasional gentle inversions.

5 The solution was extracted once with chloroform-isoamyl alcohol (24:1, v/v), by gentle but thorough mixing for 5 min.

6 The phases were separated by centifugation in a swinging bucket rotor-type centrifuge at 4000 rpm for 3 min at room temperature.

7 The aqueous layer was removed with a wide-bore pipette and transferred to a clean plastic centrifuge tube. The chloroform extraction was repeated.

8 Two to 3 volumes of ice-cold isopropanol plus 50 µL sodium acetate (pH 4.8) was added to the resulting supernatant and left at -20^{0}C for approximately 30 mins.

9 The nucleic acids were recovered by centrifugation at 4000 rpm for 5 min. The viscous layer and pellet were precipitated with 95% ethanol and centrifuged for 10 mins at 4000 rpm.

10 The pellet was dissolved in 400 µL TE buffer [10 mM Tris-HCl (pH 7.4), 1 mM EDTA] with a Pasteur pipette. 1.6 mL of 2.5 M NaCl was added to give a total concentration of 2 M NaCl, and the DNA was precipitated with 95% ethanol and centrifuged for 10 min at 4000 rpm. At this concentration of NaCl, contaminating material remained in solution.

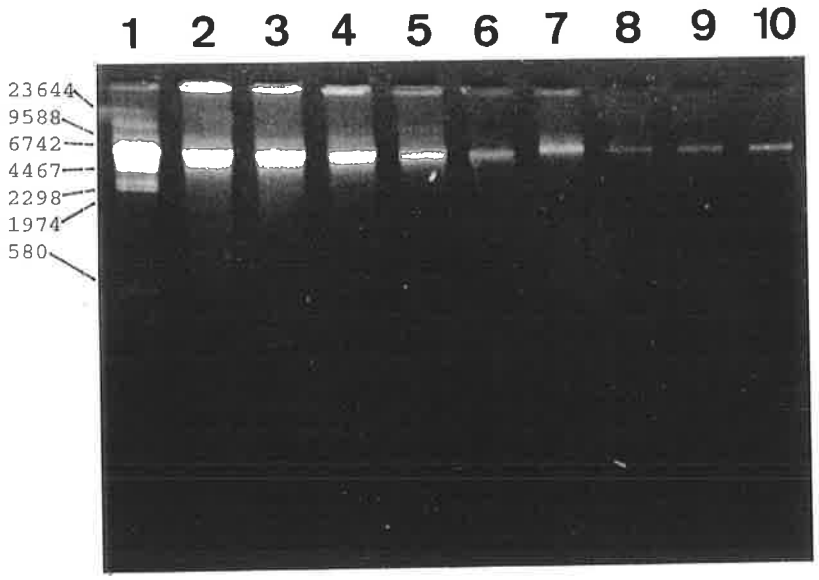
11 As much supernatant as possible was removed without disturbing the pellet. 70% ethanol was added directly to the pellet and briefly vortexed. The tube was centrifuged for 3 min at 4000 rpm. This wash procedure was repeated.

12 The supernatant was carefully poured off and the pellet allowed to air dry at room temperature for approx 5 to 10 min or until there were no visible drops or liquid in the tube.

13 The pellet was resuspended in 100 µl TE buffer [10 mM Tris-HCl (pH 7.4), 1 mM EDTA].

Using these techniques DNA yields of 8-12 µg/g fresh wt. leaf tissue were obtained.

Figure 7.1 Agarose gel (1.6%) showing DNA yield of *Banksia cuneata* individuals following DNA extraction and RNAase digestion. Standard samples of salmon sperm genomic DNA used to quantify yield. Lane 1. DNA size marker λHindIII (bp), lane 2. 100 ng/μL, lane 3. 75 ng/μL, lane 4. 50 ng/μL, lane 5. 25 ng/μL, lane 6. 10 ng/μL, lane 7. *B. cuneata*, lane 8. *B. cuneata*, lane 9. *B. cuneata*, lane 10. *B. cuneata*.


1 2 3 4 5 6 7 8 9 10
23644
9588
6742
4467
2298
1974
580

Protocol 3: Isolation of total genomic DNA from seed material (slightly modified "miniprep" method of Weining and Langridge, 1991)

1 One seed is frozen in liquid nitrogen and crushed in a 1.5 mL eppendorf tube with a steel rod

2 600 µL of DNA extraction buffer [4% sarkosyl, 100 mM tris HCl, 100 mM NaCl, 10 mM EDTA] is quickly added and mixed well

3 600 µL of phenol/chloroform [25% phenol, 24% chloroform, 1 % iso-amyl-alcohol] is then added and the tubes are mixed by thorough hand mixing. The samples are placed on ice until all samples are ready. They are continually mixed and allowed to sit for 5 mins.

4 The samples are centrifuged at 8-10 000 rpm for 2-3 mins, then the upper aqueous phase is transferred to a clean tube using a pipette

5 The phenol/chloroform extraction is repeated

6 75 µL of 3 M sodium acetate (pH 4.8) is added with 600 µL of cold isopropanol and mixed by inversion. The DNA is allowed to precipitate for 1 min

7 The sample is centrifuged at 12 000 rpm for 10 min and the supernatant is carefully poured off

8 1 mL of 70% ethanol is added to the pellet and gently vortexed

9 This is centrifuged at 12 000 rpm for 2-3 mins and the supernatant is poured off and the procedure repeated

10 The pellet is air dried by draining onto tissue, but not allowed to dry out

11 The pellet is resuspended in 50 µL of TE buffer [10 mM tris HCl pH 8, 1 mM EDTA pH 8] and 1 µL R40 [40 g/mL RNAase A in TE], with a gentle vortex and then stored at 4 ^{0}C for up to one month or -20 ^{0}C indefinitely.

Gel electrophoresis

5 µL of the DNA standards (100, 75, 50, 25, 10 ng of genomic salmon sperm DNA) were mixed with 2 µL of ficoll dye and DNA samples were electrophoresed on 1.5% agarose gels in 1X TBE buffer [5X TBE: 45 mM Tris base, 45 mM boric acid, 1 mM EDTA, ph 8.0] at 2V/cm for approximately 1.5 hours. Gels were stained with 0.5 µg/ml ethidium bromide and photographed (Polaroid 667 film) under UV light to check for purity of the preparation after RNAase treatment, and to quantify DNA yield (Figure 7.1).

Discussion

The best results of DNA extraction from *Banksia* were with seeds and fresh leaf tissue harvested from mature plants. Seedling material was more difficult as the leaves were soft and hairy, hence the development of protocol 2 suitable for seedling leaves. The DNA samples were completely digested with Hae III and Dra 1 restriction enzymes indicating low levels of DNA methylation (Nelson and McClelland 1991). The DNA has also been used successfully for the random amplified polymorphic DNA (RAPD) technique.

Chapter Eight

Genetic diversity of *Banksia* and *Dryandra* (Proteaceae) using RAPD markers

Abstract

Random amplified polymorphic DNA (RAPD) markers were investigated as a tool for estimating genetic diversity within 33 species of *Banksia* and three of *Dryandra*. Three primers were used on DNA from ten seeds per species, and band data were pooled to give between 52 and 89 bands per species, most of which were polymorphic. Genetic diversity was calculated using six published metrics on three species, for which allozyme data were also available. Based on between method consistency, three matrices were chosen for analysis of the full data set. Levels of genetic diversity in *Banksia* and *Dryandra* ranged from 0.59 to 0.90.

Introduction

Scientific approaches to the conservation and exploitation of plant genetic resources require a detailed knowledge of the amount and distribution of genetic diversity within a species. Traditionally, a combination of morphological and cultural traits was used, but these characters are influenced by the environment and may not reflect the true genetic diversity. To overcome these problems biochemical methods were introduced, and allozymes are now used extensively to characterise plant genetic resources. Allozymes have been used in *Banksia* to estimate genetic diversity and high levels have been reported, amongst the highest recorded for plants (Scott 1980, Schemske and Lande 1985, Carthew *et al.* 1988, Coates and Sokolowski 1992). Large variability is expected in *Banksia* as the mating system is predominantly outcrossing, with pollination by birds

and small mammals. There are however, widely recognised limitations of allozymes. Many species have low levels of detected allozyme diversity, because the detection of variability is limited to protein coding loci, which may underestimate levels of genetic diversity (Clegg 1989), and may not represent the entire genome (Schaal *et al.* 1991). Allozymes are also tissue specific and are influenced by environmental factors. Given these limitations many workers have moved to DNA markers such as random amplified polymorphic DNA (RAPD), based on PCR technology (Williams *et al.* 1990a). RAPDs overcome many of the limitations of allozymes and are simpler and easier to use than other DNA markers such as restriction fragment length polymorphisms (RFLPs). RAPDs have potentially unlimited numbers of markers and have been shown to be useful for population genetic studies on a number of genera (Huff *et al.* 1993, Chalmers *et al.* 1992, Russell *et al.* 1993). It has also been suggested that RAPDs may be an appropriate technique to monitor diversity in plant populations (Anderson and Fairbanks 1990, Waugh and Powell 1992). By using single random primers this technique can amplify regions within the genome without any prior sequence information, using small amounts of tissue. It is also reasonably simple and can provide a large number of potential polymorphic loci, ideal in the investigation of rare and endangered plants, where there is often little material and low allozyme variability.

Banksia and *Dryandra* are sister taxa in the tribe Banksiae, family Proteaceae, and are currently exploited for the cut flower industry. Many blooms are harvested from the wild, although *Banksia* and *Dryandra* species are increasingly being brought into cultivation. Some species are exploited for their foliage as well as flowers. Currently, selection and breeding for superior genotypes of *Banksia* is underway to produce cultivars for the cut flower industry (Sedgley *et al.*, 1994, 1996). To assist breeding knowledge of the level of genetic diversity within a species is essential for selection and conservation of genetic resources.

Banksia genetic resources are under threat due to land degradation, clearing, fire, pests and diseases, and urban development. Under the Western Australian Wildlife Conservation Act 1950-1979, seven banksias are declared rare flora. They are *B. brownii, B. chamaephyton, B. cuneata, B. goodii, B. meisneri, B. sphaerocarpa* and *B. tricuspis*. Using the criteria for designation of rare flora in Western Australia, another ten species of *Banksia* should be added to the list (George 1987). The importance of documenting the level and distribution of genetic diversity in species in order to design optimal conservation strategies is widely recognised (Hamrick 1983, Moran and Hopper 1987). The aim of this study is to investigate levels of genetic diversity within species of *Banksia* and *Dryandra* using RAPDs, and to evaluate six methods of data analysis.

Materials and methods

Plant material

Seeds of 33 species of *Banksia* and three of *Dryandra* collected from natural populations were obtained from Nindethana Seed Service. The species chosen represented the two sister genera *Banksia* and *Dryandra*, and two subgenera, two sections and thirteen series within the genus *Banksia* (George 1981, 1988) (Table 8.1). Ten seeds of each species were randomly selected for RAPD analysis.

DNA extraction

DNA was extracted from each seed using a modified method of Weining and Langridge (1991), comprising: phenol/chloroform/isopropanol extraction for 5 min on ice, DNA precipitation for 1 min with ice cold isopropanol and sodium acetate, with DNA recovered by centrifugation at 12 000 rpm for 10 min. The pellet was washed twice with 70 % ethanol, dried and dissolved in 50 µL of TE buffer, with 1.0 µL of RNAase (R40:

40 g/mL RNAase A in TE), and stored at 4 ^{0}C short term (up to 1 month) or -20 ^{0}C long term (up to 1 year).

DNA was subjected to gel electrophoresis on a 1.6% agarose gel in TBE buffer (Sambrook *et al.* 1989), and stained with ethidium bromide. DNA concentration was estimated by visual assessment of band intensities, compared to salmon sperm genomic DNA standards. The DNA content was adjusted to 10 ng μL^{-1}.

DNA amplification and documentation

DNA amplification was performed in a MJ Research Thermal Cycler. The optimal program for *Banksia* commenced with an initial denaturation step at 94 ^{0}C for 5 min, followed by 40 cycles of 94 ^{0}C for 1 min, 36 ^{0}C for 1 min, 72 ^{0}C for 2 mins, terminated with a final extension step at 72 ^{0}C for 5 min. Optimised reaction conditions were carried out in a 25 µL total volume containing 1X *Taq* buffer (Gibco-BRL), 3 mM $MgCl_2$, 200 µM of each dNTP (dGTP, dATP, dCTP, dTAP), 1 unit of *Taq* polymerase (Gibco-BRL), 0.5 µL T4 gene 32 protein (Boehringher Mannheim), 1 µM 10 mer primer (Operon Technologies Inc.) and 10 ng μL^{-1} template DNA. Each reaction mix was overlaid with PCR grade paraffin oil. DNA amplification fragments were separated by 2% agarose gel (Seakem, Promega) electrophoresis using TBE buffer (Sambrook *et al.* 1989). A DNA size marker was used (pGEM, Promega) on each gel, and gels were stained with ethidium bromide. Fragment patterns were photographed under UV light with Polaroid 667 film for further analysis. Polaroid photographs were scanned using a transmission scanner (Hewlett Packard Scanjet IIcx/T). The intensity and molecular weight of each visible band was determined using the software CREAM TM., Kem-En-Tec Software Systems, Blue Sky Scientific.

Sixty primers were evaluated for their suitability in a pilot survey (series OPA, OPB and OPC, Operon Technologies Inc.). Three primers were selected, these were OPA-20

(GTTGCGATCC), OPB-03 (CATCCCCTG) and OPB-04 (GGACTGGAGT) which gave reproducible and informative markers. Band fragments were scored as present (1) or absent (0) (Fig 8.1). A negative control was added in each run to test for contamination. In order to test reproducibility, the selected primers were tested three times on the same sample, for a random subset of three DNA samples. To aid interpretation of band homology between gels, each gel contained a standard DNA lane and pGEM DNA marker. The presence or absence of bands was determined for all individuals and a matrix of RAPD phenotypes was assembled. Each individual was represented by a vector of 1s and 0s for the presence or absence of any particular band for all the primers used in the study.

Data analysis

From the matrix of RAPD phenotypes for each DNA sample, an index of genetic distance (D) was calculated (D = 1 - F), where F is similarity calculated using six methods.

- Nei and Li (1979) matching coefficient method:
  2*n11/((2*n11)+n01+n10)
  where n=number of band positions, n11= the number of positions where x=1, y=1, n00= the number of positions where x=0, y=0, n01= the number of positions where x=0 and y=1, n10= the number of positions where x=1 and y=0. x,y = individuals being compared.
- The Jaccard coefficient (Jaccard 1901): n11/(n-n00).
- The method of Russell and Rao (1940): n11/n
- Simple matching coefficient (Apostol *et al.* 1993): (n11+n00)/n
- The method of Excoffier *et al.*, (1992): n*(1-(n11/n))
- The method of Rodgers and Tanimoto (1960):
  (n11+n00)/(n11+2*(n10+n01)+n00).

Distance matrices were calculated using the statistical package, RAPDistance (Amstrong *et al.* 1994). The mean distance value for each species was taken as an estimate of genetic diversity, and the standard deviation of the mean was calculated for each data set.

Results

Band data for the three selected primers were pooled such that each species had total band numbers ranging from 48 to 89. The number of monomorphic bands was low, with most bands being polymorphic (Table 8.1). Preliminary analysis of RAPD data for three *Banksia* species with each of the six published statistical methods gave a wide range of estimates of genetic diversity (Table 8.2). The species were chosen on the basis that there were reported estimates of genetic diversity based on allozyme electrophoresis data (Scott 1980, Coates and Sokolowski 1992). Based on between method consistency, the methods of Nei and Li (1979) , Jaccard (1901) and Russell and Rao (1940) were used for analysis of the total data set.

The estimates of genetic diversity for all species with the three chosen matrices were high, ranging from 0.59 for *Banksia praemorsa*, *B. menziesii* and *Dryandra formosa*, up to 0.90 for *B. lemanniana* and *B. cuneata* (Table 8.1).

Table 8.1 Band data and estimates of diversity for 33 species of *Banksia* and three of *Dryandra* using RAPDs (standard deviation).

Species	Number of bands			Metric		
	Poly-morphic	Mono-morphic	Total	Nei and Li (1979)	Jaccard (1901)	Russell and Rao (1940)
Genus *Banksia*						
Subgenus *Banksia*						
Section *Banksia*						
Series *Salicinae*						
B. integrifolia *	82	0	82	0.77 (0.07)	0.74 (0.09)	0.86 (0.04)
B. robur	61	0	61	0.79 (0.06)	0.77 (0.07)	0.88 (0.04)
Series *Grandes*						
B. grandis	63	0	63	0.72 (0.06)	0.68 (0.07)	0.82 (0.03)
B. solandri **	52	2	54	0.67 (0.07)	0.62 (0.09)	0.75 (0.05)
Series *Quercinae*						
B. quercifolia **	51	1	52	0.73 (0.06)	0.69 (0.07)	0.83 (0.04)
Series *Bauerinae*						
B. baueri	86	2	88	0.72 (0.06)	0.68 (0.08)	0.81 (0.05)
Series *Banksia*						
B. baxteri	84	0	84	0.71 (0.06)	0.67 (0.07)	0.82 (0.03)
B. candolleana	60	0	60	0.74 (0.06)	0.71 (0.08)	0.83 (0.04)
B. menziesii	79	5	84	0.65 (0.06)	0.59 (0.07)	0.77 (0.04)
B. serrata	53	1	54	0.73 (0.07)	0.70 (0.08)	0.84 (0.04)
Series *Crocinae*						
B. burdettii **	89	0	89	0.71 (0.08)	0.67 (0.07)	0.83 (0.04)
B. prionotes	57	1	58	0.73 (0.08)	0.69 (0.09)	0.82 (0.06)
Series *Cyrtostylis*						
B. ashbyi	69	1	70	0.67 (0.07)	0.61 (0.09)	0.77 (0.04)
B. attenuata	60	1	61	0.77 (0.07)	0.74 (0.08)	0.86 (0.05)
B. audax	69	0	69	0.79 (0.06)	0.77 (0.07)	0.88 (0.04)
B. elderiana	52	1	53	0.73 (0.07)	0.69 (0.09)	0.83 (0.06)
B. laevigata *	58	0	58	0.75 (0.07)	0.72 (0.09)	0.85 (0.05)
B. lindleyana *	69	2	71	0.72 (0.05)	0.69 (0.06)	0.82 (0.03)
B. praemorsa **	57	4	61	0.65 (0.10)	0.59 (0.13)	0.79 (0.05)
Series *Prostratae*						
B. blechnifolia **	55	4	59	0.69 (0.07)	0.65 (0.08)	0.80 (0.04)
B. repens	77	1	78	0.73 (0.07)	0.69 (0.09)	0.83 (0.04)
Series *Tetragonae*						
B. caleyi	57	1	58	0.74 (0.06)	0.70 (0.07)	0.82 (0.04)
B. lemanniana	82	0	82	0.81 (0.07)	0.79 (0.08)	0.90 (0.04)
Series *Coccineae*						
B. coccinea	65	0	65	0.78 (0.06)	0.76 (0.07)	0.85 (0.04)
Section *Oncostylis*						
Series *Spicigerae*						
B. ericifolia	51	1	52	0.76 (0.06)	0.73 (0.07)	0.85 (0.05)
B. occidentalis **	74	3	77	0.69 (0.08)	0.65 (0.09)	0.81 (0.04)
B. tricuspis **#	54	0	54	0.80 (0.07)	0.78 (0.09)	0.89 (0.05)
Series *Dryandroideae*						
B. dryandroides **	66	0	66	0.74 (0.07)	0.70 (0.09)	0.83 (0.04)
Series *Abietinae*						
B. meisneri ***#	59	0	59	0.73 (0.06)	0.69 (0.08)	0.83 (0.05)
B. pulchella	66	1	67	0.71 (0.07)	0.67 (0.09)	0.80 (0.07)
Subgenus *Isostylis*						
B. illicifolia	60	1	61	0.71 (0.08)	0.67 (0.09)	0.80 (0.05)
B. oligantha *	48	0	48	0.73 (0.07)	0.69 (0.09)	0.85 (0.04)
B. cuneata ***#	78	0	78	0.80 (0.09)	0.78 (0.10)	0.90 (0.04)
Genus *Dryandra*						
D. formosa	55	4	59	0.64 (0.09)	0.59 (0.11)	0.75 (0.05)
D. polycephela	76	1	77	0.77 (0.09)	0.74 (0.11)	0.87 (0.04)
D. carlinoides	59	3	62	0.66 (0.08)	0.61 (0.10)	0.76 (0.05)

Conservation status of *Banksia* and *Dryandra* species (George 1987).

* species which are rare but not currently considered endangered or vunerable

** vunerable species not presently endangered but at risk in the longer term

*** endangered species which may disappear from the wild within one or two decades if present land use and other causal factors continue

\# Declared rare flora under the Western Australian Wildlife Conservation Act 1950-1979.

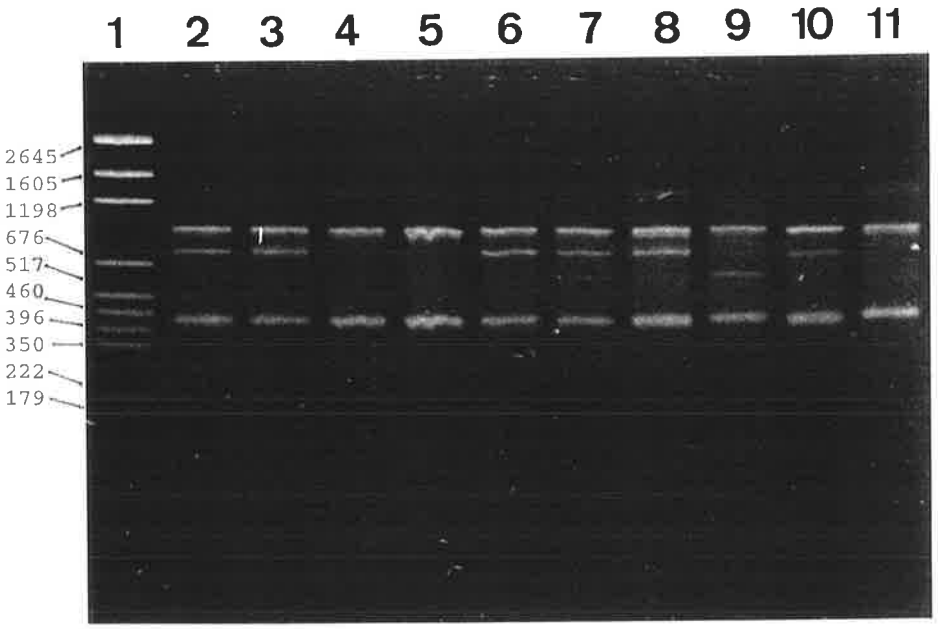
Table 8.2 Genetic diversity of three species of *Banksia* calculated from RAPD data using six different methods compared with published results from allozyme analysis.

Species	Allozymes	RAPD analysis					
		Nei and Li (1979)	Jaccard (1901)	Russell and Rao (1940)	Apostol *et al.* (1993)	Excoffier *et al.* (1992)	Rodgers and Tanimoto (1960)
B. attenuata	1.10 a	0.77	0.74	0.86	0.41	0.25	0.57
B. menziesii	1.04 a	0.65	0.59	0.77	0.35	0.29	0.51
B. cuneata	0.67-0.94 b	0.80	0.78	0.90	0.36	0.28	0.52

a. Scott (1980).

b. Coates and Sokolowski (1992).

Figure 8.1 Agarose gel showing RAPD markers produced using primer OPB-7 for 10 individuals of *B. ashbyi*. Lane 1 shows the DNA size standard pGEM (Promega), lanes 2 - 11 show the 10 individuals of *B. ashbyi*. Bands are scored as present (1) or absent (0), and a matrix of 1s and 0s are established for each individual, for all primers used in this study. The matrix is then used in subsequent genetic distance analysis.


1
2
3
4
5
6
7
8
9
10
11
2645
1605
1198
676
517
460
396
350
222
179

Discussion

This study shows that the 33 species of *Banksia* and the three species of *Dryandra* tested have high levels of genetic diversity. Comparable levels of within species diversity were detected by RAPDs and by enzyme electrophoresis. RAPDs produce large numbers of polymorphic markers, compared to the limited number available for allozyme techniques. In this study RAPD results were reproducible by using optimised standard conditions. Other workers have also shown that by using specific conditions, and taking care to avoid alteration of any of the conditions, reproducible results can be obtained (Virk *et al.* 1995).

The relationships between the six distance matrices tested have been discussed by Gower (1985). Some of the metrics are related by simple monotonic functions and the distances they produce are linearly or curvi-linearly related. Thus the metrics of Nei and Li (1979) and Jaccard (1901) form one group, and simple matching (Apostol *et al.* 1993), Excoffier *et al.*, (1992) and Rodgers and Tanimoto (1960) form another, with the metric Russell and Rao (1940) producing distances that are poorly related to those produced by any of the others. This was tested on three data sets using the different metrics, and there was agreement between the metrics of Nei and Li (1979) and Jaccard (1901), and between simple matching (Apostol *et al.*, 1993), Excoffier *et al.*, (1992) and Rodgers and Tanimoto (1960). The metric of Russell and Rao (1940) agreed with the first group, and with the previously published allozyme results (Scott 1980, Coates and Sokolowski 1992). These results are consistent with Gower's (1985) expectations, and suggest that the Nei and Li (1979), Jaccard (1901) or Russell and Rao (1940) methods are the most appropriate for RAPD data sets.

For the calculation of genetic diversity using RAPD data, it has been assumed that co-migrating fragments are allelic. Homology of co-migrating fragments has been previously demonstrated for different species of *Glycine* and *Allium* (Wilikie *et al.* 1993, Williams *et*

al. 1990b), and indirect evidence for allelism is derived from the conformity of taxonomic classifications based on RAPD data to those derived by morphology, cytology and enzyme electrophoresis which are widely accepted (Demeke *et al.* 1992, Howell *et al.* 1994). While the assumption of allelism is not unrealistic, inheritance of bands in appropriate crosses should be studied wherever possible.

Due to the large number of markers produced and the small amounts of tissue required, RAPDs have potential for use in estimating genetic diversity in populations of rare and endangered species. Estimates of genetic diversity and the distribution of this variation within or between populations of plants is important for devising effective conservation management strategies. RAPDs can also identify plant populations that should be monitored more closely for future conservation. Genetic diversity of all species tested in this study was high, indicating that none has reached dangerously low levels. More research is needed however, to target fragmented species across all remaining populations. In addition, RAPDs can identify individuals within populations, making them ideal for studies of parentage, cultivar identification and genetic mapping.

Chapter Nine

RAPD variation within and between populations of *Banksia cuneata* A.S. George (Proteaceae), a rare and endangered species.

Abstract

Banksia cuneata is a rare and endangered species with a restricted geographic distribution in south western Australia. Random amplified polymorphic DNA (RAPD) analysis was used to determine genetic diversity within and between all of the ten known populations. Estimates of genetic diversity ranged from 0.65 - 0.74, which is high considering *B. cuneata* is a rare and endangered species. Analysis of molecular variance (AMOVA) was used to partition RAPD variation within and between populations. Nearly all of the variation was attributable to individuals within populations, indicating a lack of population divergence. It is suggested that the combination of bird pollination and high outcrossing rates in *B. cuneata* maintain genetic diversity and cohesion between the populations.

Introduction

Banksia cuneata is a rare species known only in 10 populations in the central wheatbelt area of south western Australia, totalling about 550 plants, in an area of about 90 km ^{2}. It is found in deep yellow sands which occupy approximately 10 - 15 percent of this area, giving it a fragmented distribution. Associated with these soils is a rich and diverse flora dominated by species of the family Proteaceae, Myrtaceae and Leguminosae. In the last 50 - 60 years land clearing for agriculture and other disturbances have reduced the *B.*

cuneata population size to about 7% of its original distribution, and it now occurs as remnants of native vegetation.

Honeyeaters are reported to be the major pollinator of *B. cuneata* and bird populations are supported by co-existing vegetation. Many *Banksia* species are pollinated by birds and small mammals, and these pollinators move considerable distances and visit large numbers of plants (Ayre and Whelan 1989). As in the case of many Proteaceae, *B. cuneata* flowers are protandrous, and outcrossing is likely to be common (Ayre and Whelan 1989). The mating system is an important factor in determining patterns of genetic variation within and between populations (Brown 1989), and estimates of outcrossing in *Banksia* are typically high (Scott 1980, Carthew *et al.* 1988, Sampson *et al.* 1994). With high levels of outcrossing and bird pollination most genetic variation should be detected within populations, rather than between (Brown 1979, Hamrick and Godt 1989). Recently, the mating system and patterns of genetic variation for 6 populations of *B. cuneata* were determined using enzyme electrophoresis (Coates and Sokolowski 1992). Estimates of outcrossing based on 6 loci, ranged from 0.67 - 0.95, with low levels of selfing. The populations were divided into two groups, with gene flow within groups, but not between. It was suggested that an ecological barrier to the pollinators may be responsible for the restriction of gene flow between groups.

Banksia species along with many other members of the Proteaceae, show low levels of allozyme variability (Whelan 1994). More recently, random amplified polymorphic DNA (RAPD) analysis based on PCR technology (Williams *et al.* 1990) has been used to address variability within and between populations of a range of genera (Huff *et al.* 1993, Chalmers *et al.* 1992, Russell *et al.* 1993). By using single random primers this technique can amplify regions within the genome, without any prior sequence information, using small amounts of tissue. It is also reasonably simple and can provide a very large number of potential polymorphic loci. This is ideal in the conservation context of rare and endangered plants, since there is often little material and low variability

~~detected using allozymes.~~ The aim of this study is to determine patterns of genetic variation within and between all known populations of *B. cuneata* using RAPDs. Four populations are included in this study which have not been investigated previously. This information is important for devising appropriate conservation strategies for the remaining *B. cuneata* populations.

Materials and methods

Population sampling

Seed material for RAPD analysis was collected from 6 populations by the Department of Conservation and Land Management W.A. Additional seed was collected during a field trip to Western Australia (Fig 9.1). Twenty seeds were randomly selected, one per plant, from four populations with in excess of 50 plants, and ten seeds were collected, one per plant, from 3 populations with less than 50 plants. In three cases, the populations comprised less than 10 plants, so samples of one seed were taken from all remaining plants. In total 125 plants, approximately 25%, of the total number of plants remaining were sampled.

DNA isolation

DNA was extracted from each seed using a slight modification of the method of Weining and Langridge (1991), comprising: phenol/chloroform/isopropanol extraction for 5 min on ice, DNA precipitation for 1 min with ice cold isopropanol and sodium acetate, with DNA recovered by centrifugation at 12 000 rpm for 10 min. The pellet was washed twice with 70 % ethanol, dried and dissolved in 50 µL of TE buffer, with 1.0 µL of RNAase (R40: 40 g/mL RNAase A in TE), and stored at 4 ^{0}C short term (up to 1 month) or -20 ^{0}C long term (up to 1 year).

DNA was subjected to gel electrophoresis on a 1.6% agarose gel in TBE buffer (Sambrook *et al.* 1989), and stained with ethidium bromide. DNA concentration was estimated by visual assessment of band intensities, compared to salmon sperm genomic DNA standards. The DNA content was adjusted to 10 ng μL^{-1}.

DNA amplification and documentation

DNA amplification was performed in a MJ Research Thermal Cycler. The optimal program for *Banksia* commenced with an initial denaturation step at 94 ^{0}C for 5 min, followed by 40 cycles of 94 ^{0}C for 1 min, 36 ^{0}C for 1 min, 72 ^{0}C for 2 mins, terminated with a final extension step at 72 ^{0}C for 5 min. Optimised reaction conditions were carried out in a 25 µL total volume containing 1X *Taq* buffer (Gibco-BRL), 3 mM $MgCl_2$, 200 µM of each dNTP (dGTP, dATP, dCTP, dTAP), 1 unit of *Taq* polymerase (Gibco-BRL), 0.5 µL T4 gene 32 protein (Boehringher Mannheim), 1 µM 10 mer primer (Operon Technologies Inc.) and 10 ng μL^{-1} template DNA. Each reaction mix was overlaid with PCR grade paraffin oil. DNA amplification fragments were separated by 2% agarose gel (Seakem, Promega) electrophoresis using TBE buffer (Sambrook *et al.* 1989). A DNA size marker was used (pGEM, Promega) on each gel, and gels were stained with ethidium bromide. Fragment patterns were photographed under UV light with Polaroid 667 film for further analysis. Polaroid photographs were scanned using a transmission scanner (Hewlett Packard Scanjet IIcx/T). The intensity and molecular weight of each band was determined using the software CREAM TM., Kem-En-Tec Software Systems, Blue Sky Scientific.

Sixty primers were evaluated for their suitability in a pilot survey (series OPA, OPB and OPC, Operon Technologies Inc.). Five primers were selected (OPA-1, OPA-4, OPA-9, OPA-11, OPA - 16) that gave reproducible and informative markers. Band fragments included in the final analysis ranged between 2.5 kb and 100 bp in length (Fig 9.2) and were scored as present (1) or absent (0). A negative control was added in each run to test

for contamination. In order to test reproducibility, the selected primers were tested three times on the same sample, for a random subset of three DNA samples. To aid interpretation of band homology between gels, each gel contained a standard DNA sample and pGEM DNA marker. The presence or absence of bands was determined for all individuals and a matrix of RAPD phenotypes was assembled. Each individual was represented by a vector of 1s and 0s for the presence or absence of any particular band for all the primers used in the study.

Statistical analysis

The vector of presence/absence states for each individual was used to compute a measure of genetic distance for all pairs of individuals using the method of Nei and Li (1979). It was determined previously that the metric of Nei and Li was one of the most suitable for RAPD data on *Banksia* species, compared to known diversity estimates based on allozyme data (chapter eight). The index of genetic distance was calculated as D = 1-F. Where F = similarity (Nei and Li 1979), calculated by the equation 2*n11/((2*n11)+n01+n10), where n = number of band positions, n11 = the number of positions where x = 1, y = 1, n00 = the number of positions where x = 0, y = 0, n01 = the number of positions where x = 0 and y = 1, n10 = the number of positions where x = 1 and y = 0, x = individual number x, and y = individual number y.

Distances were calculated using the statistical package, RAPDistance (Amstrong *et al.* 1994). Following analysis of distance within each population, a mean estimate of genetic diversity and standard deviation was derived for each population.

Analysis of molecular variance (AMOVA) (Excoffier *et al.* 1992) was used to estimate variance components attributable to differences within and between populations. Significance levels for variance component estimates were calculated by permutational procedures. The number of permutations for significance testing was set at 100 for all

analyses. The analyses were undertaken with the AMOVA program for Windows (WINAMOVA) provided by L. Excoffier. An unweighted pair group mean average (UPGMA) clustering analysis was carried out using a distance matrix based on the Phi statistic (PhiST) produced by the WINAMOVA analysis for between population distances.

Results

The RAPD profile

After excluding bands that were greater than 2.5 kb or less than 100 bp for the whole data set, the five primers yielded a total of 169 polymorphic bands (Table 9.1). The number of markers per primer ranged from 30 (OPA - 4) to 37 (OPA - 9, OPA - 16). Of the 169 RAPD markers scored, 33 (19.5%) were present in all populations and there were no fixed differences between populations, with the remaining 136 (80.5%) bands variable between populations. Most bands occurred at a frequency of 0 - 20%, with relatively few bands in the 80 - 100% category (Table 9.2). The total number of bands present in each population was similar, with the exception of population 10, which had nearly half the number of bands (Table 9.2). Thus, for populations with between 6 and 20 individuals there was a relatively high number of bands, but population 10 with only 3 individuals, had 66 bands.

Estimate of genetic diversity

The estimate of genetic diversity (calculated as D = 1 - F) for each population of *B. cuneata*, and the whole species is shown in Table 9.3. The total genetic diversity for *B. cuneata* was 0.70, and the diversity within each population ranged from 0.65 (population 10) to 0.74 (population 2). The highest genetic diversity estimates occurred in the larger populations (population 2, 3, 4, 9), with less diversity in smaller populations (1, 5, 6, 7,

8, 10). However, genetic diversity within a population did not always depend on population size; population 6 had 6 individuals with a diversity of 0.70, which was higher than populations 1, 5, and 7 which had 10 individuals.

Results of the AMOVA analysis are shown in Table 9.4, which attributes nearly all of the variation to individuals within populations. Analysis between populations shows a small negative variance component, indicating lack of population structure at this level, so some plants between populations are more related than within populations (Excoffier *et al.* 1992). Therefore, all variation is attributable to individuals within populations and between populations (ie. among all individuals as a group), for which the corrected percentage of variation due to individuals is about 100% (Table 9.4). These results suggest that there is no distinction between populations of *B. cuneata.* This is reflected by the absence of fixed differences between populations in RAPD markers. To show relationships between the populations (Figure 9.3), UPGMA clustering was applied to the distance matrix calculated by the AMOVA analysis, based on the Phi statistic (PhiST) metric (Excoffier *et al.* 1992).

From the ~~dendogram~~ dendrogram some interesting relationships arise; geographically very close populations such as 6, 7, and 3 are in one cluster, closely related to populations 2, 4 and 10. Another cluster contains populations 5, 8 and 9, with population 1 linking the two clusters. Interestingly, population 9 and 10 are the most southerly populations, with one population in each cluster group. This clustering, with low levels of genetic diversity between populations, reflects the lack of genetic differentiation between all ten populations.

Table 9.1 Summary of data obtained by RAPD analysis for 5 primers with 125 individuals of *B. cuneata*. The table shows the sequence, total band number, number of polymorphic and monomorphic bands, and the average number of bands/primer.

Primer	Primer sequence 5' to 3'	Number of bands Total	Polymorphic	Monomorphic
OPA - 1	CAGGCCCTTC	33	33	0
OPA - 4	AATCGGGCTG	30	30	0
OPA - 9	GGGTAACGCC	37	37	0
OPA - 11	CAATCGCCGT	32	32	0
OPA - 16	AGCCAGCGAA	37	37	0
	Total	169	169	0
	Mean per primer	33.8	33.8	0

Table 9.2 Summary of band frequencies for each population of *B. cuneata*. The table shows the number of bands out of 169 (total data set), for each population that fall into frequency groups of: 0 - 20%, 20 - 40%, 40 - 60%, 60-80%, and 80 - 100%.

Population	Number of plants sampled	Number of bands	Band frequency 0-20%	20-40%	40-60%	60-80%	80- 100%
1	10	131	91	28	23	15	12
2	20	157	93	37	19	13	7
3	20	161	78	43	24	18	6
4	20	145	83	47	20	11	8
5	10	128	90	28	24	16	11
6	6	115	94	29	11	18	17
7	10	135	96	24	23	12	14
8	6	118	90	20	23	14	22
9	20	145	91	32	23	18	5
10	3	66	103	34	0	15	17
Mean		130.1	90.9	32.2	19.0	15.0	11.9

Table 9.3 Genetic diversity of each population of *B. cuneata* calculated using the similarity (F) metric of Nei and Li (1979), where distance (D) = 1 - F. The values shown are the mean distance (diversity) values for each population, and the standard deviation of the mean.

Population	Number of plants sampled	Mean distance (diversity)	Standard deviation
1	10	0.68	0.085
2	20	0.74	0.096
3	20	0.73	0.067
4	20	0.73	0.053
5	10	0.68	0.073
6	6	0.70	0.065
7	10	0.67	0.070
8	6	0.67	0.032
9	20	0.71	0.080
10	3	0.65	0.017
Total	125	0.70	0.031

Table 9.4 Analysis of molecular variance (AMOVA) for 125 individuals of *B. cuneata* using 169 RAPD bands. The 125 samples are divided into 10 populations. The data show the degrees of freedom (df), sum of squared deviation (SSD), mean squared deviation (MSD), variance component estimate, percentage of total variance contributed by each component and the probability of obtaining a more extreme component estimate by chance alone.

Source of variation	df	SSD	MSD	Variance component	% total	*P* - value
Analysis among populations						
Between populations	9	1.446	0.161	-0.0160	-4.73 %	0.57
Within populations	115	40.821	0.355	0.3549	104.73 %	< 0.01

Figure 9.1 Geographic range of *Banksia cuneata* in south-west Australia, showing locations of the ten populations from which seed was collected.

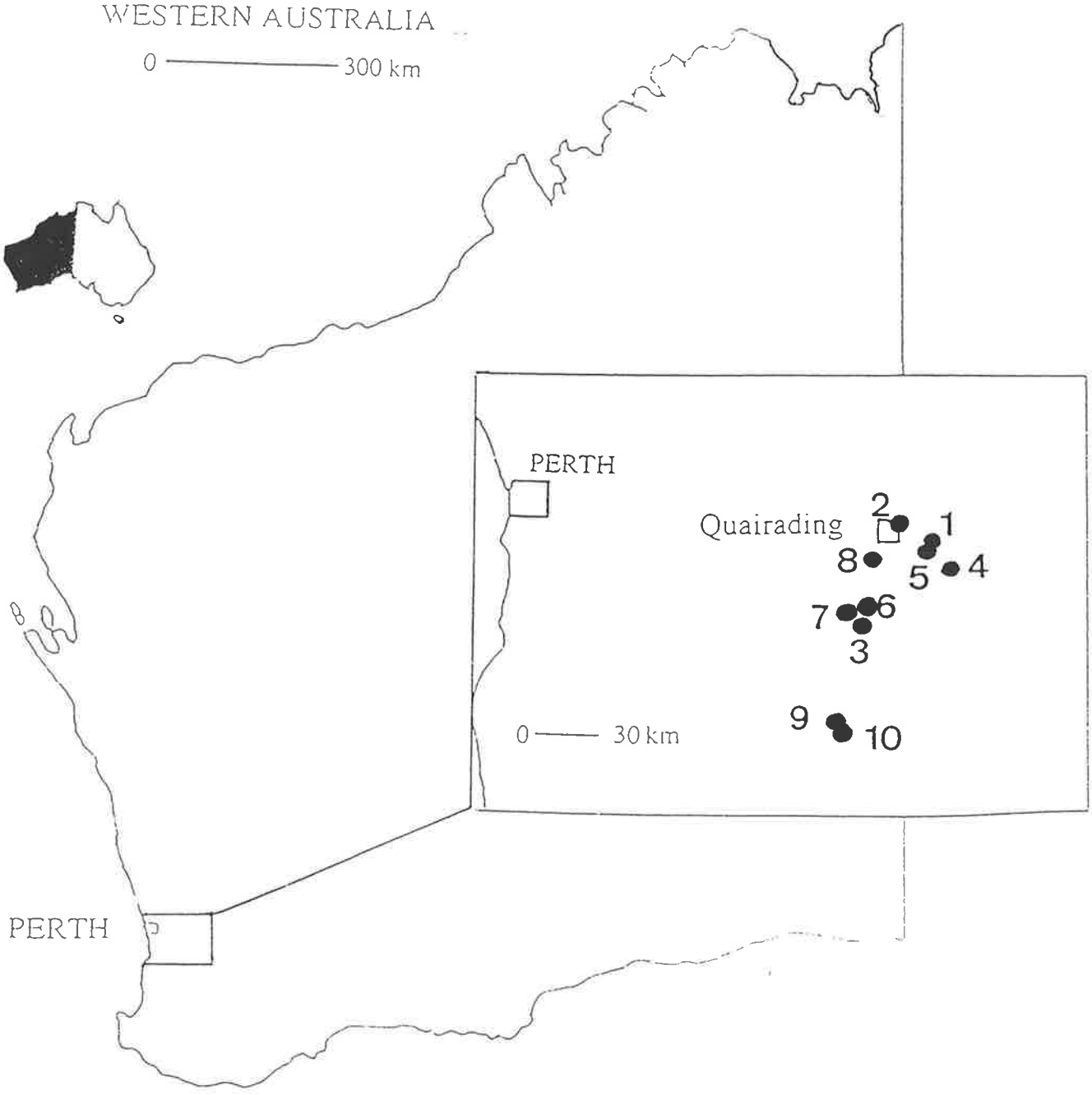

WESTERN AUSTRALIA
0 300 km
PERTH
Quairading
2
1
8
5
4
7
6
3
9
10
0 30 km
PERTH

Figure 9.2 Agarose gel showing RAPD markers produced using primer OPA - 9 for individuals of *B. cuneata*. Lane 1 shows the DNA size standard pGEM (Promega), lane 2 shows the standard individual to aid in band homology for between gel comparisons, lanes 3 - 6 show individuals in population 7, lanes 7 - 16 show individuals in population 3 and lane 17 is the negative control. Bands within the molecular size range 2.5 kb to 100 bp are scored as present (1) or absent (0), and a matrix of 1 s and 0 s are established for each individual, for all primers used in this study. The matrix is then used in subsequent genetic distance and AMOVA analyses.

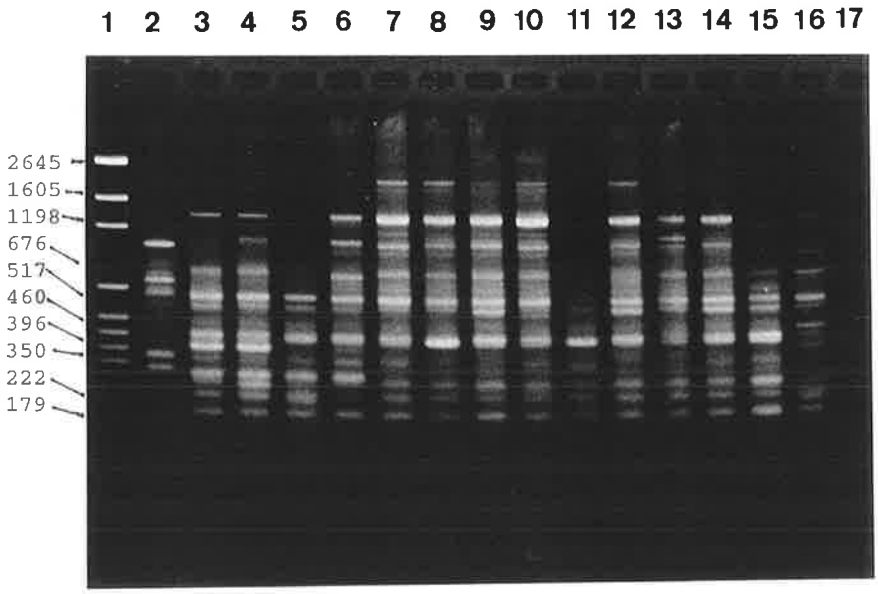
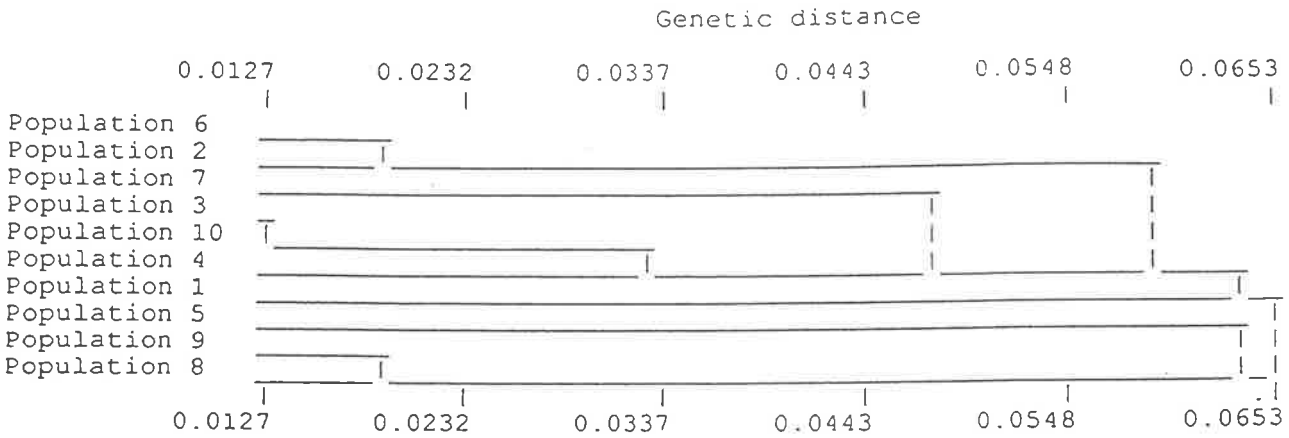

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
2645
1605
1198
676
517
460
396
350
222
179

Figure 9.3 UPGMA cluster analysis of *B. cuneata* populations based on genetic distance (PhiST) among populations calculated by AMOVA analysis.


Genetic distance
0.0127
0.0232
0.0337
0.0443
0.0548
0.0653
Population 6
Population 2
Population 7
Population 3
Population 10
Population 4
Population 1
Population 5
Population 9
Population 8
0.0127
0.0232
0.0337
0.0443
0.0548
0.0653

Discussion

Banksia species are considered to be bird and mammal pollinated, and have high levels of diversity.with outcrossing rates amongst the highest recorded for plants (Scott 1980, Carthew *et al.* 1988, Sampson *et al.* 1994). Genetic diversity in *B. cuneata* as a whole estimated by this study is quite high, considering it is a geographically restricted rare and endangered species.

The 10 known populations of *B. cuneata* show differences in the extent of disturbance, and two populations (9 and 10) have a southerly distribution. Populations 1, 6, 7, and 10 occur in highly disturbed roadside vegetation, populations 3, 4, 8 and 9 show low levels of disturbance, while populations 2 and 5 occur in virtually undisturbed vegetation. There were differences in genetic diversity calculated for each population, population 10 a highly disturbed small population of only 3 individuals, had the lowest genetic diversity of 0.65, while some of the other smaller populations had estimates ranging from 0.67 - 0.70. The larger populations with low levels of disturbance, had the highest diversity estimates ranging from 0.71 - 0.74. Variation in genetic diversity estimates between populations is not uncommon in species with mixed mating systems (Schoen 1982). Pollinator availability and activity are likely to have an influence on genetic diversity between populations. This particularly applies to bird pollinated species such as *B. cuneata*. Population structure such as plant density, and level of disturbance may also influence pollinator behaviour. This is reflected by larger populations with lower levels of disturbance, having high levels of genetic diversity. In contrast, small populations on road verges with high levels of disturbance, display lower levels of genetic diversity. The estimates of genetic diversity calculated in this study are in agreement with enzyme electrophoresis (Coates and Sokolowski 1992) methods which estimated outcrossing rates for *B. cuneata,* ranging from 0.67 - 0.95, with low levels of selfing.

Dissected population structure and geographic restriction of *B. cuneata* would be likely to influence the patterns of genetic variation within and between populations (Hamrick and Godt 1989). As *B. cuneata* is outcrossing and bird pollinated, most variation would be expected to be within populations and little between populations. The results of the AMOVA clearly demonstrate this, with nearly all variation attributable to individuals within populations, with no significant genetic differentiation between populations.

AMOVA is not strictly rigorous with non Euclidean metrics, but as Excoffier *et al.* (1992) point out the metric of Nei and Li (1979) differs only in the choice of denominator and the metric is nearly interchangeable with the Euclidean metric. The AMOVA method, designed for other molecular data, has recently been used to analyse RAPD data for buffalograss (Huff *et al.* 1993), *Eucalyptus* (Nesbitt *et al.* 1995) and *Grevillea* (Rossetto *et al.* 1995)

The lack of population differentiation in *B. cuneata* using RAPDs contrasts to a previous study by Coates and Sokolowski (1992), using six of the ten populations of *B. cuneata*, and enzyme electrophoresis. Their study using 6 polymorphic loci, found significant differentiation of populations into two groups. It was further suggested that differentiation may be due to a salt river system acting as an ecological barrier to pollinator movement. The westerly populations in particular, were more heterogenous and it was found that some populations which were geographically closer, were more related to other populations. Their study also found that in the eastern populations, gene flow was relatively high, suggesting that birds as pollinators were effective in maintaining genetic cohesion and diversity between the populations.

The results of the present study using RAPDs on all 10 known populations show no significant population differentiation, with all variation between individuals. This suggests that outcrossing and bird pollination are very effective in maintaining genetic diversity and cohesion between populations. Birds are able to travel considerable

distances between populations, and associated with areas of *B cuneata* is a rich and diverse vegetation system which can maintain bird populations. Since clearing for agriculture, which was about 50 - 60 years ago, there has been no significant differentiation between the populations, although population sizes have reduced. This could be for a number of reasons. It is possible that after clearing the populations are still visited by pollinators due to other surrounding vegetation, which support the bird population. It could also be because the populations are declining and environmental conditions are generally unfavourable to seedling recruitment, that the populations are aging and there has been few generations after clearing to see any observable amount of genetic differentiation between populations. Furthermore, it is possible that prior to clearing there were no significant ecological barriers to pollinator movement and the ecological barrier suggested by Coates and Sokolowski (1992) is relatively recent, therefore no significant divergence of the populations has occurred.

It is important for future conservation and persistence of *B. cuneata* that the remaining populations are protected. Even though they have reasonably high levels of diversity, this study shows in very small populations, such as population 10 with only 3 individuals, that genetic diversity may decline. Pollinator maintenance is essential for maintaining genetic diversity, and reserves which have other coexisting vegetation to support pollinators must also be protected. The distribution of these reserves to allow gene flow between populations, is important for the survival of *B. cuneata*, as well as maintaining genetic diversity in other plant species.

Traditionally, studies of population genetic structure have used allozyme markers (Hamrick and Godt 1989). Allozymes can provide informative markers for levels of genetic variability within a plant species. It has been shown that genetic diversity is generally greater at the population and species level for outcrossers than selfers. In addition, widespread species exhibit more variation than restricted species (Hamrick and Godt 1989). There are however, widely recognised limitations of allozymes. Many

species of the family Proteaceae have low levels of detected allozyme diversity. This is because the detection of variability is limited to protein coding loci, which may underestimate levels of genetic diversity (Clegg 1989), and may not represent the entire genome (Schaal *et al.* 1991). Allozymes are also tissue specific and are influenced by environmental factors. Given these limitations many workers have moved to using DNA markers such as RAPDs. They overcome the limitations of allozymes and are simpler and easier to use than other DNA markers such as restriction fragment length polymorphisms (RFLPs). RAPDs have potentially unlimited numbers of markers and have been shown to be useful for a variety of applications including population genetic studies on a number of genera (Huff *et al.* 1993, Chalmers *et al.* 1992, Russell *et al.* 1993).

Direct comparisons of RAPD and allozyme diversity are few. Peakall *et al.*, (1995) compared RAPD markers to allozyme markers in buffalograss. Using AMOVA analysis, they found that both markers gave similar qualitative patterns, large regional differences and significant population differentiation within regions for four populations of buffallograss. RAPDs however, detected more variation than allozymes. Dawson *et al.*, (1993) found patterns of RAPD variation in *Hordeum spontaneum* comparable to previously reported patterns using allozymes. Liu and Furnier (1993) using two species of *Populus* compared RAPD, RFLP and allozyme markers. They found that RFLP and allozyme markers gave comparable patterns within and between species, while RAPDs revealed more variation between individuals within a species than allozyme and RFLP markers, and produced reliable discrimination among clones. Our findings with *B. cuneata* are consistent with reports that RAPDs detect more variation than allozymes (Peakall *et al.*, 1995, Liu and Furnier 1993, Russell *et al.*, 1993, Dawson et al., 1993). Furthermore, our study using RAPDs shows nearly all variation is within populations of *B. cuneata* rather than between populations. Russell *et al.*, (1993) also reported RAPD diversity in *Theobroma cacao* was higher within provinces than between provinces, a pattern typical for outcrossing woody plants (Hamrick and Godt 1989). Although allozymes are valuable for many types of studies, it is clear that DNA markers such as

RAPDs will be widely used on plant population studies in the future, and will remain a valuable genetic tool.

In conclusion, *Banksia cuneata* has relatively high levels of genetic diversity detected using RAPDs, with no significant differentiation between populations over a restricted geographic range. Gene flow is high between populations, apparently due to pollinator movement and a predominantly outbreeding mating system. Future conservation of the species will depend on adequate protection of the remaining populations, collection of seed material from populations into a gene bank, and maintenance of bird populations in surrounding and co-existing vegetation areas.

Chapter Ten

Use of RAPD markers to analyse phylogenetic relationships in *Banksia* (Proteaceae)

Abstract

Random amplified polymorphic DNA (RAPD) analysis was applied to 33 species of *Banksia*, three species of *Dryandra* and *Musgravea heterophylla* in order to investigate phylogenetic relationships within genus *Banksia*, and between subgenera and genera. Fifteen primers produced 791 bands for the 37 taxa, and results were analysed using group average (UPGMA) clustering and parsimony (PAUP). The resulting analyses were in broad agreement with each other, with closely related species pairs and species groups clustering together, as in the accepted classification of *Banksia*. RAPDs were not informative between distantly related species or species groups.

Introduction

Early treatments of *Banksia* were either catalogues of species (Brown 1810), or artificial classifications (Meissner 1856). George (1981) attempted a more natural classification, using a variety of leaf, flower, follicle and seed characters to base infrageneric taxa. While George (1981) describes "possible lines of evolution" within and between taxa, his classification is difficult to interpret phylogenetically because of a number of taxa with uncertain relationships. Doust (1983) attempted a cladistic analysis of *Banksia* using 37 characters for 86 taxa, but the trees were not clearly resolved. The analysis provided a poor match with the existing taxonomy and failed to suggest a robust phylogenetic scheme. It was not used for a formal classification. More recently Thiele (1993) proposed a classification based on cladistic analysis of morphological characters. Although there is

some agreement with the currently accepted classification of George (1981, 1988), there are still uncertainties in the positions of some species such as *B. coccinea*, and support for some nodes is tenuous.

Banksia comprises two subgenera, *Isostylis* and *Banksia* (George 1981). The former group is small and the species are superficially similar to *Dryandra*. This led George (1981) to propose that *Isostylis* may be closer to *Dryandra* than to subgenus *Banksia*. Nevertheless, he placed it as a subgenus of *Banksia*, with the comment that a separate genus may be appropriate. This interpretation was based on three morphological characters. There is still speculation on the relationships within *Banksia*, between subgenus *Isostylis*, subgenus *Banksia* and genus *Dryandra*. Other data such as DNA markers may help to resolve these relationships.

The development of random amplified polymorphic DNA (RAPD) markers generated by the polymerase chain reaction (PCR) using arbitrary primers has resulted in molecular markers for the detection of nuclear DNA polymorphisms (Williams *et al.*, 1990). The technical simplicity of the RAPD technique has facilitated its use in the analysis of phylogenetic relationships in several genera (Wilkie *et al.*, 1993, Demeke *et al.*, 1992, Abo-elwafa *et al.*, 1995).

In this study RAPDs were evaluated as informative markers at different taxonomic levels within *Banksia*. In addition, this approach was used to investigate higher order relationships between related subgenera and genera.

Materials and methods

Plant material

Seeds of 33 species of *Banksia* and three of *Dryandra*, collected from natural populations, were obtained from Nindethana Seed Service. Fresh leaf material of *Musgravea heterophylla* was collected from Atherton, Queensland (QRS Arboretum No. 1372). The species chosen represented three genera *Musgravea*, *Banksia* and *Dryandra*, and two subgenera, two sections and thirteen series within genus *Banksia* (George 1981, 1988) (Table 10.2). Ten seeds of each species of *Banksia* and *Dryandra* were randomly selected for RAPD analysis, and the DNA bulked to give one sample per species. One leaf was selected for *Musgravea heterophylla*.

DNA extraction

DNA was extracted from each seed using a modified method of Weining and Langridge (1991), comprising: phenol/chloroform/isopropanol extraction for 5 min on ice, DNA precipitation for 1 min with ice cold isopropanol and sodium acetate, with DNA recovered by centrifugation at 12 000 rpm for 10 min. The pellet was washed twice with 70 % ethanol, dried and dissolved in 50 µL of TE buffer, with 1.0 µL of RNAase (R40: 40 g/mL RNAase A in TE), and stored at 4 ^{0}C short term (up to 1 month) or -20 ^{0}C long term (up to 1 year). DNA was extracted from fresh leaf material of *Musgravea heterophylla* using the extraction method for seedling material of Maguire *et al.* (1994).

DNA was subjected to gel electrophoresis on a 1.6% agarose gel in TBE buffer (Sambrook *et al.* 1989), and stained with ethidium bromide. DNA concentration was estimated by visual assessment of band intensities, compared to salmon sperm genomic DNA standards. The DNA content was adjusted to 10 ng µL $^{-1}$.

DNA amplification and documentation

DNA amplification was performed in a MJ Research Thermal Cycler. The optimal program for *Banksia* commenced with an initial denaturation step at 94 ^{0}C for 5 min, followed by 40 cycles of 94 ^{0}C for 1 min, 36 ^{0}C for 1 min, 72 ^{0}C for 2 mins, terminated with a final extension step at 72 ^{0}C for 5 min. Optimised reaction conditions were carried out in a 25 µL total volume containing 1X *Taq* buffer (Gibco-BRL), 3 mM $MgCl_2$, 200 µM of each dNTP (dGTP, dATP, dCTP, dTAP), 1 unit of *Taq* polymerase (Gibco-BRL), 0.5 µL T4 gene 32 protein (Boehringher Mannheim), 1 µM 10 mer primer (Operon Technologies Inc.) and 10 ng µL $^{-1}$ template DNA. Each reaction mix was overlaid with PCR grade paraffin oil. DNA amplification fragments were separated by 2% agarose gel (Seakem, Promega) electrophoresis using TBE buffer (Sambrook *et al.* 1989). A DNA size marker was used (pGEM, Promega) on each gel, and gels were stained with ethidium bromide. Fragment patterns were photographed under UV light with Polaroid 667 film for further analysis. Polaroid photographs were scanned using a transmission scanner (Hewlett Packard Scanjet IIcx/T). The intensity and molecular weight of each visible band was determined using the software CREAM TM., Kem-En-Tec Software Systems, Blue Sky Scientific.

Sixty primers were evaluated for their suitability in a pilot survey (series OPA, OPB and OPC, Operon Technologies Inc.). Fifteen were selected (Table 10.1) which gave reproducible and informative markers. Band fragments were scored as present (1) or absent (0) (Fig 10.1). A negative control was added in each run to test for contamination. In order to test reproducibility, the selected primers were tested three times on the same sample, for a random subset of three DNA samples. To aid interpretation of band homology between gels, each gel section contained a DNA marker (pGEM). The presence or absence of bands was determined for all individuals and a matrix of RAPD phenotypes was assembled. Each individual was represented by a vector of 1s and 0s for the presence or absence of any particular band for all the primers used in the study.

Data analysis

Two alternative methods were used to analyse the RAPD data. A genetic distance matrix (Nei and Li 1979), with group average (UPGMA) clustering was used to produce a ~~dendogram~~ dendrogram. The second method applied phylogenetic analysis using the parsimony program PAUP (Swofford 1990).

Genetic distance analysis

From the matrix of RAPD phenotypes for each species, an index of genetic distance was calculated (D = 1 - F), where F is similarity calculated using Nei and Li (1979) matching coefficient method [2*n11/((2*n11)+n01+n10)]. Where n=number of band positions, n11= the number of positions where x=1, y=1, n00= the number of positions where x=0, y=0, n01= the number of positions where x=0 and y=1, n10= the number of positions where x=1 and y=0, and x,y = individuals being compared. The distance matrix was calculated using the statistical package, GENSTAT 5 (Payne 1987), and UPGMA clustering was performed by the PATN program package (Belbin 1991).

Phylogenetic analysis using parsimony (PAUP)

RAPD data obtained from the 37 taxa, with *Musgravea* as the outgroup, were used to construct phylogenetic trees. Analysis was performed using PAUP 3.1.1 (Swofford 1990). The phylogeny was assessed using the heuristic search method of PAUP (character optimisation ACCTRAN, MULPARS and TBR branch swapping options). To ensure that all islands of most parsimonious trees were found (Maddison 1991), the search was repeated 100 times with RANDOM addition and with a maximum of 100 trees saved at each replication. The resulting most parsimonious trees produced by this process were subjected to successive weighting to minimise homoplasy. The matrix was

reweighted until the tree-length values stabilised. Equally short solutions were used to produce a consensus tree.

Results

A total of 791 bands ~~were~~ was scored, with an average of 52.7 bands per primer (Table 10.1). The banding pattern for 34 species in the study using primer OPB-11 is shown (Fig 10.1). The number of polymorphic markers was high, with few monomorphic bands, and the number of bands unique to only one species comprised 18.5 % of the total. All species generated similar numbers of bands per primer (Table 10.2), except for *B. cuneata, B. oligantha* and *M. heterophylla* which produced more than average.

In cluster analysis of genetic distance values, *Musgravea* formed a small group with *Banksia*, subgenus *Isostylis,* and the three species of *Dryandra* formed a small group with *Banksia coccinea.* For the remainder of genus *Banksia,* species pairs and closely related species generally formed groups (Fig 10.2). The cluster involving *Musgravea* and subgenus *Isostylis* is interesting, as this group is closer to many *Banksia* species, than they are to each other. UPGMA clustering forms groups with increasing genetic distance. The ~~dendogram~~ dendrogram shows approximately 7 - 8 main groups, linked together at genetic distance values ranging from 0.70 - 0.78. The higher levels of clustering between these main groups is tenuous, however clustering within these groups is likely to be real. Within the main clusters, species pairs and closely related species group together, such as *B. occidentalis* and *B. meisneri, D. polycephala, D. carlinoides* and *D. formosa, B. ericifolia* and *B. dryandroides, B. caleyi* and *B. lemanniana, B. grandis* and *B. solandri, B. audax* and *B. lindleyana, B. oligantha* and *B. cuneata,* and *B. ashbyi* and *B. elderiana.*

The PAUP program identified two minimum length trees (length = 4079) with a consistency index of 0.929. Both the strict consensus tree and the majority rule tree show

that species within series *Grandes* and *Tetragonae* grouped together, and closely related species between series grouped together (Fig 10.3; 10.4). There were close relationships between species in series *Banksia* and *Crocinae*, series *Dryandroideae*, *Spicigerae* and *Abietinae*, and series *Bauerinae* and *Cyrtostylis*. *Dryandra* was polyphyletic within *Banksia*. *D. polycephala* and *D. carlinoides*, as sister taxa, were part of a clade with *Banksia* species, whereas *D. formosa* was sister to *B. coccinea*.

Table 10.1 Primer sequence and band data for each of the 15 selected primers over the complete data set of 37 taxa producing 791 bands.

Primer	Sequence 5'-3'	Total bands	Polymorphic	Monomorphic	Unique
OPA-1	CAGGCCCTTC	49	49	0	7
OPA-3	AGTCAGCCAC	56	56	0	7
OPA-5	AGGGGTCTTG	55	55	0	9
OPA-7	GAAACGGGTG	53	53	0	6
OPA-13	CAGCACCCAC	53	53	0	9
OPA-20	GTTGCGATCC	51	51	0	15
OPB-1	GTTTCGCTCC	52	52	0	14
OPB-3	CATCCCCCTG	54	54	0	13
OPB-4	GGACTGGAGT	50	50	0	9
OPB-6	TGCTCTGCCC	45	45	0	9
OPB-7	GGTGACGCAG	50	50	0	12
OPB-10	CTGCTGGGAC	60	60	0	8
OPB-11	GTAGACCCGT	54	54	0	11
OPB-17	AGGGAACGAG	49	49	0	11
OPC-2	GTGAGGCGTC	60	60	0	7
	Total bands	791	791	0	147
	Mean per primer	52.7	52.7	0.0	9.8

Table 10.2 Band data for 33 species of *Banksia*, three of *Dryandra* and *Musgravea heterophylla* using RAPDs.

Species	Total bands	Mean per primer
Genus *Banksia*		
Subgenus *Banksia*		
Section *Banksia*		
Series *Salicinae*		
B. integrifolia	138	9.2
B. robur		
Series *Grandes*		
B. grandis	133	8.9
B. solandri	173	11.5
Series *Quercinae*		
B. quercifolia	145	9.7
Series *Bauerinae*		
B. baueri	119	7.9
Series *Banksia*		
B. baxteri	142	9.5
B. candolleana	154	10.3
B. menziesii	135	9.0
B. serrata	103	6.9
Series *Crocinae*		
B. burdettii	128	8.5
B. prionotes	163	10.9
Series *Cyrtostylis*		
B. ashbyi	131	8.7
B. attenuata	134	8.9
B. audax	128	8.5
B. elderiana	135	9.0
B. laevigata	141	9.4
B. lindleyana	150	10.0
B. praemorsa	125	8.3
Series *Prostratae*		
B. blechnifolia	135	9.0
B. repens	118	7.9
Series *Tetragonae*		
B. caleyi	113	7.5
B. lemanniana	119	7.9
Series *Coccineae*		
B. coccinea	137	9.1
Section *Oncostylis*		
Series *Spicigerae*		
B. ericifolia	122	8.1
B. occidentalis	108	7.2
B. tricuspis	120	8.0
Series *Dryandroideae*		
B. dryandroides	147	9.8
Series *Abietinae*		
B. meisneri	139	9.3
B. pulchella	164	10.9
Subgenus *Isostylis*		
B. illicifolia	133	8.9
B. oligantha	223	14.9
B. cuneata	237	15.8
Genus *Dryandra*		
D. formosa	158	10.5
D. polycephela	154	10.3
D. carlinoides	154	10.3
Genus *Musgravea*		
M. heterophylla	240	16.0

Figure 10.1 Agarose gel showing RAPD markers produced using primer OPB - 11 for bulked DNA samples of 34 species. Lane 1 shows the DNA size standard pGEM (Promega), lane 2 *B. ashbyi*, lane 3 *B. elderiana*, lane 4 *B. menziesii*, lane 5 *B. blechnifolia*, lane 6 *B. integrifolia*, lane 7 *B. quercifolia*, lane 8 *B. laevigata*, lane 9 *B. attenuata*, lane 10 *B. lindleyana*, lane 11 *B. repens*, lane 12 *B. illicifolia*, lane 13 *B. baueri*, lane 14 *B. audax*, lane 15 *B. meisneri*, lane 16 *B. praemorsa*, lane 17 *B. occidentals*, lane 18 *B. robur*, lane 19 DNA size standard pGEM (Promega), lane 20 *B. burdettii*, lane 21 *B. ericifolia*, lane 22 *B. serrata*, lane 23 *B. dryandroides*, lane 24 *B. candolleana*, lane 25 *B. prionotes*, lane 26 *B. baxteri*, lane 27 *B. lemanniana*, lane 28 *B. caleyi*, lane 29 *B. tricuspis*, lane 30 *B. grandis*, lane 31 *B. solandri*, lane 32 *B. pulchella*, lane 33 *B. coccinea*, lane 34 *D. formosa*, lane 35 *D. carlinoides*, lane 36 *D. polycephala*. Bands are scored as present (1) or absent (0), and a matrix of 1 s and 0 s are established for each species, for 15 primers. The matrix is then used in subsequent genetic distance and PAUP analyses.

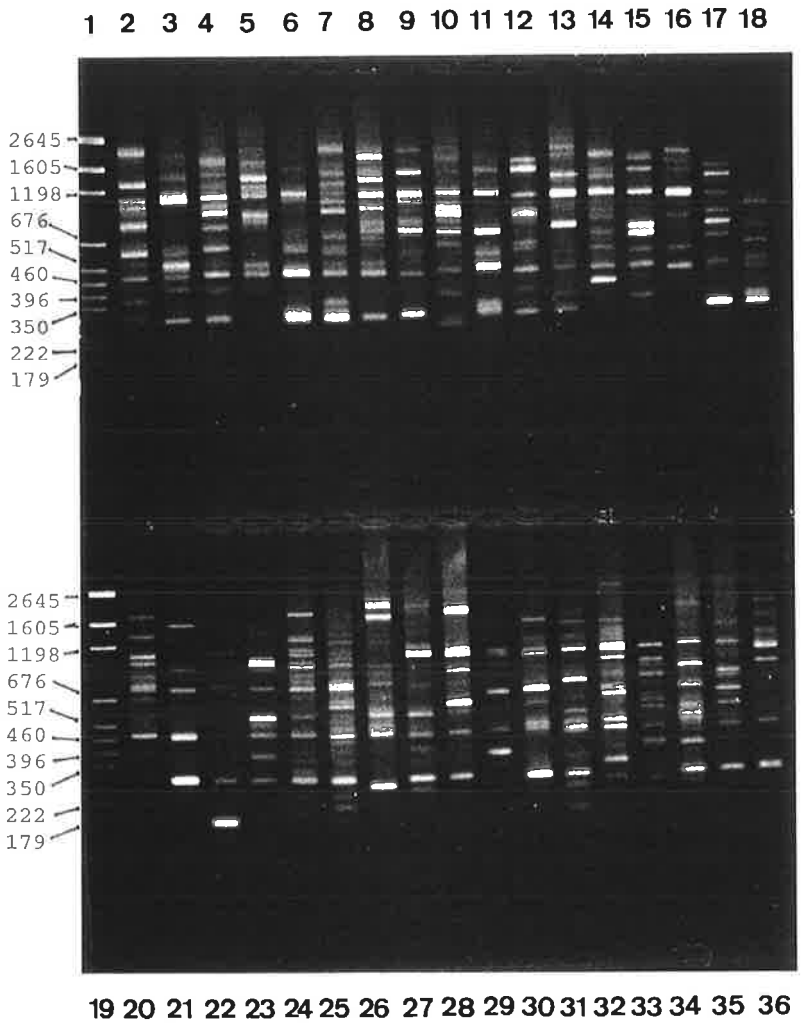

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
2645
1605
1198
676
517
460
396
350
222
179
2645
1605
1198
676
517
460
396
350
222
179
19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36

Figure 10.2 ~~Dendogram~~ Dendrogram generated by cluster (UPGMA) analysis of genetic distance values generated from 791 RAPD bands using 15 primers. Genetic distance (D) was calculated as D = 1-F, where F= similarity calculated using the method of Nei and Li (1979).

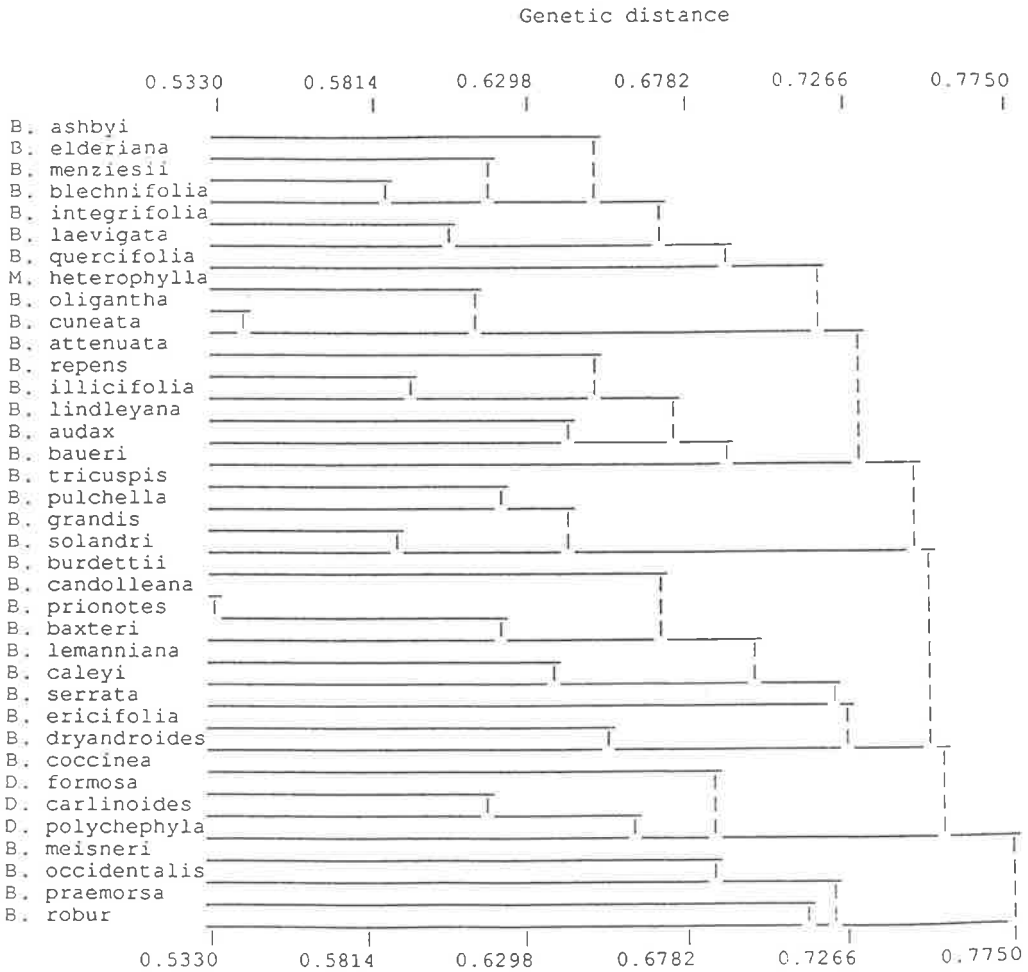

Genetic distance
0.5330
0.5814
0.6298
0.6782
0.7266
0.7750
B. ashbyi
B. elderiana
B. menziesii
B. blechnifolia
B. integrifolia
B. laevigata
B. quercifolia
M. heterophylla
B. oligantha
B. cuneata
B. attenuata
B. repens
B. illicifolia
B. lindleyana
B. audax
B. baueri
B. tricuspis
B. pulchella
B. grandis
B. solandri
B. burdettii
B. candolleana
B. prionotes
B. baxteri
B. lemanniana
B. caleyi
B. serrata
B. ericifolia
B. dryandroides
B. coccinea
D. formosa
D. carlinoides
D. polychephyla
B. meisneri
B. occidentalis
B. praemorsa
B. robur

Figure 10.3 Majority rule consensus tree obtained from PAUP, tree length = 4079; consistency index = 0.929. Taxa represent two closely related genera *Banksia* and *Dryandra*, with *Musgravea* as the outgroup in the analysis. Tree generated from 791 RAPD bands produced with 15 selected primers.

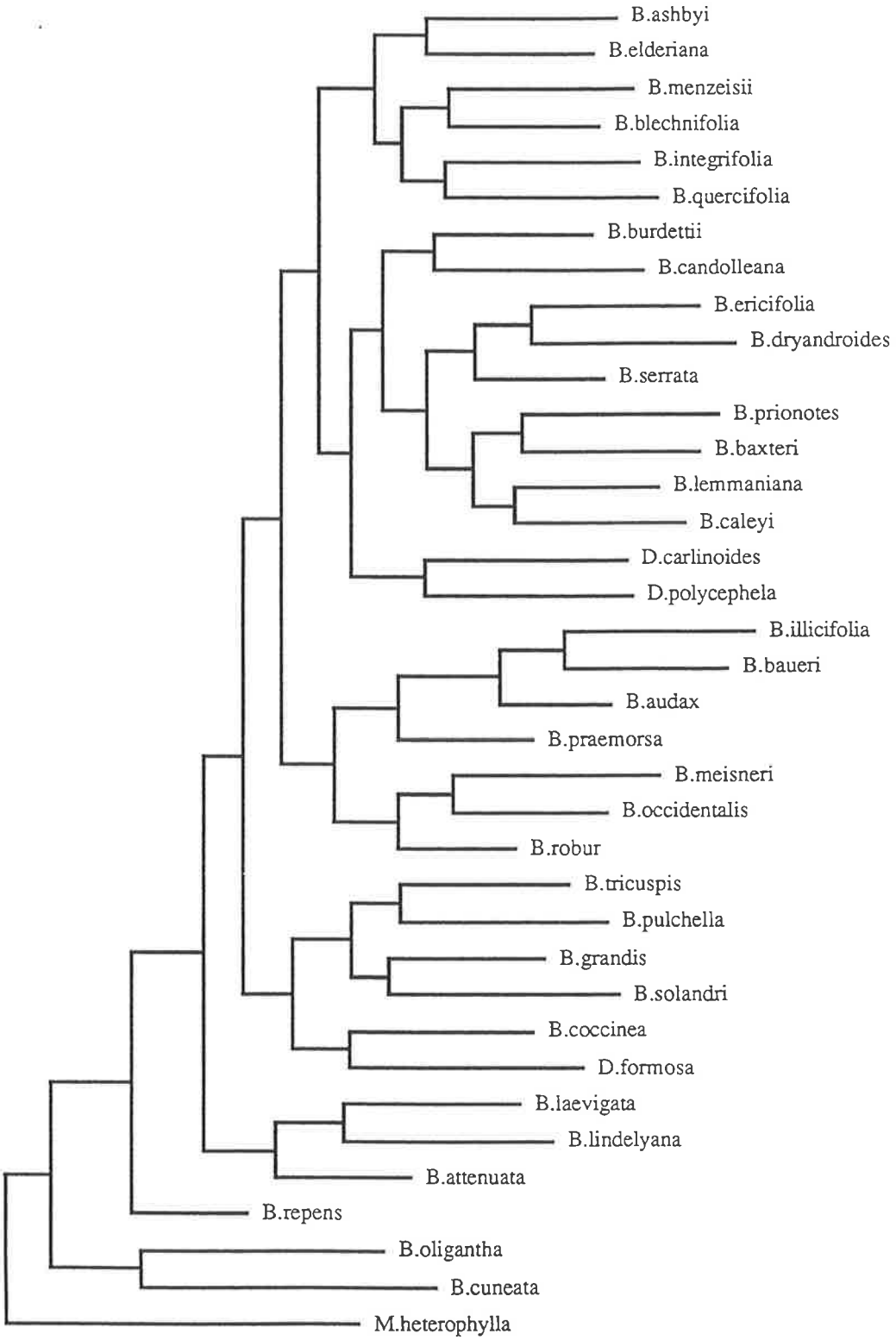
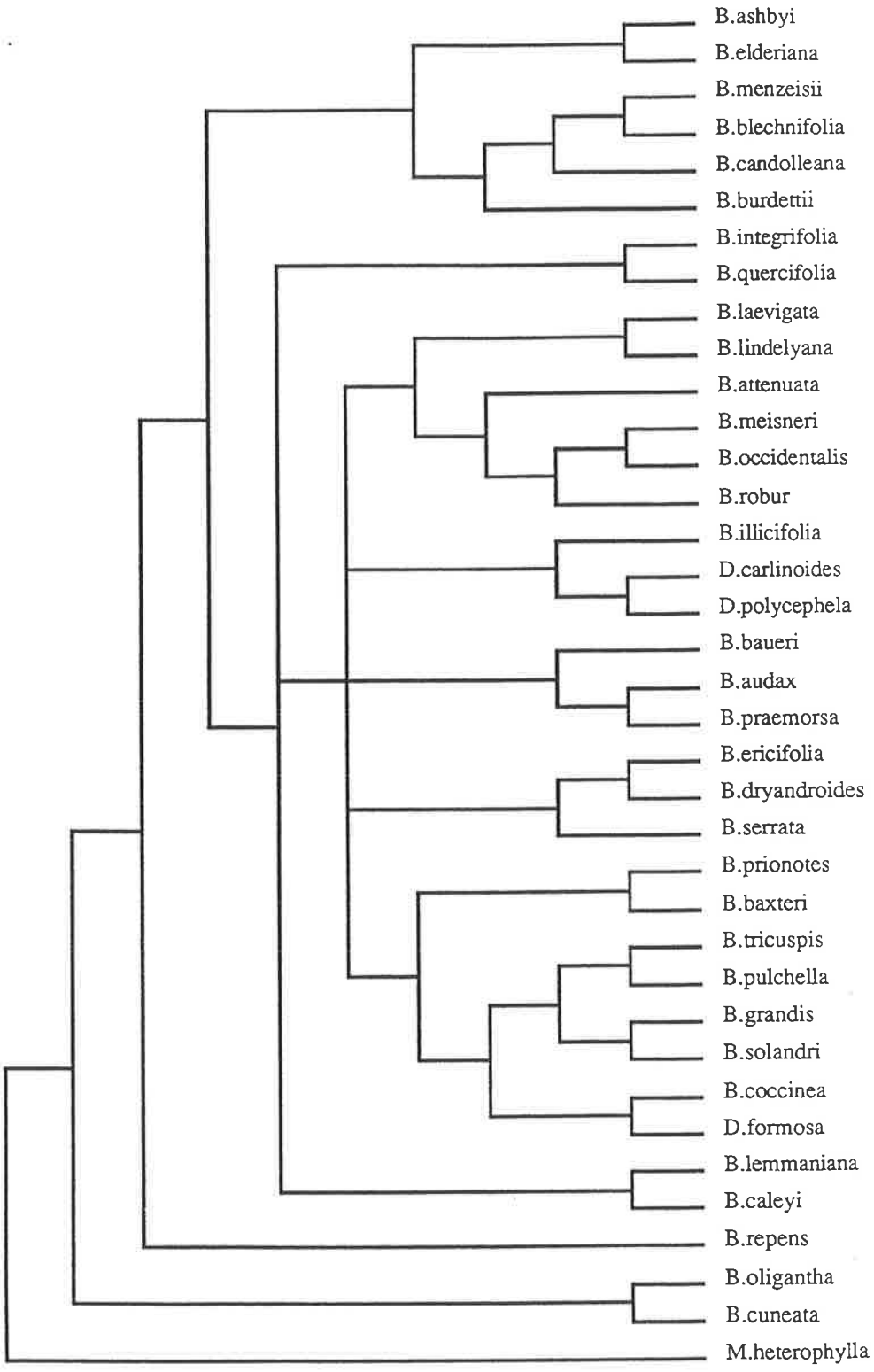

B.ashbyi
B.elderiana
B.menzeisii
B.blechnifolia
B.integrifolia
B.quercifolia
B.burdettii
B.candolleana
B.ericifolia
B.dryandroides
B.serrata
B.prionotes
B.baxteri
B.lemmaniana
B.caleyi
D.carlinoides
D.polycephela
B.illicifolia
B.baueri
B.audax
B.praemorsa
B.meisneri
B.occidentalis
B.robur
B.tricuspis
B.pulchella
B.grandis
B.solandri
B.coccinea
D.formosa
B.laevigata
B.lindelyana
B.attenuata
B.repens
B.oligantha
B.cuneata
M.heterophylla

Figure 10.4 Strict consensus tree obtained from PAUP, tree length = 4079; consistency index = 0.929. Taxa represent two closely related genera *Banksia* and *Dryandra*, with *Musgravea* as the outgroup in the analysis. Tree generated from 791 RAPD bands produced with 15 selected primers.


B.ashbyi
B.elderiana
B.menzeisii
B.blechnifolia
B.candolleana
B.burdettii
B.integrifolia
B.quercifolia
B.laevigata
B.lindelyana
B.attenuata
B.meisneri
B.occidentalis
B.robur
B.illicifolia
D.carlinoides
D.polycephela
B.baueri
B.audax
B.praemorsa
B.ericifolia
B.dryandroides
B.serrata
B.prionotes
B.baxteri
B.tricuspis
B.pulchella
B.grandis
B.solandri
B.coccinea
D.formosa
B.lemmaniana
B.caleyi
B.repens
B.oligantha
B.cuneata
M.heterophylla

Discussion

Both methods of analysis gave similar species pairs and groups, with closely related species within series, and closely related series generally grouping together, as in the classification of George (1981, 1988). It is interesting that *Banksia coccinea* grouped with species of *Dryandra* in both analyses, indicating a close relationship. In addition *Dryandra,* in the presence of an outgroup, could not be clearly distinguished as a monophyletic clade from *Banksia* using RAPDs. It is generally agreed that *Banksia* and *Dryandra* are sister taxa, with parallel developments in the two genera. Few morphological characters separate the two genera, so it could be suggested that *Banksia* and *Dryandra* may be artificial genera. Pollen - pistil data with *B. coccinea* and *Dryandra* species also show more compatibility than some *Banksia* interspecific crosses (~~Maguire *et al.*, in press~~). Using RAPDs there were close relationships between species in series *Banksia* and *Crocinae*, series *Dryandroideae*, *Spicigerae*, and *Abietinae*, and series *Bauerinae* and *Cyrtostylis*. The close relationship of series *Crocinae* and *Banksia* has been shown previously using pollen - pistil compatibility and cladistic analysis (Sedgley *et al.*, 1994, Thiele 1993). The series *Spicigerae, Abietinae* and *Dryandroideae* are grouped in section *Oncostylis*, subgenus *Banksia*, confirming the close relationship of these series (George 1981). Subgenus *Isostylis* formed a group with *Musgravea*, separate from the rest of *Banksia* showing more genetic distinctness than subgenus *Banksia* and genus *Dryandra.*

RAPDs are not generally considered to be informative at the distantly-related species level. Concerns regarding RAPD generated phylogenies at higher levels include: (a) homology of bands showing the same rate of migration; (b) causes of variation in fragment mobility; and (c) origin of sequences in the genome. Knowing the identity of shared fragments is essential, since analysis depends on the number of shared bands. The validity of the assumption that fragments of the same size are homologous is still debated, and southern hybridisation of RAPD bands in other studies have shown varying levels of

homology (Stammers *et al.*, 1995). Closely related species are expected to be more homologous than distantly related species, with more RAPD bands in close species corresponding to homologous sequences with conserved organisation. A consideration of change in fragment migration is important. The RAPD method relies on the relaxed conditions of primer annealing, and annealing is more critical at the 3' end than the 5' end, so base changes near the 3' end will significantly affect primer efficiency. It is likely that insertion/deletion events determine different fragment mobilities. The nature of the sequences detected by RAPDs are significant. Many bands contain repetitive domains (Stammers *et al.*, 1995), and it has been suggested that repetitive sequences are not suitable for phylogenetic analysis, because they can lead to concerted evolution. This may bias the phylogenetic tree due to the rapid spread of the variant repeated sequence. Although there are a number of potential problems associated with RAPD phylogeny analysis, there are also some clear advantages. Many loci are examined at the same time, which minimises the effect of loci which are under selection pressure, giving divergence of the whole genome. RAPDs are usually considered to be appropriate for closely related groups, as shown by this study and studies on *Lolium* (Stammers *et al.*, 1995), *Medicago* (Brummer *et al.*, 1995), *Lotus* (Campos *et al.*, 1994), *Brassica* (Demeke *et al.*, 1992) and *Allium* (~~Wilkie~~ Wilkie *et al.*, 1993).

Chapter Eleven

Application of non-coding chloroplast DNA sequences to *Banksia* (Proteaceae) phylogeny.

Abstract

Phylogenetic relationships within the genus *Banksia* L.f., (Proteaceae), and between *Banksia* and the related genus *Dryandra*, with *Musgravea* as the outgroup, were investigated using non-coding chloroplast DNA (cpDNA) sequences between the *trn*L (UAA) and *trn*F (GAA) gene. The phylogeny obtained is largely in accordance with the traditional classification of subgenus *Banksia*, into two main sections *Oncostylis* and *Banksia*. The relationships of *B. coccinea* remain uncertian, as it forms a polytomy with two *Dryandra* species and the two sections of subgenus *Banksia*. Subgenus *Isostylis* forms a polytomy with *D. formosa,* basal to subgenus *Banksia*, but with *B. cuneata* and *B. illicifolia* polyphyletic. *Dryandra* did not separate as a clade, and fell within *Banksia* raising questions about the currently accepted view of *Banksia* and *Dryandra* as sister genera with parallel development.

Introduction

The genus *Banksia* L.f., with over 75 taxa (George 1981, 1988) is an endemic Australian member of the family Proteaceae. Relationships amongst the Proteaceae are tentative, with *Banksia* placed in the subfamily Grevilleoideae. Within this subfamily there are six tribes including Bankisieae and Musgraveinae, the latter restricted to the rainforests of north eastern Australia. Within tribe Bankisieae there are two genera: *Banksia* and *Dryandra*, each of which is considered to be well defined, natural and monophyletic, although with some parallel morphological developments.

Early treatments of *Banksia* were either catalogues of species (Brown 1810), or artificial classifications (Meissner 1856). George (1981) attempted a more natural classification, using a variety of leaf, flower, follicle and seed characters to define infrageneric taxa. Whereas George describes "possible lines of evolution" within and between taxa, his classification is difficult to interpret phylogenetically both because it is inherantly intuitive and there are a number of taxa with uncertain relationships. Doust (1983) attempted a cladistic analysis of *Banksia* using 37 characters for 86 taxa, but the trees were poorly resolved. This analysis provided a poor match with the existing taxonomy, and failed to suggest a robust phylogenetic scheme. It was not used for a formal classification. More recently Thiele (1993) proposed a classification based on cladistic analyses of morphological characters. Although there is some agreement with the currently accepted classification of George (1981, 1988), there are still uncertainties in the positions of some species, such as *B. coccinea*, and support for some nodes is tenuous.

Banksia comprises two subgenera, *Isostylis* and *Banksia*. The former group is small and the species are superficially similar to *Dryandra*, leading George (1981) to propose that *Isostylis* may be closer to *Dryandra* than to subgenus *Banksia*. Nevertheless, he placed it as a subgenus of *Banksia*, with the comment that separate generic status may be appropriate. This interpretation was based on three morphological characters. The three species in subgenus *Isostylis* lack the prominent involucal bracts typical of *Dryandra*, but have flower subtending bracts typical of *Banksia*, their follicles are thick, woody and tomentose as in *Banksia* and they have the ovoid inflorescence axis typical of *Banksia*. However, there is still speculation on the relationships within subgenus *Banksia*, between subgenus *Isostylis*, subgenus *Banksia* and *Dryandra*. Other data, such as DNA markers, may help to resolve these relationships.

Chloroplast DNA (cpDNA) has been used extensively to infer plant phylogenies at different taxonomic levels. Direct sequencing of polymerase chain reaction (PCR) products is now becoming a rapidly expanding area of plant systematics (Clegg and

Zurawski 1991). The *rbc*L gene encoding the large subunit of RUBISCO, has been widely sequenced from many plant taxa (Chase *et al.*, 1993). These phylogenies are particularly informative at the family level (Morgan and Soltis 1993), and at higher taxonomic levels (Bousquet *et al.*, 1992). Phylogenetic relationships using *rbc*L sequences have also been used at lower taxonomic levels, indicating that it is useful at the generic level, however, in some cases the relationships remained unclear (Xiang *et al.*, 1993). The *rbc*L gene is considered to be too conservative to resolve relationships between closely related genera (Xiang *et al.*, 1993).

Analysis of non-coding regions of cpDNA can potentially overcome the poor resolution of genes such as *rbc*L at lower taxonomic levels. These non-coding regions tend to evolve more rapidly than coding sequences, by accumulation of insertions/deletions at a rate equal to that of nucleotide substitutions (Clegg and Zurawski 1991). cpDNA has been found to be extremely valuable for studying relationships between closely related species (Clegg *et al.*, 1991). Comparisons of the rates of *rbc*L and two non-coding regions of the cpDNA, the *trn*L (UAA) intron and the intergeneric spacer between the *trn*L (UAA) 3' exon and the *trn*F (GAA) gene, have been studied in several genera and have shown that these regions evolve faster, providing greater resolution at the generic and intrageneric level (Taberlet *et al.*, 1991, Ferris *et al.*, 1993, Gielly and Taberlet 1994).

This study examines the phylogenetic use of non-coding cpDNA in *Banksia*, and between *Banksia* and the closely related genus *Dryandra*, using the intergeneric spacer between the *trn*L (UAA) and *trn*F (GAA) gene.

Materials and methods

Plant material

Species included in the study are: *Banksia:* subgenus *Banksia*, section *Banksia, B. serrata* (two specimens), *B. media, B. integrifolia, B. coccinea*; section *Oncostylis, B. ericifolia, B. spinulosa, B. sphaerocarpa*; subgenus *Isostylis, B. cuneata, B. illicifolia*; *Dryandra: D. formosa, D. carlinoides, D. polycephala*; with *Musgravea heterophylla* as the outgroup. Seeds of *Banksia* and *Dryandra* were obtained from Nindethana Seed Service. Fresh leaf material of *M. heterophylla* was collected from Atherton, Queensland (QRS Arboretum No. 1372).

DNA extraction

DNA was extracted from each seed using a modified method of Weining and Langridge (1991), comprising: phenol/chloroform/isopropanol extraction for 5 min on ice, DNA precipitation for 1 min with ice cold isopropanol and sodium acetate, with DNA recovered by centrifugation at 12 000 rpm for 10 min. The pellet was washed twice with 70 % ethanol, dried and dissolved in 50 µL of TE buffer, with 1.0 µL of RNAase (R40: 40 g/mL RNAase A in TE), and stored at 4 ^{0}C short term (up to 1 month) or -20 ^{0}C long term (up to 1 year). DNA was extracted from fresh leaf material of *Musgravea heterophylla* using the seedling leaf extraction method of Maguire *et al.* (1994).

The DNA was subjected to gel electrophoresis on a 1.6% agarose gel in TBE buffer (Sambrook *et al.* 1989), and stained with ethidium bromide. DNA concentration was estimated by visual assessment of band intensities, compared to salmon sperm genomic DNA standards. The DNA content was adjusted to 10 ng µL $^{-1}$.

DNA amplification

DNA amplification was performed in a MJ Research Thermal Cycler. The optimal program commenced with an initial step of 94 ^{0}C for 2 min, followed by 35 cycles of 1 min at 94 ^{0}C, 1 min 58 ^{0}C, 2 min 72 ^{0}C. Optimised reaction conditions were carried out in a 25 µL total volume containing 1X *Taq* buffer (Gibco-BRL), 3 mM $MgCl_2$, 1 µM of each primer, 200 µM of each dNTP (dGTP, dATP, dCTP, dTAP), 1 unit of *Taq* polymerase (Gibco-BRL), and 10 ng µL $^{-1}$ template DNA. Each reaction mix was overlaid with PCR grade paraffin oil. DNA amplification fragments were separated by 2% agarose gel (Seakem, Promega) electrophoresis using TBE buffer (Sambrook *et al.* 1989). A DNA size marker was used (pGEM, Promega) on each gel, and gels were stained with ethidium bromide. The intergeneric spacer between the *trn*L (UAA) 3' exon and *trn*F (GAA) gene was amplified using the primers e (GGTTCAAGTCCCTCTATCCC) and f (ATTTGAACTGGTGACACGAG) (Taberlet *et al.*, 1991)

DNA sequencing

Following amplification, excess primers and deoxynucleotide triphosphates were removed from samples using polyethylene glycol (PEG) precipitation in magnesium chloride (Nicoletti and Condorelli 1993). Direct PCR sequencing of the purified fragment was carried out using standard conditions in the DyeDeoxy Terminator Sequencing Kit of Applied Biosystems, then automatically sequenced on an Applied Biosystems Model 373A. Both directions of the non-coding region were sequenced, and a consensus sequence determined for each species.

Data analysis

Multiple alignments of the sequences of the non-coding region were performed manually. Phylogenetic relationships within *Banksia*, and between *Banksia* and *Dryandra,* using *Musgravea* as the outgroup, were analysed via a parsimony approach using PAUP 3.1.1 (Swofford 1990). The phylogeny was assessed using the heuristic search method of PAUP (character optimisation ACCTRAN, MULPARS and TBR branch swapping options). To ensure that all islands of most parsimonious trees were found (Maddison 1991), the search was repeated 100 times with RANDOM addition and with a maximum of 100 trees saved at each replication.

Results

Double stranded DNA amplification products were obtained for all species. The consensus sequence of the spacer region between the *trn*L (UAA) 3' exon and the *trn*F (GAA) gene is shown (Fig 11.1); the size of the aligned sequence for all species is 413 bp. There was no intraspecific variation between the two individuals of *B. serrata* .

The single most parsimonious tree found using PAUP had a consistency index of 0.983, tree length 60, and a retention index of 0.909 (Fig 11.2). Generally, most *Banksia* species fell into the two main sections of *Banksia*, subgen. *Banksia*, based on morphological characters: *B. ericifolia, B. spinulosa,* and *B. sphaerocarpa*, of section *Oncostylis* forming a clade with *B. integrifolia.* A second clade comprised *B. media* and the two individuals of *B. serrata*, representing section *Banksia. B. coccinea* along with two *Dryandra* species *D. carlinoides* and *D. polycephela* were unresolved at the polytomy with the two sections of subgenus *Banksia. B. illicifolia, Dryandra formosa* and *B. cuneata* were basal to this polytomy with the related two members of *Banksia* subgen. *Isostylis* separated.

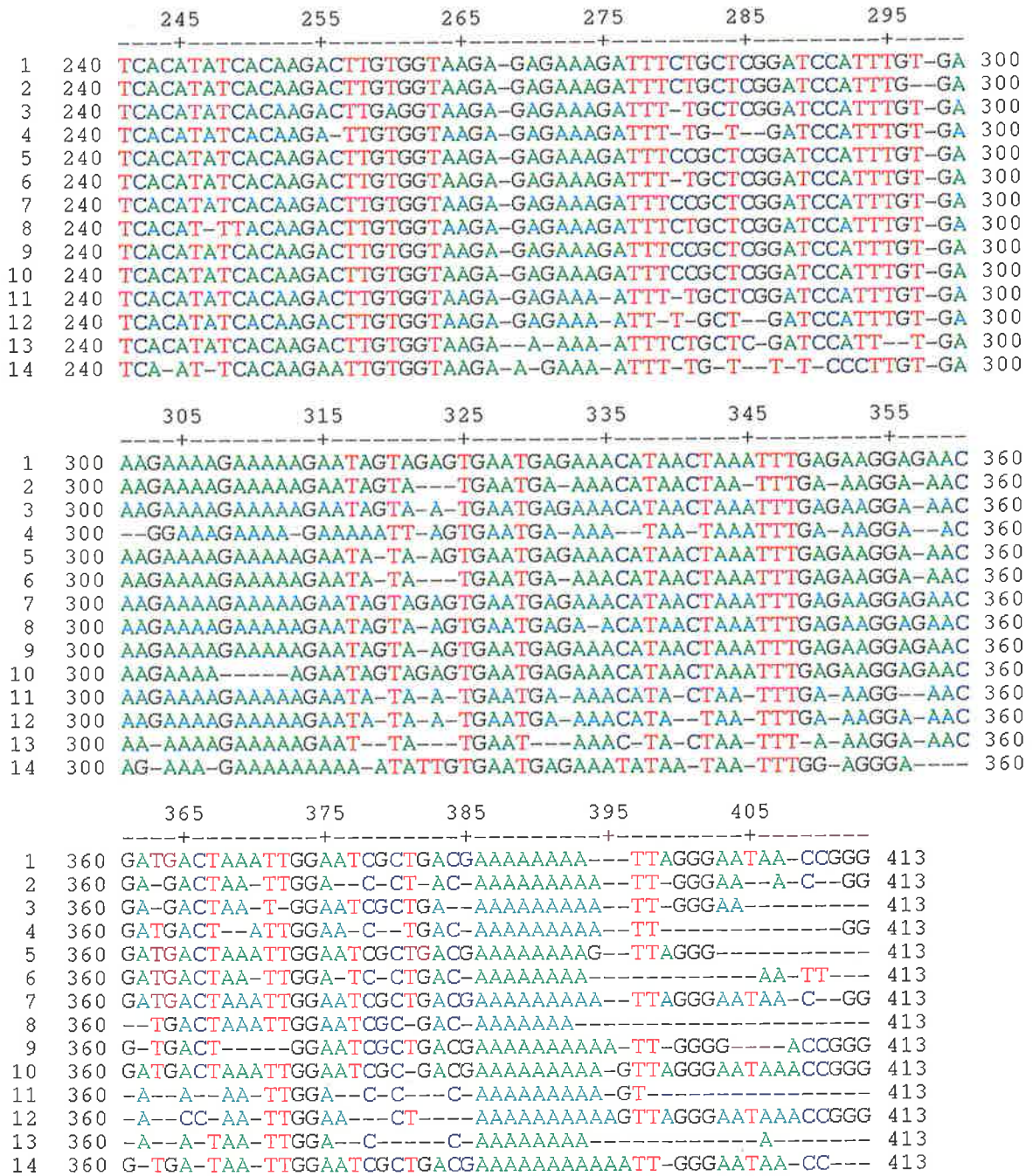
Figure 11.1 Complete nucleotide sequences of the spacer between the *trn*L (UAA) and *trn*F (GAA) gene in *Banksia, Dryandra* and *Musgravea* (length of alignment 413 bp). 1. *B. serrata* (individual 1), 2. *B. serrata* (individual 2), 3. *B. coccinea,* 4. *B. cuneata*, 5. *B. ericifolia*, 6. *B. illicifolia,* 7. *B. integrifolia*, 8. *B. media*, 9. *B. sphaerocarpa,* 10. *B. spinulosa*, 11. *D. carlinoides*, 12. *D. formosa*, 13. *D. polycephala*, 14. *M. heterophylla.*

```
                5         15         25         35         45         55
               ----+---------+---------+---------+---------+---------+-----
1       0 T-TCCCGA--ATTCC-G-GC-TCATCCTCATT--A-TAG-GTTCTTGGG--TATGTCAAT  60
2       0 T-TCCCGACTATTCC-G-GCATCAT-CTCATT--ACTAG-GTTCTTGGGTCTATGTCAAT  60
3       0 T-TCC-GACTATTCCTGTGC-TCATCCTCATT-TA-TAGAGTTCTTGGGTCTATGTCAAT  60
4       0 T-TCCCGACTATTCCTGTGC-TCATCCTCATT-TA-TAGAGTTCT-GGGT-TATGTCAAT  60
5       0 TATCCCGACTATTCCTG-GCATCATCCTCATT-TACTAGAGTTCT-GGGTCTATGTCAAT  60
6       0 T-TCCCG--TATTCC-GTGCATC-TCCTCATT-TA-TAG-GTTCTTGGGT-TATGTCAAT  60
7       0 TATCCCGACTATTCC-GTGCATCATCCTCATT-TA-TAGAGTTCTTGGGTCTATGTCAAT  60
8       0 ---CCC-ACTATTCC-G-G--TCATCCTCATTTTACCA-AGTTCT-GGGTCTATGTC-AT  60
9       0 T-TCCCGACTATTC--G-GCATCATCCTCATT-T-ATAG-GTTCT-GGGT-TATGTCAAT  60
10      0 TATCCCGACTATTCCTGTGCATCATCCTCATTCTACTAGAGTTCT-GGGTCTATGTCAAT  60
11      0 ----CCGA--ATTCC-G-GC-T-ATCCTCATT--A-TAGA-TT-TTGGGT-TATGTCAAT  60
12      0 -----CGA-T-T-CC-G-G--T--TC-T-ATT-TA-T---G-T--TGGGT-TA-GTCAAT  60
13      0 T-TCC-GA-TATTCC-G-GCAT-ATCCT-A-GA-A-TAG-GTT--TGGGT-TATGTCAAT  60
14      0 G-TCCCGA---TT-C-G-G--TC-TCCTC-TT-TA--A-AGG-T-TTGGG-TATG-C-AT  60

                65         75         85         95        105        115
               ----+---------+---------+---------+---------+---------+-----
1      60 TAAAGGATTAAAAAAACATTACAAAGTCTTA-CCCAGGCCCCGGAAATTCTTGGATCTTC 120
2      60 TAAAGGATTAAAAAAACATTACAAAGTCTTA-CCCAGGCCCCGGAAATTCTTGGATCTTC 120
3      60 TAAAGGACTAAAAAAACATTACAAAGTCTTA-CCCAGGCCCCGGAAATT-TTGGAT-TTC 120
4      60 TAAAGGACTAAAAAAACATTACAAAGT-TTATCC-AGGCCCCGGAAATT-TTGGAT-TTC 120
5      60 TAAAGGACTAAAAAAACATTACAAAGTCTTA-CCCAGGCCCCGGAAATT-TTGGATCTTC 120
6      60 TAAAGGACTAAAAAAACATTACAAAGTCTTATCC-AGGCCCCGGAAATT-TTGGAT-TTC 120
7      60 TAAAGGACTAAAAAAACATTACAAAGTCTTA-CCCAGGCCCCGGAAATTCTTGGATCTTC 120
8      60 TAAAGGATTAAAAAAACATTACAAAGTCTTA-CCC--GCCCCGGAAATTCTTGGATCTTC 120
9      60 TAA-GGACTAAAAAAACATTACAAAGT-TTA-CCCAGGCCCCGGAACTT-TTGGAT-TTC 120
10     60 TAAAGGACTAAAAAAACATTACAAAGTCTTA-CCCAGGCCCCGGAAATTCTTGGATCTTC 120
11     60 TAAAGGACTAAAAAAACATTACAAAGTCTTA-CCCAGGCCCCGAAAATT-TTGGATCTTC 120
12     60 T-A-GGA-TAAAAAAACATTACAAA-T-TTA-CCCAGGCCCCGGAA-TT-TTGGATCTTC 120
13     60 TAAAGGACTAAAAAAACATTACAAAGTCTTACCCCAGGCCCCCGAAATTCTTGGATCTTC 120
14     60 TAAAGGGCTAAAAAAACATTACAAAGT-TTA-CCCAGACCCCGGGAATT-TTGGGTTTTC 120

               125        135        145        155        165        175
               ----+---------+---------+---------+---------+---------+-----
1     120 AAAAAGAAGACTTTGTAAGTTTCATTAAGATTAAGAGTAATTATATGGATGGTAAATGAT 180
2     120 AAAAAGAAGACTTTGTAAGTTTCATTAAGATTAAGAGTAATTATATGGATGGTAAATGAT 180
3     120 AAAAAGAAGACTTTGTAAGTTTCATTAAGATTAAGAGTAATTATATGGATGGTAAATAAT 180
4     120 AAAAA-AAGACTTTGTAAGTTTCATTAA-ATTA--A-TAATTATATGGATGGTAAATGAT 180
5     120 AAAAAAAAGACTTTGTAAGTTTCATTAAGATTAAAAGTAATTATATGGATGGTAAATGAT 180
6     120 AAAAAGAAGACTTTGTAAGTTTCATTAAGATTAAGAGTAATTATATGGATGGTAAATGAT 180
7     120 AAAAAGAAGACTTTGTAAGTTTCATTAAGATTAAGAGTAATTATATGGATGGTAAATGAT 180
8     120 AAAAA-AA-ACTTTGTAAGTTTCATT--GATTAA-AGTAATTAT-TGGATGGT--ATGAT 180
9     120 AAAAAGAAGACTTTGTAAGTTTCATTAAGATTAAGAGTAATTA------ATGGTAAATGAT 180
10    120 AAAAAGAAGACTTTGTAAGTTTCATTAAGATTAAAAGTAATTATATGGATGGTAAATGAT 180
11    120 AAAAAGAAGACTTTGTAAGTTTCATTAAGATTAAGAGTAATTATATGGATGGTAAATGAT 180
12    120 AAAAAGAAGACTTTGTAAGTTTCATTAAGATTAAGAGTAATTATATGGATGGTAAATGAT 180
13    120 AAAAAGAAGACTTTGTAAGTTTCATTAAGATTAAGAGTAATTATATGGATGGTAAATGAT 180
14    120 CAAA-GG--A--TTGTAAGTTT-ATTAAGGTTAAGGGT-AT-ATATGG-TGGTAAATGAT 180

               185        195        205        215        225        235
               ----+---------+---------+---------+---------+---------+-----
1     180 TTAGATTCAAATGGGAATTCCTTGCTCATAGATGTTCATTTGTACGTATATCTTAATATA 240
2     180 TTAGATTCAAATGGGAATTCCTTGCTCATAGATGTTCATTTGTACGTATATCTTAATATA 240
3     180 TTAGATTCAAATGGGAATTCCTTGCTCATAGATGTTCATTTGTACGTATATCTTAATATA 240
4     180 TTA-ATTCAAATGGGAATTCCTTGCTCATAAATGTTCATTTGTA-GTATAT-TTAATATA 240
5     180 TCAGATTCAAATGGGAATTCCTTGCTCATAGATGTTCATTTGTACGTATATCTTAATATA 240
6     180 TTAGATTCAAATGGGAATTCCTTGCTCATAAATGTTCATTTGTACGTATAT-TTAATATA 240
7     180 TCAGATTCAAATGGGAATTCCTTGCTCATAGATGTTCATTTGTACGTATATCTTAATATA 240
8     180 -TAGATTC---TGGGAATTCCTTGCTCATAGATGTGCATTTGTACGTATATCTTAATATA 240
9     180 TTAGATTCAAATGGGAATTCCTTGCTCATAGATGTTCATTTG-A---ATAT-TTAAT-TA 240
10    180 TCAGATTCAAATGGGAATTCCTTGCTCATAGATGTTCATTTGTACGTATATCTTAATATA 240
11    180 TTAGATTCAAATGGGAATTCCTTGCTCATAGATGTTCATTTGTACGTATATCTTAATATA 240
12    180 TTAGATTCAAATGGGAATTCCTTGCTCATAGATGTTCATTTGTA-GTATAT-TTAATATA 240
13    180 TTAGATTCAAATGGGAATTCCTTGCTCATAGATGTTCATTTGTACGTATATCTTAATATA 240
14    180 T-AGGTT-AAATGGGGATTCC-TG-T-ATAGA-GTT-ATTTGTA-G--TAT-TTAGTATA 240
```


245 255 265 275 285 295
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2 240 TCACATATCACAAGACTTGTGGTAAGA-GAGAAAGATTTCTGCTCGGATCCATTTG--GA 300
3 240 TCACATATCACAAGACTTGAGGTAAGA-GAGAAAGATTT-TGCTCGGATCCATTTGT-GA 300
4 240 TCACATATCACAAGA-TTGTGGTAAGA-GAGAAAGATTT-TG-T--GATCCATTTGT-GA 300
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6 240 TCACATATCACAAGACTTGTGGTAAGA-GAGAAAGATTT-TGCTCGGATCCATTTGT-GA 300
7 240 TCACATATCACAAGACTTGTGGTAAGA-GAGAAAGATTTCCGCTCGGATCCATTTGT-GA 300
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9 240 TCACATATCACAAGACTTGTGGTAAGA-GAGAAAGATTTCCGCTCGGATCCATTTGT-GA 300
10 240 TCACATATCACAAGACTTGTGGTAAGA-GAGAAAGATTTCCGCTCGGATCCATTTGT-GA 300
11 240 TCACATATCACAAGACTTGTGGTAAGA-GAGAAA-ATTT-TGCTCGGATCCATTTGT-GA 300
12 240 TCACATATCACAAGACTTGTGGTAAGA-GAGAAA-ATT-T-GCT--GATCCATTTGT-GA 300
13 240 TCACATATCACAAGACTTGTGGTAAGA--A-AAA-ATTTCTGCTC-GATCCATT--T-GA 300
14 240 TCA-AT-TCACAAGAATTGTGGTAAGA-A-GAAA-ATTT-TG-T--T-T-CCCTTGT-GA 300
305 315 325 335 345 355
1 300 AAGAAAAGAAAAAGAATAGTAGAGTGAATGAGAAACATAACTAAATTTGAGAAGGAGAAC 360
2 300 AAGAAAAGAAAAAGAATAGTA---TGAATGA-AAACATAACTAA-TTTGA-AAGGA-AAC 360
3 300 AAGAAAAGAAAAAGAATAGTA-A-TGAATGAGAAACATAACTAAATTTGAGAAGGA-AAC 360
4 300 --GGAAAGAAAA-GAAAAATT-AGTGAATGA-AAA--TAA-TAAATTTGA-AAGGA--AC 360
5 300 AAGAAAAGAAAAAGAATA-TA-AGTGAATGAGAAACATAACTAAATTTGAGAAGGA-AAC 360
6 300 AAGAAAAGAAAAAGAATA-TA---TGAATGA-AAACATAACTAAATTTGAGAAGGA-AAC 360
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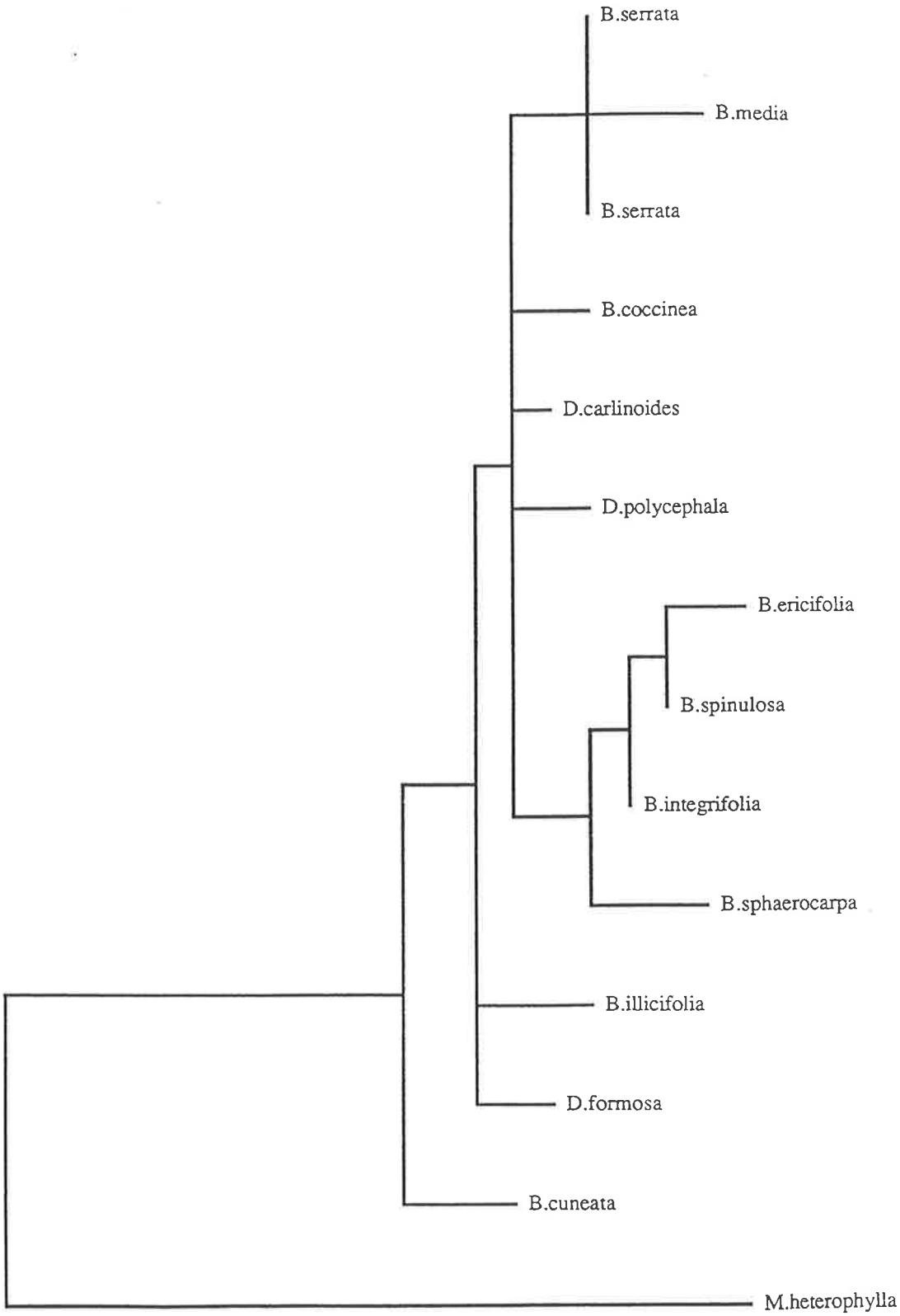
12 300 AAGAAAAGAAAAAGAATA-TA-A-TGAATGA-AAACATA--TAA-TTTGA-AAGGA-AAC 360
13 300 AA-AAAAGAAAAAGAAT--TA---TGAAT---AAAC-TA-CTAA-TTT-A-AAGGA-AAC 360
14 300 AG-AAA-GAAAAAAAAA-ATATTGTGAATGAGAAATATAA-TAA-TTTGG-AGGGA---- 360
365 375 385 395 405
1 360 GATGACTAAATTGGAATCGCTGACGAAAAAAAA---TTAGGGAATAA-CCGGG 413
2 360 GA-GACTAA-TTGGA--C-CT-AC-AAAAAAAAA--TT-GGGAA--A-C--GG 413
3 360 GA-GACTAA-T-GGAATCGCTGA--AAAAAAAAA--TT-GGGAA--------- 413
4 360 GATGACT--ATTGGAA-C--TGAC-AAAAAAAAA--TT----------GG 413
5 360 GATGACTAAATTGGAATCGCTGACGAAAAAAAAG--TTAGGG---------- 413
6 360 GATGACTAA-TTGGA-TC-CTGAC-AAAAAAAA----------AA-TT--- 413
7 360 GATGACTAAATTGGAATCGCTGACGAAAAAAAAA--TTAGGGAATAA-C--GG 413
8 360 --TGACTAAATTGGAATCGC-GAC-AAAAAAA-------------------- 413
9 360 G-TGACT-----GGAATCGCTGACGAAAAAAAAAA-TT-GGGG----ACCGGG 413
10 360 GATGACTAAATTGGAATCGC-GACGAAAAAAAAA-GTTAGGGAATAAACCGGG 413
11 360 -A--A--AA-TTGGA--C-C---C-AAAAAAAAA-GT---------------- 413
12 360 -A--CC-AA-TTGGAA---CT----AAAAAAAAAAGTTAGGGAATAAACCGGG 413
13 360 -A--A-TAA-TTGGA--C-----C-AAAAAAAA----------A------- 413
14 360 G-TGA-TAA-TTGGAATCGCTGACGAAAAAAAAAAATT-GGGAATAA-CC--- 413

Figure 11.2 Phylogenetic relationships within *Banksia* and related genera. Single most parsimonious tree retained by the heuristic search algorithm of PAUP based on sequences of the spacer between *trn*L and *trn*F. Tree length 60, consistency index 0.983, retention index 0.909.


B.serrata
B.media
B.serrata
B.coccinea
D.carlinoides
D.polycephala
B.ericifolia
B.spinulosa
B.integrifolia
B.sphaerocarpa
B.illicifolia
D.formosa
B.cuneata
M.heterophylla

Discussion

The *trn*L intron and the spacer between *trn*L and *trn*F are valuable tools for preliminary studies in closely related species groups that have not been investigated previously. These primers are universal and amplify DNA from a wide range of species, and due to the small size of this region, no internal primers are needed for sequencing, so information is easily obtained.

The phylogeny obtained using cpDNA sequence data show broad species grouping into the two sections of subgenus *Banksia*, section *Banksia* and section *Oncostylis*. *B. coccinea*, whose relationships are still uncertain, is distinct from either section grouping instead in a polytomy with two species of *Dryandra* at the node with the two sections of subgenus *Banksia,* thus suggesting that a third section containing *B. coccinea* may be more appropriate. This separation has already been proposed recently, based on morphological characters and pistil - pollen compatibility data (Chapter 5 ~~Maguire *et al.*, 1996~~). The node between the two sections of *Banksia* with *B. coccinea* and the two *Dryandra* species is unresolved, and the relationship of *B. coccinea* to the *Dryandra* species is unclear. The non-coding region between *trn*L and *trn*F appears to be too conservative to clearly resolve these relationships, and a faster evolving region might be more appropriate.

Subgenus *Isostylis* and *D. formosa* were basal to the remainder of *Banksia* and *Dryandra*. George (1981) indicated that subgenus *Isostylis* may be more closely related to *Dryandra* than *Banksia*, but placed it in *Banksia* with a note that a new genus may be appropriate. Interestingly, *Dryandra* in the presence of *Banksia*, did not separate as a monophyletic clade based on its cpDNA sequence data. These data suggest that the currently accepted view of two sister genera, *Banksia* and *Dryandra,* with parallel morphological development, may be inappropriate. Based on cpDNA sequence data, it can be suggested that *Banksia* and *Dryandra* are possibly artificial genera. Similarly, molecular data have suggested that widely accepted views of separate genera may be

artificial in other plant groups such as *Eucalyptus* (Sale *et al.*, 1996, Ladiges *et al.*, 1995).

The results of this study do not represent a definitive study of the relationships between the genera *Banksia* and *Dryandra*, but it raises important questions about the currently accepted views of the classification. It is not easy, for many reasons, to establish the correct choice of a region of the chloroplast genome for resolving phylogenies. *rbc*L has been shown to be valuable at the family level (Morgan and Soltis 1993), and at higher levels (Bousquet *et al.*, 1992). Phylogenetic relationships using *rbc*L sequences have also been used at lower taxonomic levels, however in some cases the relationships remained unclear (Xiang *et al.*, 1993). Therefore, the *rbc*L gene is sometimes too conservative to clarify relationships between closely related genera. For this reason, non-coding regions of the cpDNA such as the *trn*L intron, the spacer between the *trn*L and *trn*F gene, and the spacer between *rbc*L and *atp*B (Taberlet *et al.*, 1991, Goldenberg *et al.*, 1993) have been advocated as being more appropriate for working at lower taxonomic levels.

For large scale phylogenies of a genus or genera, a preliminary study, such as the one presented here, is advisable. This is because sequence divergence of these regions at the intrageneric level can vary greatly. In some cases the evolution of these regions is scarcely faster than the *rbc*L gene (Gielly and Taberlet 1994), and few comparisons of the evolutionary rates of both cpDNA and nuclear ribosomal DNA (nrDNA) have been conducted. Gielly *et al.*, (1996) compared the rates of the non-coding region of nuclear internal transcribed spacer regions (ITS) to the non-coding regions of cpDNA *trn*L (UAA) intron sequences in the genus *Gentiana*. Comparisons of the evolutionary rates found that sequence divergence in the ITS regions were higher than for the *trn*L introns. However, the cpDNA intron and the ITS of the nrDNA gave largely concordant phylogenetic trees. At the intrageneric level in *Gentiana,* ITS sequences appeared to be more appropriate in the assessment of plant phylogeny, but the cpDNA *trn*L intron was preferable at the intergeneric level. In our study, we similarly found *trn*L to be

conservative at the intrageneric level in *Banksia* and *Dryandra*. This again suggests the two may be artificial genera, challenging the accepted views of two monophyletic, sister taxa, with considerable parallel morphological development. This work shows that further investigation both into the taxonomic relationships within *Banksia* and between *Banksia* and *Dryandra* is needed.

Chapter Twelve

General discussion

Banksia *breeding*

Research is essential to understanding the biology of banksias and to develop strict selection criteria for crop improvement. A breeding program has been established for *Banksia* (Sedgley *et al.* 1991) which focuses on the species *B. coccinea, B. menziesii, B. hookeriana,* and *B. prionotes*. Breeding programs on other members of the Proteaceae have been established in South Africa and America, and have used interspecific hybridisation as the basis for cultivar development (Parvin 1981, Brits 1985). The *Banksia* program (Sedgley *et al.* 1991, 1994) also aims to use interspecific hybridisation within the genus to produce novel cultivars and combine characters from other species. Hybridisation methods have been developed for *Banksia* based on knowledge of the breeding biology of the genus (Fuss and Sedgley 1991). In addition, research into the structure of the pollen presenter and the stigmatic groove has shown that there is more than one type of pollen presenter, and the location of the stigmatic groove is different depending on the species (Sedgley *et al.* 1993). This is important in interspecific hybridisation to make sure the target is clearly identified for deposition of interspecific pollen into the stigmatic groove.

This thesis describes an extensive interspecific pollination program based on *B. coccinea* (chapter 4). To focus breeding efforts, species relationships need to be known in order to increase the probable success of hybridisation. It is generally assumed that closely related species will hybridise, and distantly related species will not. Interestingly, *B. coccinea* is unique in the genus with distinct morphological characters and unclear relationships to other species. Interspecific hybridisation was conducted with a broad range of species with *B. coccinea* as the male parent in order to determine its relationships, and secondly, to

identify species combinations that may be successful for hybridisation. A number of important conclusions arose from this work.

Hybridisation success generally depends on species relationships, and only closely related species have compatible pollinations, as assessed by fluorescence microscopy. *B. coccinea* was most compatible with species of section *Oncostylis*, with some compatibility with species of section *Banksia* and subgenus *Isostylis*. In addition, pollen tube growth was controlled in the pollen presenter and upper style regions, which has been demonstrated previously in intra and interspecific crosses (Sedgley *et al.*, 1994, Fuss and Sedgley 1991). Pollen germination is also controlled on the stigma, perhaps a more severe display of an interspecific incompatibility mechanism. Interspecific crosses with *B. coccinea* displayed pollen tube abnormalities not previously described in *Banksia.* These types of abnormalities have also been noted in the interspecific crosses of other genera (Ellis *et al.*, 1991, Fritz and Hanneman 1989). Unilateral incompatibility was not observed in crosses with *B. coccinea.* Unilateral cross incompatibility is commonly seen in other crops where a self incompatible female rejects the pollen of a self compatible male. It was suggested that unilateral incompatibility was not present in *Banksia* interspecific crosses, as most species of *Banksia* are largely outcrossing and thought to be self incompatible (Scott 1980, Carthew *et al.*, 1988, Goldingay *et al.*, 1991).

Species combinations identified as potentially compatible were assessed for seed set. *B. coccinea* was crossed with species from section *Banksia*, section *Oncostylis* and subgenus *Isostylis*. Crosses failed to produce follicles and seed with species of section *Banksia* and subgenus *Isostylis*. Crosses with species of section *Oncostylis* formed follicles with aborted seed, and follicles with seed. Seed were tested for viability and seedling growth. The seed had low viability, with only two seedlings produced, one of which died. Based on early seedling morphology the remaining seedling was indistinguishable from the female parent. As it reaches maturity, it may display more intermediate characters, although the balance of probability suggests that it arose from self pollination. The cross that initiated

follicle set, but not seed, indicates that post fertilisation selection occurs in *Banksia,* as well as pre fertilisation barriers in the pollen presenter and upper style. The breeding systems operating in *Banksia* appear complex, with pre and post fertilisation selection occurring in interspecific crosses. *Banksia* is a very ancient genus, which has barely changed anatomically over millions of years. Breeding systems maintain species purity and survival, and few *Banksia* interspecific hybrids occur in the wild. Interspecific hybrids are generally intermediate between closely related species, occur in the same area, where species range can be extended and may be beneficial for species persistence.

Pollen storage and viability testing methods are important adjuncts to a plant breeding program. Stored pollen can be used to hybridise species that do not flower at the same time, or are geographically isolated, increasing flexibility of the breeding program. Pollen storage and viability testing methods were developed for *B. menziesii*, which are also applicable to other species (chapter 3). Two methods of viability testing were investigated, staining with FDA and *in vitro* germination. Staining methods were found to be unreliable and *in vitro* germination was preferable, with optimal conditions for *in vitro* germination developed for *B. menziesii*. Pollen storage above zero degrees was found to be unsuccessful, with pollen retaining good viability at temperatures below zero. Pollen, after drying over silica gel, can be stored up to six months in an ordinary 'fridge or freezer to suit breeding purposes.

Genetic diversity

The continued interest in Australian species, particularly those from family Proteaceae, for cut flowers provides a challenge to plant breeders to supply new cultivars. Therefore, continued selection and cultivation of new variants and species is essential to expand current markets and maintain high quality products. Conservation of natural populations of indigenous plants will ensure there are sufficient resources to tap for further crop development and improvement in the future.

Traditionally, studies of genetic diversity have used allozyme markers. Allozymes can provide informative markers for levels of genetic variability within a plant species, but there are widely recognised limitations. Many species of the family Proteaceae have low levels of detected allozyme diversity. This is because the detection of variability is limited to protein coding loci, which may underestimate levels of genetic diversity (Clegg 1989), and may not represent the entire genome (Schaal *et al.* 1991). Allozymes are also tissue specific and are influenced by environmental factors. Given these limitations many workers have moved to using DNA markers such as RAPDs. RAPDs overcome the limitations of allozymes and are simpler and easier to use than other DNA markers such as restriction fragment length polymorphisms (RFLPs). RAPDs produce potentially unlimited numbers of markers and have been shown to be useful for a variety of applications including population genetic studies on a number of genera (Huff *et al.* 1993, Chalmers *et al.* 1992, Russell *et al.* 1993).

Genetic diversity has not been widely studied in *Banksia*. Allozyme methods have estimated high levels of genetic diversity in *Banksia*, amongst the highest levels recorded for plants (Scott 1981, Carthew *et al.*, 1988). Genetic diversity for a number of species of *Banksia* using RAPD markers were investigated, in particular *B. cuneata*, a rare and endangered species. High levels of genetic diversity were detected in 33 species of *Banksia*, and three species of *Dryandra* using RAPDs (chapter 8), and levels detected using RAPDs were comparable to levels of within species diversity detected by allozymes. RAPDs also produced large numbers of polymorphic markers, compared to the limited number available for allozyme techniques. Genetic diversity of all species tested including threatened, or rare species, was high indicating that none have reached dangerously low levels. More research is needed however, to target fragmented species across all remaining populations.

B. cuneata is declared a rare species under the WA Wildlife Conservation Act, and is under threat of extinction due to small population sizes and fragmented distribution over

private and public land. Genetic studies including all known remaining populations have not previously been conducted. Levels and patterns of genetic diversity were investigated using RAPDs (chapter 9), which is important for the future conservation and management of *B. cuneata*. Genetic diversity of *B. cuneata* estimated using RAPDs was quite high, considering it is a geographically restricted rare and endangered species. The 10 known populations of *B. cuneata* showed differences in the extent of disturbance and levels of genetic diversity. Variation in genetic diversity estimates between populations is not uncommon in species with mixed mating systems (Schoen 1982). Pollinator availability and activity are likely to have an influence on genetic diversity between populations, and this particularly applies to bird pollinated species such as *B. cuneata*. Population structure such as plant density, and level of disturbance may also influence pollinator behaviour, and this is reflected by larger populations with lower levels of disturbance, having high levels of genetic diversity. In contrast, small populations on road verges with high levels of disturbance, display lower levels of genetic diversity. The estimates of genetic diversity within populations were in agreement with allozyme methods conducted on 6 *B. cuneata* populations (Coates and Sokolowski 1992).

Dissected population structure and geographic restriction of *B. cuneata* is likely to influence the patterns of genetic variation within and between populations (Hamrick and Godt 1989). As *B. cuneata* is outcrossing and bird pollinated, most variation would be expected to be within populations and little between populations. Analysis of molecular variance (AMOVA) was used to partition RAPD variation within and between populations of *B. cuneata*. The results clearly demonstrated that nearly all variation was attributable to individuals within populations, with no significant genetic differentiation between populations. The lack of population differentiation in *B. cuneata* using RAPDs contrasts to a previous study by Coates and Sokolowski (1992), using six of the ten populations of *B. cuneata*, and enzyme electrophoresis. Their study using 6 polymorphic loci, found significant differentiation of populations into two groups. It was further

suggested that differentiation may be due to a salt river system acting as an ecological barrier to pollinator movement.

Direct comparisons of RAPD and allozyme diversity are few. Peakall *et al.*, (1995) compared RAPD markers to allozyme markers in buffalograss. Using AMOVA analysis, they found that both markers gave similar qualitative patterns, large regional differences and significant population differentiation within regions for four populations. RAPDs however, detected more variation than allozymes. Dawson *et al.*, (1993) found patterns of RAPD variation in *Hordeum spontaneum* comparable to previously reported patterns using allozymes. Liu and Furnier (1993) using two species of *Populus* compared RAPD, RFLP and allozyme markers. They found that RFLP and allozyme markers gave comparable patterns within and between species, while RAPDs revealed more variation between individuals within a species than allozyme and RFLP markers, and produced reliable discrimination among clones. The findings reported here with *Banksia* are consistent with reports that RAPDs detect more variation than allozymes (Peakall *et al.*, 1995, Liu and Furnier 1993, Russell *et al.*, 1993, Dawson *et al.*, 1993). Furthermore, the study using RAPDs on *B. cuneata* shows nearly all variation is within populations rather than between. Russell *et al.*, (1993) also reported that RAPD diversity in *Theobroma cacao* was higher within provenances than between, a pattern typical for outcrossing woody plants (Hamrick and Godt 1989). Although allozymes are valuable for many types of studies, it is clear that DNA markers such as RAPDs will be widely used on plant population studies in the future, and will remain a valuable genetic tool.

Species relationships

For focused and efficient breeding efforts, species relationships need to be known in order to increase the probable success of hybridisation. Species relationships were investigated using three approaches, interspecific hybridisation (chapter 4), RAPDs (chapter 10) and DNA sequencing (chapter 11).

Interspecific hybridisation found that *B. coccinea* was more closely related to species in section *Oncostylis*, than species in section *Banksia*, where *B. coccinea* is currently placed. Due to the distinct morphology of *B. coccinea*, and the lack of compatibility with section *Banksia*, it was proposed to move *B. coccinea* into a new section *Coccinea*, containing only *B. coccinea*. Furthermore, it was suggested that the new section *Coccinea* is the sister section to *Oncostylis*, given the close compatibility of *B. coccinea* to this section, shown by fluorescence microscopy and seed set data. The close relationship of *B. coccinea* to section *Oncostylis* has been previously suggested based on morphological characters and cladistic analysis (George 1981, Thiele 1993).

RAPDs were assessed for phylogenetic signal at various levels within *Banksia*, and also between *Banksia* and the closely related genus *Dryandra*. RAPDs have been recently used in other genera, and have produced phylogenies which are in agreement with existing classifications based on morphology, cytology, and allozyme electrophoresis methods (Demeke *et al.*, 1992, Wilikie *et al.*, 1993). Debate continues as to which is the best method of analysis for RAPD data in phylogeny assessment, so two common methods of analysis were used on *Banksia* data. Both cluster analysis and phylogenetic inference using parsimony gave similar patterns in *Banksia*. Closely related species within series formed groups, and closely related species between series formed groups. These groups generally agreed with the current classification of *Banksia* based on morphology (George 1981, 1988). RAPDs were uninformative at higher levels between distantly related species. Interestingly, *B. coccinea* formed a group with species of *Dryandra*, indicating a close relationship to this genus. Interspecific hybridisation also showed some compatibility with *Dryandra*, with one cross showing more compatibility than some *Banksia* crosses. Subgenus *Isostylis* is thought to be more closely related to *Dryandra* than *Banksia*, however it was placed in genus *Banksia* with a note that a new genus may be appropriate (George 1981). Few morphological characters separate subgenus *Isostylis* from *Banksia* and *Dryandra*. Using RAPDs subgenus *Isostylis* grouped with *Musgravea*, included as the out-group, indicating more genetic distinctness than subgenus*Banksia* and

species of *Dryandra*. The three species of *Dryandra* did not separate as a group, and were indistinguishable from *Banksia*.

DNA sequencing of cpDNA (chapter 11) also showed the same trends observed with interspecific hybridisation and RAPD data. Using DNA sequence data, genus *Dryandra* was indistinguishable from *Banksia*. *B. coccinea* grouped with species of *Dryandra*, between the two sections of subgenus *Banksia*, section *Banksia* and *Oncostylis*. Resolution at the node between the sections with *B. coccinea* and *Dryandra* was poor using the region studied. A faster evolving region may be more appropriate to resolve the relationship between *B. coccinea* and *Dryandra*. DNA sequence data support the placement of *B. coccinea* into its own section, however its relationship to *Dryandra* is still unclear. DNA sequence data also support subgenus *Isostylis* as distinct to subgenus *Banksia*, with the two related species of subgenus *Isostylis* and *Dryandra formosa,* basal to subgenus *Banksia*. Both molecular and sexual compatibility data suggest that *Banksia* and *Dryandra* may be artificial genera, challenging the accepted view of two natural monophyletic, sister genera, with parallel morphological development.

In conclusion, this research contributes information important for conservation and exploitation of natural genetic resources for plant breeding. The relationship of *B. coccinea* to other species of *Banksia* is more clearly resolved, however this work raises important questions about the currently accepted view of *Banksia* and *Dryandra*.

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Appendix

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