Horticultural Management and Population Biology of several *Banksia* species.

By

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Horticultural management and population biology of several *Banksia* species.

ABSTRACT

The aims of this research are to improve understanding of the control of flowering in relation to photoperiod and temperature to increase knowledge of the floral initiation trigger for *Banksia*. An exploration of population biology in relation to genetic variation present in commercial and natural populations of *Banksia* will provide more information on the pool of variation available for breeding programs. Using molecular biology techniques to explore areas such as pollen competition and gene flow may improve our understanding of these important processes.

The floral initiation of *Banksia coccinea* and *Banksia hookeriana* were examined in relation to controlled environment conditions. Both species were subjected to four different treatments long (16h) and short (8h) daylengths at high (25/20°C) and low (15/10°C) temperature. During these treatments vegetative flushing, vegetative extension growth and flowering were measured. *Banksia coccinea* showed a significant response to photoperiod while *Banksia hookeriana* responded to temperature. The flushing pattern for the two species was similar with most flushing occurring in late spring. The most significant vegetative extension growth for both species occurred during the flush period of late spring/summer. *Banksia coccinea* under 16 hours and 25/20°C produced maximum average vegetative growth of 6 cm. This treatment also resulted in maximum flowering for *Banksia coccinea*. Contrastingly, maximum vegetative growth for *Banksia hookeriana* was observed at both daylengths and 25/20°C. The two high temperature treatments resulted in flowering at both 16h and 8h, but no flowering was observed for low temperature treatments.

A survey of genetic variation of *Banksia coccinea* and *Banksia menziesii* in eleven South Australian commercial and fourteen Western Australian natural populations was

conducted. DNA was extracted from leaf material and the random amplified polymorphic DNA (RAPD) technique was utilised. Individuals and populations were compared using the simple matching similarity index. Dendrograms were developed to establish relationships between natural and cultivated populations. Within cultivated populations similarity comparisons between individuals of *Banksia coccinea* ranged from 70.1 - 93.5%, while within natural populations values ranged from 63.5 - 93.4%. The highest similarity value between populations in South Australia was 96.0% while the highest similarity value between populations within Western Australian was 73.7%. The dendrogram constructed from the between population similarity values indicated that the South Australian populations are closely linked but also indicated some links to Western Australian populations. Within cultivated population comparisons of *Banksia menziesii* ranged from 54.5 - 80.8%, while within natural populations values ranged from 53.3 - 98.0%. The similarity value between populations in South Australia ranged from 65.9 - 91.8%, while the highest between natural population value was 93.9% with all other values above 80%. The dendrogram constructed from the between population similarity values indicated that the Western Australian populations are closely related but there were links indicated between the South Australia cultivated and some natural Western Australia populations.

Gene flow in a small population was examined for two species, *Banksia praemorsa* and *Banksia prionotes*. Open pollinated seed was collected from a number of inflorescences and the DNA extracted. The RAPD technique was used to identify the father of each seed and hence the frequency and direction of pollen and therefore gene flow. A survey of potential pollinators was also conducted to establish if pollinators were transferring pollen. Honeyeaters were the most common agents of the pollen transfer. Pollen competition experiments were carried out on *Banksia menziesii*. On a total of six plants, six inflorescences per plant were hand pollinated. One inflorescence was selfed, one was

crossed, two inflorescences had self pollen followed by cross pollen applied and in the last two the latter process was reversed. Mature seeds were collected after twelve months and the DNA extracted. The RAPD technique was applied to determine the status of the double pollinated seeds and confirm the status of the self and crossed seed.

DECLARATION

I declare this thesis contains no material which has been accepted for an award of another degree in any University or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I am willing to make this thesis available for loan and photocopy, when deposited in the University Library.

M A RIEGER

March 1996

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Chapter 1: Literature Review

1.1 Introduction

In recent years, due to increased industry demands, research has been initiated to further develop Australian native plants for the overseas floriculture market. Quality, consistency and reliability are all fundamental factors which affect any fresh horticultural product. To achieve these objectives, genetically selected plants or cultivars are essential with selection of important characteristics, followed by breeding. This procedure demands an intimate understanding of many plant processes including reproductive biology. Understanding reproductive processes such as pollination, self-incompatibility and seed set all enhance the likelihood of producing successful cultivars. Aspects of flowering, which includes flower initiation and development, are important with a floricultural product to maintain reliability of product supply. An understanding of plant genetics is also valuable when developing a cultivar. A measure of the genetic variability present enables relationships within and between populations to be assessed, but can also demonstrate the level of variability in existing cultivated plantations. Cultivar development is fundamental to improving all horticultural products and involves seemingly diverse fields, but when combined development is advanced.

An examination of the Australian floriculture industry is included in this review along with factors that affect floral initiation and development, reproductive biology and the application of DNA technology to all these areas. An exploration of Australian floriculture is presented to outline the important challenges faced by this expanding industry. Floral initiation and development is discussed and aspects which affect this process are considered. The integral problem of low fruit to flower ratio in

many hermaphrodite plants is discussed in relation to reproductive biology theories. Finally the structure of DNA, its discovery and some of the numerous molecular techniques which have been developed are presented.

1.2 Floriculture in Australia

Floriculture in Australia is divided into two separate markets; domestic and export. Domestic demand is dominated by exotic species with some consumer interest in native products. By contrast, native flora is the basis of the growing Australian export industry. Australian research investment in floriculture is minimal in relation to the potential profit from this lucrative industry. The various challenges for research include; cultivar development, improvement in post-harvest handling and storage, transport and product promotion, in addition to reduction in pests and diseases. Within the context of this discussion floriculture refers only to cut flowers; other floriculture products are not considered.

In 1990, the domestic floral trade within Australia was valued at $A270 million dollars (James, 1991). The domestic market is dominated by the production of exotic species such as roses, carnations, chrysanthemums and bulbs (Barth, 1986; Curtis, 1986; Maddock, 1986; Wrigley, 1988). Native flowers are also produced but the majority is exported (Lake, 1991).

1.2.1 Floriculture statistics

Limited statistical information is available in the area of floriculture, particularly for the domestic market. Western Australia has the largest proportion of the floriculture trade, earning $A11.2 million dollars in 1990/91, 55% of this derived from the domestic market (Fuss, 1992, per. comm.). Other states where information is available includes Queensland and Tasmania. In 1991, $A520,000 was earned

domestically in Queensland with the total industry estimated at $A2 million (Carson, 1992, per. comm.), while Australian native flowers and proteas earned Tasmania a total of $A2.2 million dollars in 1992 (Wemyss-Smith, 1992, per. comm.). The types of native flower available domestically appear to be very similar in these three States. Geraldton wax, *Chamelaucium uncinatum*, dominates in Queensland with $A270,000 dollars earned domestically (Carson, 1992, per. comm.); Tasmania and Western Australia grow a mixture of *Banksia* spp, kangaroo paw, Geraldton wax and *Telopea* spp (Fuss, 1992, per. comm.; Wemyss-Smith, 1992, per. comm.).

The international floriculture market was valued at approximately $A5.4 billion dollars in 1991, and Australia had 0.05% share of this market (James, 1991). The potential exists for a considerable expansion of the Australian share of this lucrative market. In the financial period '82/'83 to '85/'86 Australian exports trebled in volume, and exports to Japan increased rapidly, from $A458,000 in '85/'86 to $A4.5 million in the '89/'90 period. The overall export earnings from floriculture in Australia during the '84/'85 financial period was $A1.3 million compared with $A24 million in 1990.

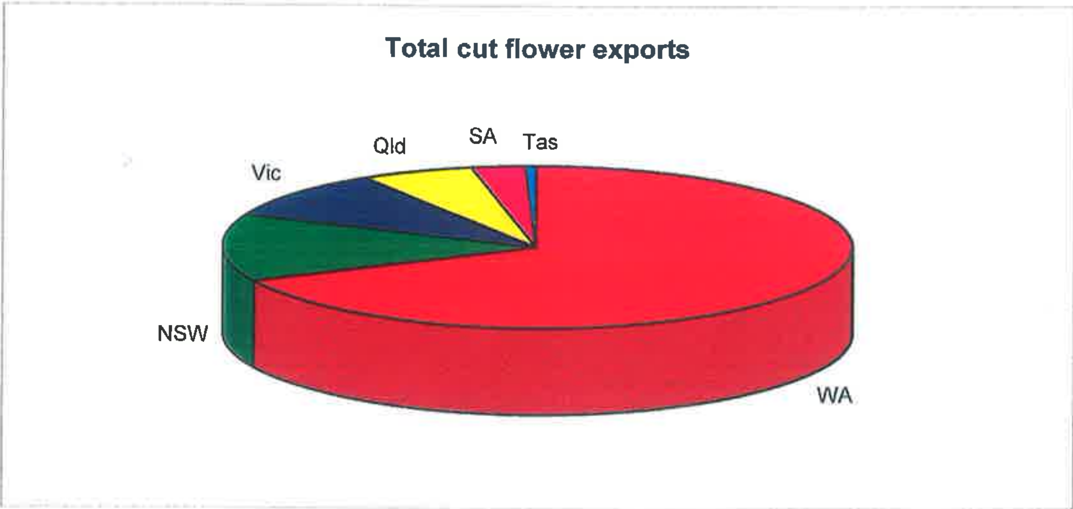
The majority of the $A24 million dollars of cut flowers exported in 1990 came from Western Australia (Figure 1.1). The largest export market for fresh material is Asia, followed by Europe and North America (Figure 1.2). For dried flowers Europe is the largest market, closely followed by Asia and North America (Figure 1.3).

Australian natives are not grown only in Australia. Many other countries including Israel, Germany, Italy, USA (California) and South Africa have also recognized the horticultural potential of these plants (Frith, 1990; Lake, 1991; Sharman, 1991).

Figure 1.1: Percentage of all cut-flowers exported from Australia (James, 1991).

Figure 1.2: The destination of exports of all fresh flowers from Australia expressed as a percentage (James, 1991).

Figure 1.3: The destination of exports of dried flowers from Australia expressed as a percentage (James, 1991).



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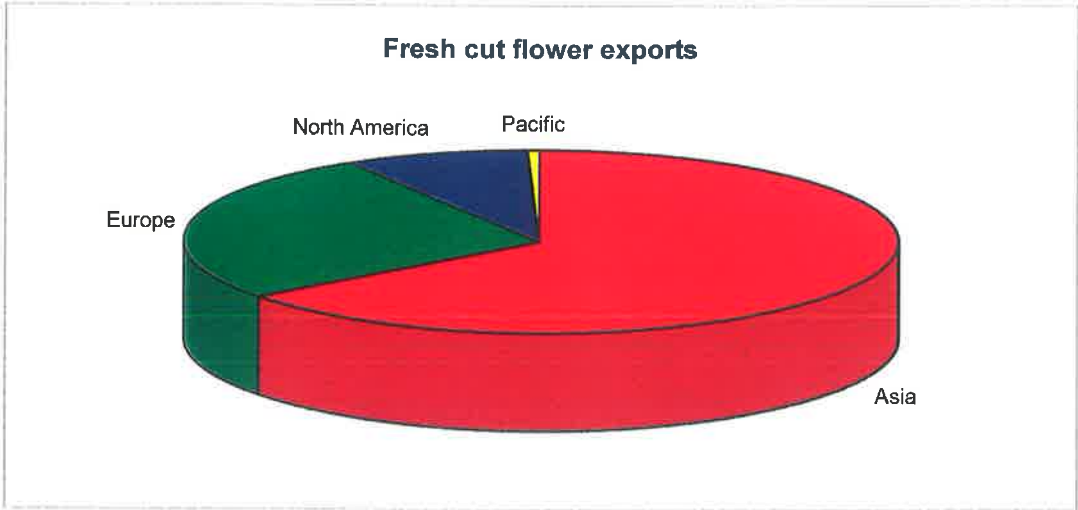

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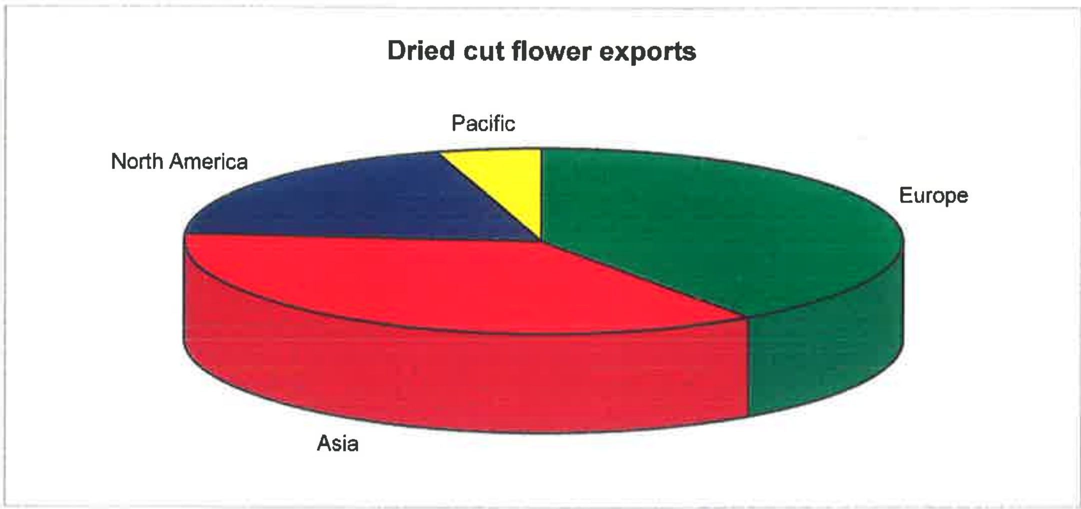

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1.2.2 Flower consumption in Australia

The consumption of flowers in Australia is low (Watkins, 1991) in comparison with the Europeans and Japanese. Australian consumers generally buy flowers only for special occasions, whereas Europeans tend to buy flowers regularly for home decoration. Increase in awareness, reduced costs and production of high quality flowers may increase Australian consumption in future. In the early 1980s Australians spent a per capita annual average of $A5 on flowers, which increased to $A12 by the mid 80's and reached a peak of $A25 in 1990 (Watkins, 1991). If this is compared with European countries, where the average per capita consumption of flowers has reached $A100 (Watkins, 1991) the low level of consumption in Australia is clearly illustrated.

1.2.3 Challenges in floriculture

Floriculture in Australia is a growing industry, problems still exist in both the domestic and export markets. Many research groups around Australia are attempting to address these challenges by developing new cultivars (Bowden, 1990; Oliver, 1990), improved harvesting techniques and post-harvest handling (Jacobs, 1982), improving transport and packaging techniques (Joyce, 1988) and reducing pests and diseases (Wood, 1988; Woods, 1988). The latter factors are particularly important in the export industry due to the strict demands of customs and quarantine regulations overseas. Consumers also expect high quality for the increased price paid for imported products (Faragher, 1983).

1.2.3.1 Cultivar development

Cultivar development of exotic species such as roses and chrysanthemums via breeding and selection has been underway for hundreds of years, resulting in a wide variety of choice to the consumer. Conversely the Australian native flower trade has

developed only in the last twenty years (Pegrum, 1988). The recent demand for this unusual flora prompted commercial utilization, with plants grown from wild seed and without cultivar development (Pegrum, 1988). The resultant product variability in the export industry led to low quality shipments and some plantation failures (Frith, 1990). This highlights the need for breeding and selection research to produce clonally propagated cultivars which consistently produce high quality flowers. This type of research has two main objectives:

(1) Improving the yield of the product.

(2) Improving the quality of the product. Quality includes criteria such as flower size, colour, growth habit and stem length. Quality is also subjective and depends on the consumer's perception.

A range of cultivars of Australian natives has already been produced including kangaroo paw (Oliver, 1990), paper daisy *Helichrysum bracteatum* (Knight, 1990), brachyscomes (Salmon, 1992) and some *Banksia* species (Stackhouse, 1990). Several others are being examined to assess their potential for cultivar selection including several *Banksia* spp. (Fuss and Sedgley, 1991c), sturt's desert pea, *Swainsona formosa* (Williams and Taji, 1991) and the fire daisy, *Ixodia achillaeoides* (Bennell and Jusatis, 1989). A preliminary investigation is underway on sturt's desert pea in relation to cultivar development. Areas such as plant propagation, reproductive biology and the collection of germplasm are being conducted by several different groups (Williams and Taji, 1991; Williams and Taji, 1987). An extensive selection program has been undertaken using the fire daisy at the Black Hill Flora Centre in Adelaide. Variation in this species is very high, in attributes such as plant form, leaf and floral characteristics. Superior forms will be selected, depending on consumer demand (Bennell and Jusaitas, 1989). Variation is also present in *Banksia coccinea*

and *Banksia menziesii*, as investigated by Fuss and Sedgley (1991c), enabling selection for superior forms. Others have study examined reproductive biology and hybridization techniques in relation to cultivar development (Fuss and Sedgley, 1991a; Fuss and Sedgley, 1991b).

1.2.3.2 Post-harvest techniques

Extensive information is available on the post-harvest requirements of the most common exotic species (Mayak *et al*, 1973; Veen, 1979; Halvey and Mayak, 1981; Woltering and Van Doorn, 1988; Spikman, 1989). Much of this early work relates to water stress, ethylene production and reasons for flower senescence (Halvey and Mayak, 1981). Specific problems have also been considered such as abscission of floral buds in *Delphinium* and *Lupinus* species, yellowing and darkening of foliage, and the problem of geotrophic bending which occurs in many flowers including gladiolus, lupin and some rose cultivars (Halvey and Mayak, 1981). Alternatively not much information is known about the post-harvest behaviour of the many Australian natives which make up the bulk of our exports (Faragher, 1989; Lake, 1991a). Research is being conducted on the carbohydrate status of several native species (Joyce, 1988). Other factors such as storage, effects of ethylene gas, rate of cooling and packing are also being investigated (Joyce, 1988). Shattering, or abscission of whole flowers from a stem is a major problem with Geraldton wax. Floral absission has been attributed to the accumulation of ethylene gas, and this problem has been overcome by pulsing the flowers with silver thiosulphate (STS) (Joyce, 1988). Also it was found that the vase-life of Geraldton wax was improved by a preservative solution containing sucrose and 8-hydroxyquinoline sulphate (Joyce, 1988). The vase-life of three different kangaroo paw cultivars was assessed in relation to STS and sucrose pulsing. A maximum extension to a vase-life of five weeks was achieved by

pulsing the cultivar 'Gold Fever' with 2.5% sucrose solution. STS was not as beneficial as sucrose pulsing in improving the vase-life of kangaroo paw following dry storage (Teagle *et al*, 1991). Preliminary trials with sturt's desert pea have shown that STS and sucrose are effective in extending vase-life (Barth, 1990). Dried material must also be handled post-harvest. Safe methods of drying and dying, ensuring retention of flower shape, are also be investigated by research (Joyce, 1988).

1.2.3.3 Pest and disease control

An additional challenge in floriculture in relation to post-harvest handling is the presence of pests and diseases in commercial plantations. The presence of insects in native export floral consignments is still a significant problem and methods to safely remove insects without damaging the floral product are being investigated (Joyce, 1988; Japan praises quarantine, 1990). Insects can cause whole shipments of flowers to be rejected (Joyce, 1988; Woods, 1988; Japan praises quarantine, 1990). A major cause of rejection of *Banksia* flowers is the presence of moth and weevil larvae. *Banksia* inflorescences are attacked by larvae of the moth *Arothcphora arcuatails*. The larva chews through into the inflorescence leaving several rows of flowers destroyed and thus unsuitable as a saleable product (Woods, 1988). Many types of insect use flowers as a major food source, and insects found in flowers include bees, wasps, ants, beetles and thrips (Woods, 1988; Bennell *et al*, 1989). There are various methods available to remove pests, such as fumigation, gamma irradiation, temperature and storage treatments, controlled atmospheres and aerosols. To minimise insect damage and reduce quarantine problems, insect species need to be identified in relation to the damage caused, and seasonal changes in insect numbers monitored (Seaton and Woods, 1990).

More serious damage can be caused by insects before harvest causing plant death. For example, Myrtaceous plants, particularly *Chamelaucium* and *Verticordia* are attacked by an as yet unidentified native weevil. The larva ring-barks the plants below the ground level, by feeding on the stem. Damage is not initially obvious, but death results if not treated (Wood, 1988; Woods, 1988). Research is urgently needed in this area to minimize plant damage and death from pests.

Diseases are just as important a threat to production as pests. Examples of diseases affecting the floral industry include *Phytophthora cinnamomi* and foliage diseases such as *Botrytis* and powdery mildew, *Oidium* spp. (Wood, 1988). *Phytophthora* spp. are fungi which attack the roots of many plants including banksias, boronias, geraldton wax and proteas from South Africa (Elphick, 1985; Lamont, 1985; Wood, 1988; Bennell *et al*, 1991). Limited control of this disease is possible, but cost is prohibitive. In badly affected areas, eradication is not possible without removal of all plants and soil fumigation (Elphick, 1985). Other soil-borne fungi such as *Verticillum* spp. and *Fusarium* spp. can cause stunting, yellowing and wilting and can ultimately kill some crops such as the fire daisy (Bennell *et al*, 1991). Foliage diseases are also prevalent in plants like kangaroo paw, for example ink disease caused by *Alternaria alternata*, and rust caused by *Puccinia haemadori* (Watkins, 1985), and in Geraldton wax *Botrytis cinerea* (Lamont, 1985). These diseases are promoted by warm, moist conditions, but fungicides can provide effective control (Watkins, 1985). Foliage diseases are less serious than soil-borne fungal diseases due to their ease of treatment and earlier detection. The major challenge in this area is the development of biological control and integrated pest management methods, which will reduce dependence on potentially dangerous chemicals.

1.2.4 The Proteaceae

The family Proteaceae is important in the Australian floriculture industry. The genera which have the highest profile include *Banksia, Telopea, Protea, Leucospermum* and *Leucadendron*. Australia is not the only location where flowers of the family Proteaceae are produced, as industries exist in California, Israel (Ben-Jaacov, 1986), Hawaii and South Africa (Elphick, 1985). This group of genera produce spectacular and unusual inflorescences which are much in demand around the world. World-wide sales of Proteaceae in 1989 amounted to $US12 million dollars, with Australia earning $US2.5 million, and North America and South Africa earning $US4 million each (Parvin, 1991). Increasingly growers are planting more proteas, and more research on these species is necessary to ensure high quality products to render Australia competitive. Until recently little was known about the reproductive biology of commercially produced banksias. This was addressed by Fuss and Sedgley (1990) in the areas of floral development in *Banksia coccinea* and *Banksia menziesii*, and of self-incompatibility and hybridization (Fuss and Sedgley, 1991a; Fuss and Sedgley, 1991b). Work on flowering in *Leucospermum* has been conducted to determine the best conditions for flowering (Zieslin and Gottesman, 1986), to delay flowering (Jacobs *et al*, 1986) and to improve quality and stem length using growth regulators (Napier *et al*, 1986).

Another area which can increase the quality of cut flowers is the improvement of post-harvest techniques. The quality of *Telopea speciosissma* was shown to be increased by refrigeration after harvesting (Faragher, 1986; Lill and Denis, 1986) and picking inflorescences at the immature stage extends the vase-life (Lill and Denis, 1986). Picking early, at the "soft-bud" stage, not only increases vase-life but can also reduce insect damage (Coetzee and Wright, 1986). Insects are a concern with all cut

flowers but have been continuing to trouble protea growers for 20 years or more (Seaton and Woods, 1990). In 1988 12% of *Leucadendron* spp., 57% of *Leucospermum* spp. and 59% of *Protea* spp. did not pass quarantine inspection in Japan due to the presence of insects (Seaton and Woods, 1990).

The future prospects of the Proteaceae in floriculture looks promising at the present time, with a high level of interest in the unique nature of the flora and premium prices. As emphasised above continuing research is needed to ensure consistent flower quality once the novelty and high prices have both diminished.

1.3 Ecology and conservation of native Australian flora

Native Australian flora is important to the continuing survival of the Australian floriculture industry. This growing industry is dependent on conservation of native flora to maintain its existence and enable the development of new varieties and cultivars. Four fundamental arguments have been developed which illustrate why all species should be conserved (McMicheal, 1982; Ellyard, 1987; Briggs and Leigh, 1988; Benson, 1990).

(1) To preserve the right of all species to exist;

(2) The beauty, symbolic value and interest of all species should be preserved;

(3) The economic value of species should be considered, for example drugs and/or food may be discovered from yet unexamined plants and animals;

(4) The ecological benefit of species has an indirect benefit to humans. Other species are part of the overall ecosystem in which we live, and without this system humans would be unable to survive.

In the past five to ten years there has been a growing realisation in Australia that the native flora is important, and that steps need to be taken to conserve this unique vegetation.

1.3.1 The state of Australia's flora

The total number of native vascular plant species in Australia is estimated at about 20-25,000 (Briggs and Leigh, 1988; Benson, 1990), of which only 16,000 species have yet been described in 262 families (Benson, 1990). Few of the plant families are unique to Australia, but 80% of species are endemic (Good and Lavarack, 1981; Benson, 1990). The addition of the non-vascular plants elevates this number to 50,000 native plant species occurring in Australia.

In 1988 a total of 3329 taxa were listed as rare or threatened on the Australian continent (Briggs and Leigh, 1988). The majority of these plants are from Western Australia (Table 1.1), closely followed by Queensland and New South Wales, while the other States, and the Northern Territory, have about the same level of threatened species. Of the 176 families (Table 1.1), most have one or more species listed and sixteen families account for the majority (65%) of the species presented (Table 1.2). The number and area of nature conservation reserves is important when discussing the present state of native flora. Table 1.3 shows that Western Australia and South Australia have the largest areas of conservation reserves, with between 1 and 4 million hectares elsewhere and the smaller states and territories, Tasmania and the ACT, having a lesser area in reserves (Mobbs, 1988). Of all the species listed as rare or threatened, 1719 (53.2%) occur in national parks or reserves. Of these 231 (13.4%) are inadequately conserved and for 1160 (67.5%) there is no estimate available of the population size (Briggs and Leigh, 1988). Thus there are problems with the

Table 1.1: Number of rare or threatened families, genera and species listed for Australia and for each state and territory.

	Families	*Genera*	*Species*
Australia	176	876	3329
New South Wales	87	236	532
Northern Territory	51	109	179
Queensland	144	529	1059
South Australia	45	98	189
Tasmania	46	85	136
Victoria	44	100	180
Western Australia	79	327	1442

Table 1.2: Families which have the highest proportion of species listed on the rare and threatened list (Briggs and Leigh, 1988).

Family	*Species number*	*Family*	*Species number*
Myrtaceae	441	Poaceae	97
Proteaceae	300	Cyperaceae	74
Fabaceae	212	Euphorbiaceae	71
Mimosaceae	173	Laminaceae	67
Orchidaceae	170	Goodeniaceae	54
Asteraceae	135	Rhamnceae	54
Rutaceae	122	Sterculiaceae	54
Epacridaceae	103	Liliaceae	51

Table 1.3: The total number of reserves and the area in hectares in each state and territory in Australia (Mobbs, 1988).

State	*Total numer of reserves*	*Area (hectares)*
Austrailan Capital Territory	6	112 242
New South Wales	438	3 812 165
Northern Territory	90	4 023 564
Queensland	574	3 663 769
South Australia	279	11 117 167
Tasmania	227	966 997
Victoria	362	1 829 983
Western Australia	1 247	15 252 213

conservation of the species listed as rare or threatened, but the first step of identifying these species has been taken.

1.3.2 Threats to Australian flora

The most visible effects on the Australian vegetation have been agriculture, and grazing by both domestic and feral animals (Scarlett and Parsons, 1982; Leigh *et al*, 1984). Other threats include introduced plants, animals and diseases, forestry and logging, roadworks, mining, urbanisation, fire, small scale commercial exploitation and the pollution of watercourses (Leigh *et al*, 1984; Harris, 1986; Witkowski *et al*, 1990; Benson, 1990).

Agriculture and grazing affects approximately 65% of the Australian continent (Leigh *et al*, 1984; Harris, 1986), and clearing has resulted in the removal of trees and the loss of native grasses and bushes. These have been replaced in South Australia by introduced pasture grasses, annual legumes (clover, medics), and weeds such as silver grass *Vulpia bromoides* and barley grass *Hordeum leporinum* (Harris, 1986). The main domestic grazers are sheep and cattle but feral rabbits, goats, camels, donkeys and horses have also substantially affected the native vegetation (Leigh *et al*, 1984; Benson, 1990). Overall, 72% of extinct species, and 85% of endangered species, are believed to have been adversely affected by agriculture and grazing (Leigh *et al*, 1984).

The introduction of exotic plants and diseases over the years is a further threat (Leigh *et al*, 1984). Examples of introduced plants include gorse *Ulex europaeus*, privet *Ligustrum* spp., prickly pear *Opuntia* spp., and blackberry *Rubus procerus*. These plants were first introduced by the early settlers for various reasons such as for fruit, in the case of blackberry, or for hedging, in the case of privet and prickly pear (Leigh *et al*, 1984). Once released into the bush, these plants competed successfully

with native plants. gorse and blackberry still cause problems in conservation reserves (Leigh *et al*, 1984). The major introduced disease, which has caused immeasurable devastation, is the soil-borne fungus *Phytophthora*. Seven species have been identified but the main problem is caused by *Phytophthora cinnamomi* (Shearer *et al*, 1991). This species has affected jarrah forests, banksias and the heath communities in Western Australia. The fungus has been spread by off-road vehicles and the construction of fire breaks and roads (Shearer *et al*, 1991), and has the potential to destroy many of the important species in unique ecosystems which occur only in Western Australia.

Unfortunately, increasing interest in native flora has put some species under further pressure (Good and Lavarack, 1981; Witkowski *et al*, 1990; Benson, 1990). Material is collected from the bush for cut-flowers, cones, seeds and landscape plants. There are sixty-eight species which are known to be commercially exploited; of these thirteen are endangered all in the south-western area of Western Australia (Good and Lavarack, 1981). An example of plants taken whole for landscaping is tree ferns from the families Cyathaceae and Dicksoniaceae (Good and Lavarack, 1981).

There are many threats to the continuing existence of the current diversity of native flora. In some of the areas discussed, some efforts are being made by government and other bodies to decrease these threats, such as limits to the exploitation of wild plants, and limiting the importation of exotic plants and diseases. At the present time, bush picking in Western Australia is strictly controlled by the Department of Conservation and Land Management .

1.3.3 The role of commercial floriculture in conservation and ecological research

Floriculture tends to raise the profile of a particular species and in turn stimulates research on that species. This can clearly be seen with the *Banksia* genus. In recent years research has included examination of pollinators, breeding systems, nutrient allocation, insect control and some work on horticultural management (Scott, 1980; Kuo *et al*, 1982; Mitchell and Allsopp, 1984; Ramsey, 1989; Vaughton, 1990a; Vaughton, 1990b; Cunnigham, 1991; Fuss and Sedgley, 1991a; Vaughton and Ramsey, 1991). Studies directly related to the conservation issue have also been stimulated (Witkowski *et al*, 1990; Low and Lamont, 1986; Cowling and Lamont, 1986) and the harvesting of *Banksia* inflorescences and cones from wild populations has generated interest in the management and health of these communities. Cowling and Lamont (1986) used preliminary data to stress that harvesting from plant communities should be dependent on seed bank dynamics, variation in the production of cones, and seed viability of the individual species. These authors also showed that the fire regime can profoundly affect plant recruitment, and incorrect fire management has the capacity to drive some species to extinction. The long-term impact that harvesting from the bush has on a species is crucial when trying to exploit but conserve at the same time. *Banksia hookeriana* was examined to determine how harvesting affects long-term community viability (Witkowski *et al*, 1990). The distribution of the species was examined along with phenology, seed bank dynamics, and the post-fire establishment and survival of seedlings. The preliminary data showed that there was an impact on subsequent inflorescence and seed production, with a great reduction in seeds available for the next generation. Another important impact that the removal of plant material can have on a plant community is reduction in

available nutrients (Low and Lamont, 1986). Once again only preliminary data is available, but they suggest that wildflower picking and frequent burning combined, can severely deplete nutrient reserves. A moderate picking level of about 10% per season may remove 4.1% nitrogen and 9.6% phosphorous from individual bushes of *Banksia hookeriana* (Low and Lamont, 1986), and thus from the ecosystem.

This research has shown that floriculture has been an important stimulus to understand more about native plant communities and to change practices to help conserve a valuable resource.

1.4 Reproductive biology

The fact that many out-crossing animal-pollinated hermaphroditic flowering plants have a relatively low fruit to flower ratio, or fruit set, has interested many workers in the field of reproductive biology (Lovett-Doust and Caver, 1980; Stephenson, 1979; Stephenson, 1981; Sutherland and Delph, 1984; Sutherland, 1986). When first examined this phenomenon appears to be a paradox, as resources appear to be wasted on excess flowers which do not develop fruits. A number of hypotheses has been developed which suggest that these excess flowers can nevertheless be important in reproduction:

(1) **Pollinator limitation:** The production of fruits may be limited by insufficient pollen transfer, indicating low pollination success which could be due to pollinator limitation (Stephenson, 1979; Stephenson, 1981; Snow, 1982; Sutherland, 1986; Lalonde and Roitberg, 1989).

(2) **Biological limitation**: Low pollination success may be due to biological aspects such as low stigma receptivity, inviable pollen, and self-incompatibility. (Fuss and Sedgley, 1991a; Ramsey and Vaughton, 1991; Vaughton and Ramsey, 1991)

(3) **Pollinator attraction**: An extensive floral display provides a strong signal advertising potential reward to the pollinator. This may explain the frequency of plants with excess flowers (Stephenson, 1979; Willson and Schemske, 1980; Stephenson, 1981; Sutherland, 1986; Brunet, 1992). The out-crossing nature of many hermaphroditic plants means that there is a dependence on pollinators moving between male and female floral organs to ensure cross-pollination. This may result in the need for a large visible floral display.

(4) **Bet-hedging**: This hypothesis proposes that when a large number of flowers is produced, this allows plants to compensate for variations in environmental conditions such as resource availability, and pollinator number or visitation rates (Stephenson, 1979; Stephenson, 1981; Sutherland, 1986; McGinley *et al*, 1987; Fox and Stevens, 1991). If the conditions are right a large number of flowers will be available to be pollinated.

(5) **Selective abortion**: When the success of pollinators is high and more fruits are initiated than can be matured, it is hypothesized that plants can selectively abort some fruits. Thus the plant matures only fruits which are of the highest quality either in terms of the number of ovules or the genetic constitution of the seeds (Stephenson, 1979; Stephenson, 1981; Stephenson and Winsor, 1986; Sutherland, 1986; Marshall and Folsom, 1991). Key points to note with this hypothesis are the requirement for a high level of pollination success, and the fact that resources and spatial limitation may affect the number of fruits matured.

(6) **Pollen donation**: There are two components which determine the fitness of a hermaphroditic plant; female- fruit maturation and male- pollen donation. Flowers which produce fruits contribute to both female and male function, but flowers which do not produce fruits contribute only to male function, through pollen donation. The

production of excess flowers may be favoured if the male function is increased through pollen donation and outweighs the loss of female function (Stephenson, 1979; Stephenson, 1981; Sutherland, 1986). Limited information is available on the extent of male function (Horovitz, 1978; Charnov, 1979; Willson, 1979; Roach and Wulff, 1987; Brunet, 1992), which is difficult to accurately measure compared with female function.

Considerable research has been conducted concerning these hypotheses, the emphasis being focussed on examining northern hemisphere species (Snow, 1982; Sutherland and Delph, 1984; Stanton *et al*, 1986; Nybom, 1987; Sutherland, 1987; Newport, 1989; Zimmerman and Aide, 1989; Rocha and Stephenson, 1991; Brunet, 1992). Preliminary work has been carried out in the southern hemisphere and many researchers have chosen to concentrate on the family Proteaceae which displays extremely low levels of fruit set (Lewis and Bell, 1981; Scott, 1982; Whelan and Goldingay, 1986; Enright and Lamont, 1989).

1.5 Reproductive biology of the Proteaceae

Plants of the family Proteaceae are noted for their extremely low level of fruit set, particularly the genus *Banksia*. Table 1.4 presents a summary of a number of species which have been examined for fruit set. The percentages range from a maximum of 20.0% down to 0.2% fruit set. Two alternative methods were used to determine fruit set, the first method involved measuring natural productivity. The parameters were the mean number of follicles per infructescence and the mean number of flowers per inflorescence. The mean number of follicles is divided by the

Table 1.4: Follicle and seed set of various Australian Proteaceae expressed as a percentage.

Species name	*Fruit set %*	*Source*
Banksia attenuata	0.51	Lewis and Bell (1981)
Banksia attenuata	0.33	Scott (1982)
Bankisa attenuata	0.9	Whelan and Burbridge (1980)
Banksia baxteri	0.3	Witkowski *et al* (1991)
Banksia coccinea	0.9	Witkowski *et al* (1991)
Banksia cuneata	1.79	Lamont *et al* (1991)
Banksia grandis	2.61	Scott (1982)
Banksia grandis	3.4	Whelan and Burbridge (1980)
Banksia ilicifolia	2.03	Scott (1982)
Banksia ilicifolia	1.6	Whelan and Burbridge (1980)
Banksia littoralis	6.95	Lewis and Bell (1981)
Banksia littoralis	3.9	Scott (1982)
Bankisa littoralis	6.5	Whelan and Burbridge (1980)
Banksia menziesii	0.81	Lewis and Bell (1981)
Banksia menziesii	0.45	Scott (1982)
Bankisa menziesii	0.4	Whelan and Burbridge (1980)
Banksia paludosa	20.0	Whelan and Goldingay (1986)
Banksia speciosa	0.2	Witkowski *et al* (1991)
Banksia spinulosa	6.4	Vaughton (1988)
Banksia telmatiaea	6.68	Lewis and Bell (1981)
Banksia telmatiaea	4.29	Scott (1982)
Grevillea leucopteris	5.0	Lamont (1982)

mean number of flowers and multiplied by 100 giving an estimate of the potential fruit set. The second method involved experimentation using hand pollination. Four standard treatments were used, these included automatic self (autogamy), self (xenogamy), cross (geitogamy), and open pollination. Inflorescences were pollinated multiple times during the flowering period to ensure maximum pollination and the subsequent seeds were used to determine fruit set. This method was used by Whelan and Goldingay (1986) and Vaughton (1988). Variations in fruit set, for the same species, are shown by this comparison, and this may be the result of the different methods employed, or varying habitats and environmental conditions when fruit set was determined. Having established the low level of fruit set observed in this southern hemisphere family, an examination of research in this area will discuss the validity of the previously presented hypotheses, in relation to the family Proteaceae. Hypotheses to be discussed include pollinator and biological limitation, bet-hedging, pollen donation, and selective abortion.

1.5.1 Nutrients and fruit set

The resources which are required by plants to produce both inflorescences and subsequent infructescences and seeds will be considered under the bet-hedging hypothesis, as variation in the resources available may result in variable fruit set. Members of the Proteaceae generally produce seeds which are rich in organic reserves and essential mineral nutrients, particularly phosphorus (Mitchell and Allsopp, 1984). The natural habitat of the Proteaceae has been characterized as being nutrient poor sandy soil. For example, in south-western Australia, which has over 500 proteaceous species, many are restricted to sandy soils which are extremely deficient in nutrients, particularly phosphorus (Kuo *et al*, 1982). Eight species from this area were sampled for levels of organic and inorganic nutrients present in their seeds. All the seeds

carried large reserves of oil, protein, essential mineral nutrients, especially phosphorus and trace elements (Kuo *et al*, 1982). Similarly ten species of *Grevillea* also proved to have high concentrations of phosphorus in their seeds, with over 90% of the phosphorus content of the infructescence located in the seeds (Hocking, 1986). Approximately 50-70% of the nitrogen, zinc and copper content was also located in the seeds. By comparison, leaves and the leathery follicles which enclose the *Grevillea* seeds were typically very low in these nutrients (Hocking, 1986). *Hakea sericea* was also found to have considerable phosphorus reserves in the seeds. The level was comparable to *Banksia* and *Grevillea* but higher than that found in *Protea, Leucodendron* and *Leucospermum* (Mitchell and Allsopp, 1984). These studies suggest that the seeds of most plants belonging to the family Proteaceae accumulate high levels of nutrients in their seeds, despite low soil levels.

Is fruit set affected by the level of nutrients provided to the plant, and how does this relate to the bet-hedging hypothesis? Stock *et al* (1989), using *Banksia laricina,* found that addition of nutrients in 1985/86 increased the number of infructescences produced in 1986/87, but this increased number was not statistically significant. The variation in infructescence number may have been the result of factors unrelated to nutrient application. Factors such as pollinator number, flower number and environmental conditions such as rainfall and predation could result in a variation in infructescence number. This highlights the difficulties of determining if excess flowers are produced to cope with environmental variation, as specific factors are difficult to isolate. Stock *et al* (1989) also suggested that there may be competition for resources between inflorescences, and ultimately infructescences. Greater reproductive success, 74% fruit set, occurred with only one inflorescence per plant,

compared with two or more inflorescences per plant, which achieved only 34% fruit set.

Further work is needed on nutrient addition and how this affects fruit set under controlled conditions, as no conclusive results have yet been achieved. The results which are available are confusing due to uncontrolled factors which may be affecting the experimental outcome. Also, nutrient addition needs to target a wide range of different species from the Proteaceae family which inhabit different environments to establish the level of response exhibited by different species. As Stock *et al* (1989) illustrates, natural yearly variation in inflorescence and hence infructescence number also need to be considered.

1.5.2 Insect and bird damage and fruit set

Damage by insects and predation on infructescences indirectly affects fruit set of plants and is related to the bet-hedging hypothesis. Producing more inflorescences and infructescences ensures that some seeds may escape predation. The level of damage to fruiting structures has been assessed by various workers. Vaughton (1990a) determined that damage to seeds and inflorescences halved the number of seeds each plant of *Banksia spinulosa* var. *neoanglica* could produce. Up to 60% of follicles from six *Banksia* spp. were destroyed by insects, and almost one third of inflorescences were destroyed, preventing any fruits being set (Scott, 1980). An average of 84% of the stored seed reserves of *Protea repens* were destroyed by insects in a two year period, and in the third year of the study the number of seeds surviving predation declined to almost zero (Coetzee and Giliomee, 1987). In contrast, there are some reports of minimal or negligible damage resulting from insect predation (Lamont and Barker, 1988; Lamont and Barrett, 1988; Stock *et al*, 1989).

Insect damage, or the presence of insect larvae in infructescences, can result in predation by birds, mainly cockatoos. Lamont and Van Leeuwen (1988) examined the rare Western Australian species *Banksia tricuspis* to assess factors limiting the occurrence of this species and found that 58% of inflorescences were damaged by both insect larvae and cockatoos, while a further 12% per plant were damaged by birds alone. The authors felt that, although this damage appears high, the predation by the cockatoos is beneficial as the population of insects is controlled (Lamont and Van Leeuwen 1988). Scott and Black (1981) also studied cockatoo predation and found that these birds selectively destroyed infested infructescences when searching for weevil larvae, even though some infructescences without larvae were also damaged.

Researchers have established that high levels of insect damage can affect the overall level of fruit set (Zammitt and Hood, 1986; Vaughton, 1988, Wallace and O'Dowd, 1989). The easiest way to decrease insect damage is by application of insecticide. When insects were completely excluded from *Banksia spinulosa* var. *neoanglica* by insecticide a 45% increase in the number of inflorescences which produced fruit was observed (Vaughton, 1988), with an increase of 28% more follicles per infructescence. Interestingly the proportion of aborted seeds did not differ between these two treatments. Vaughton, (1988) established that the insecticide treatment did not affect the seed weight or the reproductive output in the next flowering season. Even though insecticide treatment increased fruit set, 18% of inflorescences still failed to produce seeds (Vaughton, 1988). A similar result was achieved using *Banksia ericifolia*, where the number of inflorescences setting fruit doubled and a 40% increase in the number of seeds produced per inflorescence was achieved. Once again there was a percentage (50%) which remained barren, despite this treatment (Zammit and Hood, 1986).

A more involved study coupled two factors affecting fruit set, nutrients and insect damage in the species *Banksia spinulosa* (Wallace and O'Dowd, 1989). Insecticide and nutrient treatments were applied separately. The insecticide treatment eliminated damage to the developing inflorescences, while the micronutrient application increased the level of nutrients in the leaves. Only the joint application of micronutrients and insecticide increased fruit set per inflorescence from 2.5% to approximately 8%, which proved to be statistically significant (Wallace and O'Dowd, 1989). This suggests there is an interaction between nutrients and insect damage in this particular situation.

These studies support the bet-hedging hypothesis which theorises that numerous factors are combining to produce excess flowers to compensate for variation in seasonal conditions.

1.5.3 Breeding system and fruit set

The breeding system of a plant is important in a number of the proposed hypotheses. A plant's breeding system is relevant when discussing pollen donation, as the pollen donation hypothesis infers an out-crossing breeding system. Indirectly, breeding system relates to all the hypotheses involving pollination, including pollinator and biological limitation. A summary of a number of breeding system studies is presented in Table 1.5, with the presence of autogamy (automatic self-fertilization), geitongamy (self-pollination from a different flower on the same plant) and xenogamy (cross pollination) expressed as either positive or negative. In a number of cases partial self-compatibility occurs, which is expressed as a combination of positive and negative. The method by which the authors gained the information is also presented, to enable comparison of the information from the different studies.

Table 1.5: Breeding systems of some Australian members of the family Proteaceae. Symbol describtion; Not investigate (NI).

Plant species	*Autogamy*	*Geitongamy*	*Allogamy*	*Methods*	*Source*
Banksia attenuata	-	-	+	Allozymes	Scott (1980)
Banksia coccinea	-	-/+	-/+	Hand pollination and pollen tube growth	Fuss and Sedgley (1991)a
Banksia elegans	-	-	+		Lamont and Barrett (1988)
Banksia ericifolia	-	-	+	Hand pollination and seed set	Paton and Turner (1985)
Banksia ericifolia	-	-	+	Hand pollination and pollen tube growth	Goldingay ***et al*** (1991)
Banksia menziesii	-	-	+	Allozymes	Scott (1980)
Banksia menziesii	-	-	+	Hand pollination and seed set	Ramsey and Vaughton (1991)
Banksia paludosa	-	NI	+	Hand pollination and seed set	Whelan and Goldingay (1986)
Banksia paludosa	-	-	+	Allozymes	Carthew ***et al*** (1988)
Banksia paludosa	-	-/+	-/+	Hand pollination and seed set	Goldingay and Whelan (1990)
Banksia prionotes	-	-	+	*In vivo* pollination and pollen tube growth	Collins and Spice (1986)
Banksia spinulosa var. *neoanglica*	+	+	+	Hand pollination and seed set	Vaughton (1988)
Banksia spinulosa var. *neoanglica*	+	NI	NI	Hand pollination and seed set	Vaughton and Ramsey (1991)
Banksia spinulosa var. *spinulosa*	-	NI	+	Hand pollination and seed set	Whelan and Goldingay (1986)
Banksia spinulosa var. *spinulosa*	-	-	+	Hand pollination and seed set	Goldingay and Whelan (1990)
Banksia spinulosa	-	-	+	Allozymes	Carthew ***et al*** (1988)
Grevillea leucopteris	+	+	NI	Exclusion experiments and seed set	Lamont (1982)
Macadamia cv. Kakea	-	-/+		Hand pollination and pollen tube growth	Sedgley (1983)
Macadamia cv Keahou	-	-/+		Hand polliantion and seed set	Sedgley (1983)
Macadamia cv Hinde	-	-/+		Hand pollination and seed set	Sedgley (1983)
Macadamia cv Renown	-	-/+		Hand pollination and seed set	Sedgely (1983)
Telopea speciosissma	-	-		Hand pollination and seed set	Whelan and Goldingay (1989)

In this group of species from the family Proteaceae autogamy appears to be uncommon. *Banksia spinulosa* var. *neoanglica* and *Grevillea leucopteris* show a relatively high seed set of around 6% due to automatic self-fertilization and in a number of species a combination of selfing and out-crossing is possible. The majority of the species presented in this table however are fully xenogamous or self-incompatible. These species are therefore completely reliant on pollinator activity to achive fruit set.

Variability of methods and populations used to determine the breeding system is very important, as illustrated by *Banksia paludosa*. Carthew *et al*, (1988) determined that this species was completely xenogamous using allozymes, but Goldingay and Whelan (1990) concluded that this species was partially self-compatible using hand pollinations and seed set to determine the breeding system. Allozymes are very reliable when large samples are taken. Carthew *et al* (1988) sampled 10-20 progeny using 4 polymorphic enzymes and Scott (1980), used lower numbers of polymorphic enzymes but up to three hundred progeny were assessed. Allozymes can be used to determine if autogamy or xenogamy is the predominant breeding system, but cannot clearly assess the level of partial compatibility. Hand pollination treatments may also have difficulty assessing partial compatibility. The final seed product may be influenced by uncontrollable factors such as resources available, quality of the pollen applied, problems with the pollination technique, including pollen inviability, or the stigma not being receptive at the time of pollination. Some of these problems have been shown to occur, particularly in *Banksia* (Fuss and Sedgley, 1991a; Fuss and Sedgley, 1991b; Ramsey and Vaughton, 1991; Vaughton and Ramsey, 1991). A close examination of pollen tube growth can determine the level of pollen-pistil self-compatibility. The method of assessment used by Fuss and

Sedgley (1991a), using *Banksia coccinea* included investigation using scanning electron microscopy, controlled hand pollinations, to examine the timing of stigma receptivity and pollen tube growth of different pollen types. A 5x5 diallel experiment was also used to assess incompatibility and combining ability, and showed a considerable level of follicle production following selfing, 27.9% compared with 40.7% following cross pollination. A combination of all available methods (hand pollination, examining pollen tube growth, seed set and allozymes) would give the most reliable assessment of breeding system. Once the breeding system has been accurately determined, some idea about the potential cause of low fruit set can be addressed. For example, in the cases of *Banksia coccinea*, which was found to show partial self-compatability (Fuss and Sedgley, 1991a), problems which may arise from this are pollen competition and, ultimately, competition between self and crossed zygotes for limited resources. In the case of a completely xenogamous species, number or pollinator activity may be limiting fruit set by inefficient transfer of compatible pollen. So, breeding system analysis is important when assessing the problem of low fruit set in any situation.

1.5.4 Pollinators and fruit set

The members of the family Proteaceae are pollinated by a variety of pollinators, which includes birds, honeyeaters of many species (Paton and Ford, 1977; Collins and Spice, 1986; Whelan and Burbridge, 1980; Ramsey, 1989; Vaughton, 1990), small non-flying mammals (Rourke, 1980; Goldingay *et al*, 1987; Cunningham, 1991), insects and introduced honeybees (Paton and Turner, 1985; Ramsey, 1988a). This discussion will be confined to the behaviour of pollinators which relates to the low fruit set observed in this family.

Several researchers have attempted to investigate the hypothesis that low fruit set is due to pollen limitation. Pyke (1982) found that when additional pollen was applied to *Lambertia formosa*, the level of fruit set was not increased. Supplementary pollination of *Banksia ericifolia* and *Banksia spinulosa* also failed to increase fruit set above the natural level (Paton and Turner, 1985; Whelan and Goldingay, 1986; Goldingay and Whelan, 1990). Two separate studies using the same species, *Banksia ericifolia*, by the same authors found similar results. In contrast *Banksia paludosa* displayed increased fruit set with additional pollen which increased the number of inflorescences that set seed from 20% in the control to 45% (Whelan and Goldingay, 1986) and similar results four years later (Goldingay and Whelan, 1990), when 15% of the controls produced fruit while an increase to 30% was achieved by supplementary pollination. The pollen addition experiments indicate that the natural level of pollination of this species limits fruit set (Whelan and Goldingay, 1986). Interestingly the number of follicles per non-barren inflorescence is not altered significantly by any of the treatments. *Telopea speciosissima* also exhibited an increase when naturally pollinated inflorescences were given additional pollen (Whelan and Goldingay, 1989) resulting in 91% fruit set of supplemented inflorescences compared with 52% in the open-pollinated controls. Even though supplementary pollination increased fruit set, only 2.9% of the total number of inflorescences produced fruit. This indicates that pollen limitation is not the only factor which is affecting fruit set.

1.5.5 Spatial limitations

A number of factors may be affecting pollen supplementation experiments and therefore producing misleading results. The finding by Whelan and Goldingay (1986) that the number of follicles of *Banksia paludosa* per non-barren inflorescence as not altered significantly by the treatments suggest a physical limit to follicle number. The number of follicles which are able to develop on an inflorescence may be spatially limited regardless of the level of pollination received above a certain threshold. This was found for *Banksia menziesii*, the maximum number of follicles which would fit being 35 per infructescence from an inflorescnece with up to 700 flowers (Fuss and Sedgley, 1991b). This value was calculated after a number of measurements including infructescence length, infructescence diameter, follicle length and follicle diameter were statistically analysed (Fuss and Sedgley, 1991b).

1.5.6 Stigma receptivity

Another factor which appears to be important in the process of pollination, and therefore pollen and biological limitation, is the receptivity of the stigma. The importance of stigma receptivity in *Banksia* species has been investigated by a number of authors (Fuss and Sedgley, 1991a; Fuss and Sedgley, 1991b; Vaughton and Ramsey, 1991). Three species, *Banksia coccinea*, *Banksia menziesii* and *Banksia spinulosa* var. *neoanglica* were found to achieve maximum receptivity three to four days after the flowers open. All these studies examined pollen tube growth at various times. Vaughton and Ramsey (1991) continued this experiment to a peak at three days, whereas both of the other studies continued to observe stigma receptivity until twelve days after anthesis. This approach clearly shows a peak by three days, which declines rapidly by nine days. Observations of the stigmatic groove, using scanning electron microscopy, of *Banksia coccinea* and *Banksia menziesii* found the widest

opening at three days after anthesis, which coincides with peak stigma receptivity (Fuss and Sedgley, 1991a; Fuss and Sedgley, 1991b).

1.5.7 Pollen viability

Pollen donation, or male function, has interested some researchers in the northern hemisphere (Ockendon and Currah, 1977; Horovitz, 1978; Willson, 1979; Marshall and Ellstrand, 1986; Stanton, 1986; Stanton *et al*, 1986; Roach and Wulff, 1987; Thomson, 1989; Aizen *et al*, 1990; Richards and Stephenson, 1991; Rocha and Stephenson, 1991; Snow and Spira, 1991a; Snow and Spira, 1991b), with recent interest in this fundamental process in the southern hemisphere. Vaughton and Ramsey (1991) and Ramsey and Vaughton (1991), examined biological aspects of the pollen itself. The viability of the pollen is important when discussing pollen donation as if viability is limited this will affect the pollination process. The viability of *Banksia spinulosa* var. *neoanglica* pollen was found to drop from 80% when the flowers first opened to 58% by eight days, with a further drop to only 33% by twelve days (Vaughton and Ramsey, 1991). This drop in viability suggests that only a short time is available for successful pollen movement between plants. Pollen viability was assesed by using different aged pollen to pollinate numerous flowers on an inflorescence and the number of germinated grains within the stigma cavity as assessed. Four age classes of pollen were used, and the recipient flowers were used at peak receptivity, 72 hours after opening (Vaughton and Ramsey, 1991). The only problem with this method may be if any incompatibility is present between the recipient and the tested pollen. A more rapid reduction in pollen viability was found when *Banksia menziesii* was investigated (Ramsey and Vaughton, 1991). Mixed pollen was collected from one hundred flowers per inflorescence, with a total of ten inflorescences being used. When the flowers first opened, over 90% of the pollen was viable, but 24 hours later most of the pollen

appeared to be inviable. The viability in this study was determined by an indirect method, fluorescien diacetate staining. Viable pollen grains fluoresce while inviable grains do not.

The quality of the pollen available to an inflorescence is a central theme in both the pollen donation and the selective abortion hypotheses. Several groups have discussed various aspects of pollen quality in the family Proteaceae. The main emphasis of these discussions centres on the proposal that cross-pollination results in seeds of a higher quality than self-pollination (Paton and Turner, 1985; Whelan and Goldingay, 1986; Goldingay *et al*, 1991). Many of these authors have assumed that cross-pollinated zygotes are of a higher quality, and that selfed or closely related zygotes will have a greater chance of being aborted (Whelan and Goldingay, 1986). This point of view may be correct and has a theoretical basis. Cross-pollinated zygotes are less likely to be homozygous for deleterious lethals, common to closely related individuals, and out-crossing produces more genetic variability (Campbell and Waser, 1987; Waser and Price, 1989). These facts are not in dispute, for some plants, but the assumption that cross-pollinated zygotes are of a higher quality should be tested experimentally in the Proteaceae. Previous research in the northern hemisphere demonstrates quite clearly that there is competition between pollen tubes (Thomson, 1989; Snow and Spira, 1991a) and that the identity of the pollen donor may have an effect on the fate of the resultant zygote (Bertin, 1986; Newport, 1989; Aizen *et al*, 1990; Snow and Spira, 1991b). This possibility is important in the family Proteaceae and should be thoroughly investigated through experimentation.

1.5.8 Gene flow

Gene flow is another important aspect of the hypothesis of pollen donation. The distance pollen moves, which pollen is being moved and which pollen is the most

successful at producing offspring, all play an important role in the evolution of male function. For many years research and discussion has been directed at this fundamental reproductive process (Schaal, 1980; Levin, 1981; Turner *et al*, 1982; Slatkin, 1985; Goldenberg, 1987; Melampy, 1987; Thomson and Thomson, 1989; Fenster, 1991; Rasmussen and Brodsgaard, 1992). None of these crucial factors has yet been explored in the family Proteaceae, even though an enormous amount of information is available on types of pollinators and their behaviour.

1.5.9 Conclusions

Research which has been conducted on the reproductive biology of the family Proteaceae has given an insight into numerous areas such as the large requirement of nutrients needed to produce seeds, ways to combat predation and its variable effect on fruit set, and why breeding system assessment is important when examining fruit set. More fundamental research dealing with pollen and the pollination processes is needed. A great deal of information has already been gathered on various members of the Proteaceae family, but in some areas fundamental information is lacking. These areas include maternal and paternal control in seed production, and understanding the fundamentals of the gene flow process. These two areas have been studied in other plants and have been shown to be key elements in the overall process of reproductive biology.

1.6 Flowering and Environmental growth conditions

1.6.1 Photoperiod, temperature and light intensity

It has been known since the 1920s that many plants require specific environmental conditions under which floral initiation and development occurs (Vince-Prue, 1975; Kinet and Sachs, 1984; Hart, 1988). Two factors are of prime

importance in this process, daylength and temperature (Vince-Prue, 1975; Hart, 1988). All plants have been classified into various groups depending on the photoperiod which they require to flower.

(1) Short-day plants (SDP) which will only flower, or flower most rapidly, with fewer than a certain number of hours of light in each 24 hour period.

(2) Long-day plants (LDP) which will only flower, or flower most rapidly, with more than a certain number of hours of light in each 24 hour period.

(3) Day-neutral plants (DNP) which flower at the same time irrespective of the photoperiod conditions.

Each of these groups are then further subdivided into

(1) Absolute photoperiodic response, where a given daylength is essential for flowering

(2) Facultative photoperiodic response, where a given daylength is not essential for flowering but does promote it.

A few plants are known to exhibit a dual photoperiodic response, for example, flowering will occur in short days but only after exposure to long days; long day-short day plants (LD-SDP), and conversely, with short day-long day plants (SD-LDP) (Vince-Prue, 1975; Salisbury, 1982). An important point to remember with these classifications is that the photoperiodic response is to an arbitrary daylength which may be increasing or decreasing (Salisbury, 1982).

Plants which have a daylength response and are wholly independent of temperature are extremely rare (Vince-Prue, 1975). The interaction between temperature and photoperiod introduces a complication into the examination of floral initiation and development as extensive studies show that photoperiodic responses are readily modified by temperature change. A very common response is that a plant may

be day-neutral at one temperature, and have a facultative response at another (Salisbury, 1982).

The spectral quality and amount of light which a plant receives can affect the response to a particular photoperiod (Vince-Prue, 1975; Hart, 1988). In some cases shading plants affects the accumulation of assimilates and can also interrupt the plant's timing mechanism. Low intensity light has been shown to delay flowering in some plants (Vince-Prue, 1975). The photoperiodic response of plants, and aspects of the quality and quantity of light are closely related variables which make individual aspects difficult to examine closely.

1.6.2 The interaction between daylength, temperature and light intensity in the Proteaceae

Limited information is available on all aspects of floral initiation and development in the family Proteaceae. The only group which has been extensively studied is the genus *Leucospermum* (Jacobs, 1985; Wallerstein, 1989b; Malan and Jacobs, 1990). This group of plants begins vegetative growth in spring and summer, in the southern hemisphere, with reproductive development beginning in autumn, once shoot extension has ceased. The inflorescences then develop during winter and flowers open in spring (Jacobs, 1985). Research on the cultivar "Red Sunset" has shown that short days are necessary to initiate flowering (Malan and Jacobs, 1990). However Wallerstein (1989b) suggests that *Leucospermum patersonii* is an absolute long day-short day plant under a moderate temperature regime. Long days had an inductive effect, whereas floral differentiation occurred under short days (Wallerstein, 1989b).

Light intensity appears to interact with daylength in the floral development process. Low light intensity during the spring and summer vegetative phase of the

cultivar 'Red Sunset' results in fewer shoots flowering. The percentage which fail to produce flowers increased as the light intensity decreased (Napier and Jacobs, 1989). High light intensity during the summer months appears to be required by this cultivar to respond to the inductive short days in autum and winter. Very low light intensity in winter also reduces the responsiveness of shoots to inductive short days (Napier and Jacobs, 1989). Under these circumstances light intensity is apparently interacting with photoperiod. Conversely Jacobs and Minnaar (1980) found that floral development in *Leucospermum cordifolium* was not affected by a decrease in light intensity.

Light intensity has been discovered to be important in *Leucospermum*, but temperature is thought to play a key role in the floral initiation of *Macadamia* flowers (Trochoulias and Lahav, 1983; Moncur *et al*, 1985; Storey, 1985; Stephenson and Gallagher, 1986). During spring/summer in Australia the commercial macadamia tree, *Macadamia integrifolia* grows rapidly in a series of 50-100 cm vegetative flushes. Inflorescences are thought to be initiated in the last flush of the late summer/autumn period, appearing at the resumption of vegetative growth in the following spring (Storey, 1985). Conversely, Moncur *et al* (1985) concluded that floral initiation occurred under short day conditions and low temperatures. This work was conducted under orchard conditions however, and others believe that *Macadamia* is a day-neutral plant (Storey, 1985). Floral initiation appears to occur when the temperature begins to fall, and inflorescences appear in spring when the temperature is increasing (Storey, 1985). Stephenson and Gallagher (1986) determined that warm nights (20°C) just prior to and after floral initiation followed by low night temperatures (10°C) prior to anthesis promote floral bud production. It appears that temperature plays an important role in the floral initiation and development of *Macadamia* but more controlled experimentation is required to clarify the interaction with daylength.

The control of floral initiation and development in *Telopea* is unknown, but indirect evidence is available. Observations have suggested that competition and shade reduce flowering (Worrall, 1983; Faragher, 1989). Floral initiation occurs naturally at times of high light intensity and long daylength (Faragher, 1989). Supplementary lighting which extends the daylength increases flower initiation. This suggests that light may be effecting floral initaition by increasing assimilates, or by the action of the long daylength. Observations indicate that assimilate accumulation is important; flowering only occurs after vegetative growth ceases, shoots which continue to grow through summer do not flower, and weak and thin shoots tend not to flower. Once initiated observations indicate that the developing inflorescence is affected by both light and temperature. Flowering is slowed by two to four weeks in natural shade and by a few days of artificial shade which is about 40% full sunlight (Worrall, 1983). Field observations have also shown that flowering in cooler areas is delayed about six weeks compared with warmer areas (Worrall, 1983; Faragher, 1989). Observations have been conducted on floral initiation and development of *Banksia coccinea* and *Banksia menziesii* (Fuss and Sedgley, 1990). Under South Australian conditions these species initiate floral apices in late spring, which suggests a response to increasing daylength and temperature (Fuss and Sedgley, 1990). This is in contrast to most other observations in the Proteaceae family (Moncur *et al*, 1985; Malan and Jacobs, 1990; Wallerstein, 1989a), where short daylength and low temperature appear to result in floral initiation.

These examples of environmental conditions affecting flower initiation and development of members of the Proteaceae highlight the possible interaction between daylength, temperature and light intensity. In the genus *Leucospermum* different species can respond to different daylength conditions and in the cultivar "Red Sunset"

there is an interaction between daylength and light intensity. The work on *Macadamia* suggests an interaction between daylength and temperature, whereas in *Telopea* species all three factors appear to interacting. These examples illustrate not only the interesting potential interactions, but also the complexity of studying environmental conditions in relation to flower initiation and development.

1.6.3 Delaying flowering in the family Proteaceae

The ability to advance or extend the flowering period of any commercial species is advantageous to the floriculture industry. Extension of flower availability may result in increased consumer familiarity and thus demand, depending on the flower type and the time of year. Another academic aspect is that greater understanding of the timing of floral initiation may be gained.

The ability to delay the flowering period of several species of the genus *Leucospermum* has received some attention over recent years due to the short flowering season and great demand for cut stems of these species on the European market (Brits, 1977; Jacobs and Honeyborne, 1979; Jacobs, 1983; Jacobs *et al*, 1986). The primary bud of *Leucospermum* inhibits growth of axillary buds (Jacobs, 1983; Jacobs *et al*, 1986). The 6-10 axillary buds directly below the primary inflorescence are able to develop to about 5cm in size. These buds are refered to as secondary buds (Jacobs, 1983). Brits (1977) attempted to delay the flowering of *Leucospermum cordifolium* by removing the primary bud, which results in the development of the secondary buds. Flowering was delayed by 4-8 weeks. Disbudding, or the removal of the primary bud, was considered to be too laborious for a commercial situation, so Brits (1977) experimented with a chemical pruning agent, ethephon. The application of this chemical proved successful, resulting in

inhibition of the primary bud, which in turn stimulated secondary bud development (Brits, 1977).

Jacobs and Honeyborne (1979) removed the primary bud from the *Leucospermum* cultivar " Golden Star" at four different dates, resulting in various lengths of delay. Removal of the primary bud up to late autumn resulted in delaying flowering, but removal later than the first week in October (spring) resulted in vegetative growth. The limit to disbudding of the cultivar 'Red Sunset' without crop loss was about mid-September (Jacobs, 1983). This demonstrates that different plants in the same genus have quite specific requirements when the procedure of disbudding is used.

Jacobs and Honeyborne (1979) demonstrated that development of the secondary inflorescence was quicker at higher temperatures. A linear relationship was shown between the mean daily temperature and the rate of development of secondary inflorescences. Using this information Jacobs and Honeyborne (1979) hypothesized that shading may affect the development of secondary inflorescences and hence delay flowering further. Shading at three different levels, combined with disbudding, did not result in an additional delay of flowering but did affect floral initiation and flower quality (Jacobs, 1983).

1.7 Molecular biology techniques for floriculture and reproductive ecology research

1.7.1 The structure of deoxyribonucleic acid

A new era of genetics began when Watson and Crick presented their double helix model of deoxyribonucleic acid (DNA) in 1953 (Watson and Crick, 1953). This model presented DNA as a spiralling two stranded symetrical molecule. The Watson-

Crick model of DNA was the first to offer a plausible explanation for the molecule's self-replication mechanism. The two complementary strands of DNA are held together by weak hydrogen bonds to form the distinctive double helix shape. Four subunits make up these separate strands of DNA, these units being referred to as nucleotides. Each type of distinctively shaped nucleotide is composed of a sugar, a phosphate and one of four kinds of nitrogen-containing bases; adenine (A), guanine (G), cytosine (C) and thymine (T). The spiralling double stranded spine of DNA is formed by strong bonds between the sugars and the phosphates. The inwardly projected bases are joined by weak hydrogen bonds which bridge the gaps between the two strands. The distinctive shapes of the nucleotides making up the DNA results in exclusive pairing, thymine bonding only with adenine and cytosine with guanine. This base-pairing preference ensures the capacity of DNA to act as the heritable material for most living organisms (Blackburn and Gait, 1990).

The discovery of the structure of DNA revolutionised the field of molecular biology. In quick succession new properties and ways of manipulating DNA were determined. The genetic code of DNA was deciphered 10 years after the modelling of its structure (Blackburn and Gait, 1990). By 1973 the first recombinant DNA molecule was created in a laboratory (Blackburn and Gait, 1990). In 1974 restriction fragment length polymorphisims (RFLP) were first used as a tool for genetic anaylsis (Botstein *et al,* 1980). A further leap occurred in 1981 when the first human disease was diagnosed prenatally by DNA analysis (Kan and Dozy, 1978). By 1985 several new technologies of manipulating DNA were discovered. One of these technologies was the polymerase chain reaction (PCR), first performed by scientists at the Cetus Corporation (Erlich, 1989) The reaction involves the in-vitro synthesis of many copies of a specific DNA fragment. The two techniques, which have rapidly advance

molecular biology, are RFLP DNA fingerprinting and the polymerase chain reaction. Their contributions to molecular biology ranges from identifying genetic variation in man (Jefferys, 1979), identifying plant cultivars (Nybom *et al*, 1990; Nybom, 1990), diagnosing diseases (Baker *et al*, 1984; Wong *et al*, 1987; Lee *et al*, 1988) and advancing forensic science (Gill *et al*, 1985; Lander, 1989).

The analysis of DNA rather than isozymes or morphological markers has many significant advantages. The genotype rather than the phenotype is being examined, which eliminates the problems of pleiotrophic effects (Harvey and Muehlbauber, 1989; Hillis and Mortiz, 1990). The other advantages of using DNA techniques include uniformity of methology for all DNA types, preparation from small amounts of tissue, and relative stability for long periods of time (Hillis and Mortiz, 1990).

1.7.2 Restriction fragment length polymorphism DNA fingerprinting

The basic steps of RFLP DNA fingerprinting are DNA extraction, followed by the preparation of probes, cleavage of the DNA with restriction enzymes, sorting of these fragments by gel electrophoresis and finally visualizing the sorted fragments via probes, radioactivity and autoradiographic exposure (Beckman and Soller, 1983; Hillis and Moritz, 1990; Gupta and Tschiya, 1991). This process involves comparison of the number and size of DNA fragments produced by digestion with restriction enzymes (Hillis and Mortiz, 1990; Gupta and Tschiya, 1991) which are isolated from bacteria and cleave at a constant position within a specific recognition site on the DNA molecule. The cleavage specificity of the restriction enzyme means that a complete digest of a sequence of DNA will produce consistently reproducible results (Hillis and Mortiz, 1990; Gupta and Tschiya, 1991). The fragments produced by the restriction enzymes are sorted by gel electrophoresis. The smaller fragments move through the gel pores more quickly and travel the greatest distance (Beckman and

Soller, 1983; Hillis and Mortiz, 1990). When the DNA of higher organisms is used in this process, a large number of different sized fragments results, making a continuous smear on the gel. In order to detect a specific fragment a cloned DNA sequence homologous to a specific fragment of DNA is used as a probe (Beckman and Soller, 1983). The radioactive probe is exposed to the DNA under conditions which promote DNA-DNA hybridization. The unhybridized radioactivity is washed away and autoradiographic exposure produces a photographic DNA fingerprint (Beckman and Soller, 1983).

Several factors result in unique RFLP patterns occuring in individuals but this is also dependent on the restriction enzyme and probe used. Different individuals have base changes and different sequences in their DNA so a particular restriction enzyme cannot always recognise its recognition site. These slight changes in the DNA between individuals may either abolish or create a restriction enzyme site and so alter the DNA fingerprint produced (Beckman and Soller, 1983; Hillis and Mortiz, 1990). This is the reason why different individuals, with the exception of identical twins or clonal organisms, will give unique DNA fingerprints.

The uses of RFLPs are wide and varied, from detecting plant genetic variation (Nybom, 1990) to population genetics of poultry strains (Kuhnlein *et al*, 1989). Using RFLP technology has become quite common when identifying plant cultivars or varieties (Hosaka, 1986; Nybom *et al*, 1990). Economically important plant species have been investigated by RFLPs, including soybean (Apuya *et al*, 1988; Keim *et al*, 1989), lentil (Harvey and Muehlbauber, 1989), members of the Roseaceae family (Nybom, 1990; Nybom *et al*, 1990), *Brassica* crops (Erikson *et al*, 1986; Figdore *et al*, 1988), rye (Tudzynski *et al*, 1986) maize and tomatoes (Helentjaris *et al*, 1986).

RFLPs are most commonly used to construct genetic linkage maps. A genetic map provides a summary of a species' genetic information and a linkage map extends this by showing the linkages between genes. This procedure helps in mapping economically important genes and in determining the inter-relationships and genomic organization of a particular species (Figdore *et al*, 1988). When using RFLPs for this purpose a high level of genetic variation is required (Helentjaris *et al*, 1986). Genetic linkage maps have been constructed for maize and tomato (Helentjaris *et al*, 1986) and Figdore *et al*, (1988) determined that it was feasible to construct a genetic linkage map of three *Brassica* species. Lentil provided enough variability to produce a linkage map by combining isozymes, morphological and RFLP markers (Harvey and Muehlbauber, 1989), but there was low genetic variation in soybean, which makes the construction of a linkage map very difficult (Apuya *et al*, 1988; Keim *et al*, 1989). A genetic linkage map of the human genome has also been constructed using 403 polymorphic loci, which includes 393 RFLPs, with DNA from 21 three-generation families (Donis-Keller *et al*, 1987). Chloroplast and mitochondrial DNA have been examined using RFLPs to help determine the origin of cytoplasmic male sterility, which is important in the production of hybrid crops such as rapeseed (Erikson *et al*, 1986) and rye (Tudzynski *et al*, 1986).

Many problems have become apparent in the last five years; the technique is time consuming (Weber and May, 1989), expensive (Hillis and Moritz, 1990), uses radioactive materials and problems may arise with reliability, (Erlich, 1989; Steane *et al*, 1991) and sensitivity (Weber and May, 1989).

1.7.3 The polymerase chain reaction

The polymerase chain reaction is based on the process of DNA self-replication involving denaturing, extension and re-annealing of DNA molecules. Three major

steps constitute one PCR cycle; denaturation, primer binding, DNA replication. Repetitions of this cycle rapidly result in an exponential increase in the amount of the initial DNA fragment (Erlich, 1989; Erlich *et al*, 1991). The DNA fragment to be copied is denatured to form single stranded DNA and two oligonucleotide primers are hybridized to the 3' end of each single stranded fragment. The primer's function is to provide a starting point for the polymerase to begin the replication process on the single stranded DNA template. The polymerase enzyme functions by aiding the nucleotides to be positioned correctly on the new complementary DNA strand. The original polymerase used to develop PCR was the Klenow fragment of *Escherichia coli* DNA polymerase I. This polymerase is inactive at the high temperatures needed to denature the DNA, so new enzyme was added after each cycle. This tedious step was eliminated by the discovery of a thermostable DNA polymerase from the bacterium *Thermus aquaticus*, hence the name Taq polymerase (Saiki *et al*, 1988; Erlich, 1989; Erlich *et al* , 1991).

The majority of research which has been conducted using PCR technology has centred on human disease research (Erlich, 1989). Some of the diverse range of diseases diagnosed by PCR include sickle cell anaemia (Saiki *et al*, 1985), thalasemia (Wong *et al*, 1987), muscular dystrophy (Chamberlain *et al*, 1988), and the skin disease pemphigus vulgaris (Sihna *et al*, 1988). Forensic analysis is another area of application of the PCR process (Erlich, 1989). Very small and highly contaminated samples of DNA can be analysed (Saiki *et al*, 1986; Li *et al*, 1988) including DNA from a single hair (Erlich, 1989; Higuchi *et al*, 1988). Additional uses may include identification of missing persons, determining the identity of rapists, and paternity verification.

Evolutionary and developmental biology have also benefited from this technique (Erlich, 1989). Ancient mitochondrial DNA from a fossilised skull has been amplified successfully (Paabo *et al*, 1988). Other sources of ancient DNA, such as from museum specimens, analysed in the same manner may lead to revised phyla analysis, or comparison between different populations of flora and fauna around the world, which may in turn increase our knowledge of the prehistoric environment. Developmental biology has proven a difficult field to study, but with the help of PCR new aspects are being addressed. For example the activity of macrophages at an actively healing wound site has been partially ellucidated using PCR technology (Rappolee *et al*, 1988).

1.7.4 Randomly amplified polymorphic DNA technique

The PCR process has been shown to be very useful in many diverse areas of science. Recently an extension of the basic PCR process was developed (Williams *et al*, 1990; Welsh and McClelland, 1990; Welsh *et al*, 1991; Caetano-Anolles *et al*, 1991), termed randomly amplified polymorphic DNA (RAPD). The basic PCR technique is altered by adding random primers to the reaction mixture and the stringent cycling requirements are relaxed. These random primers will amplify specific sections of the target DNA. Once the sample is amplifed and resolved using an agarose or acrylamide gel the result is similar to an individual specific DNA fingerprint.

RAPD analysis was developed in 1990 by two groups of researchers (Williams *et al*, 1990; Welsh and McClelland, 1990) who independently discovered this methology. Williams *et al*, (1991), concentrating on the area of genetic mapping, demonstrated the technique by using human, corn, soybean and *Neurospora* DNA, whereas eleven strains of *Strepococcus pyogenes* and three varieties of rice were

identifyed by Welsh and McClelland (1990). This technique has been used to distinguish between different strains of mice using only one primer (Welsh *et al*, 1991), while a subsequent paper described how multiple primers were used to generate several different genomic fingerprints in the same mice strains (Welsh and McClelland 1991). Other uses for this technique include detecting fungal infection in wheat (Schesser *et al*, 1991), genetic mapping of humans and plants (Williams *et al*, 1991), and identifying bacterial strains (Welsh and McClelland, 1990)

1.7.5 Advantages of randomly amplified polymorphic DNA technique

Whereas RFLP DNA fingerprinting is relatively expensive, time consuming and technically difficult, the RAPD technique overcomes the majority of these drawbacks. Using RAPDs a research program can commence without much background knowledge of the DNA and without the identification of RFLP probes (Welsh *et al*, 1991). Any probe which is available can generate a large amount of polymorphism (Sommer and Tautz, 1989), which reduces both time and cost. Automation of PCR was one of the great attractions for many researchers. A PCR reaction takes less than a day, with a large number of samples, whereas a single RFLP analysis may take several weeks to complete (Weber and May, 1989). An enormous advantage of RAPDs, particularly for plant scientists, is that a high level of contamination may not adversely affect the performance of PCR. Other techniques, like isozymes and RFLP may be sensitive to the numerous contaminants present in plant tissue.

When detecting fragments of amplified DNA on a gel, several alternative detection methods are effective. The fluorescence method of ethidium bromide staining is commonly used (Welsh *et al*, 1991) and another method, with less toxic

reagents, is silver staining of the gel (Caetano-Anolles *et al*, 1991). DNA on a gel can also be isolated and reamplified directly from the gel for use in other processes like restriction mapping or genome walking (Welsh *et al*, 1991).

1.8 Conclusions

Floriculture is a competitive industry with constant demand for new cultivars, which enhances consumer choice. Improvement in quality, reliability and diversity are also important requirements of new cultivars. Development of cultivars is a complex process involving a detailed understanding of the particular plant species, ecological conservation, reproductive biology, flowering and molecular biology.

Conservation of habitats is important, as wild communities are the source of valuable genetic variation. This aspect of cultivar development has not been as well recognised in floriculture as for food crops (Ford-Lloyd and Jackson, 1986), although some studies have been done, mainly in Western Australia, which illustrate the importance of preserving enough genetic variation to enable a population to exist and survive (Coates, 1988). Much greater emphasis on the link between floriculture and conservation is needed to assist the advancement of both areas.

Another important area to floriculture, which is central to cultivar development, is reproductive biology. This is a large dynamic and complex field of study, which at present is dominated by northern hemisphere species. Investigations on the Australian flora have been initiated, mainly concentrating on breeding systems and pollination. Other important areas have been neglected and this needs to be addressed. These areas include pollen and zygote competition, male function, pollen quality and maternal/paternal interaction. Investigations into these areas will further our understanding of processes involved in reproduction and thus plant breeding.

An understanding of reproduction enables selections and hybrids to be developed, but in the floriculture industry an understanding of the initiation and development of the flower is also essential. Extensive information and experiments have been performed on traditional floriculture crops. In contrast there is an overall lack of information on the initiation and development of flowers in most of the Australian flora. More information is needed involving aspects of the conditions which stimulate initiation and maintain development.

Molecular biology techniques can be applied to aspects of reproductive biology and conservation of species. This enables difficult areas of reproductive biology such as pollen competition, gene flow and pollen quality to be more fully explored. Aspects of conservation are also enhanced by molecular biology such as genetic variation of populations thus benefiting conservation and floriculture simultaneously.

Chapter 2: Introduction

2.1 Family Proteaceae, Tribe Banksieae, Genus *Banksia*

A total of 73 genera make up this family with more than 1500 species. The greatest diversity of genera is found in Australia; with 42 genera and 860 species (Wrigley and Fagg, 1989). The family Proteaceae is divided into five subfamilies, including the Grevilleoieae, to which the genus *Banksia* belongs. A total of 73 species make-up the genus *Banksia*, with the greatest concentration, 58 species, occurring naturally in south-west Western Australia (George, 1987; Wrigley and Fagg, 1989). Fourteen species occur in south-eastern and eastern Australia, from Eyre Peninsula to Cape York. Two of these species are also present in Tasmania. The one tropical species, *Banksia dentata*, is found in northern Australia, as well as Papua New Guinea, Irian Jaya and the Aru Islands. A number of botanical features are characteristic of the genus *Banksia* (Salinger, 1985; George, 1987; Wrigley and Fagg, 1989). All are evergreen shrubs or trees, trees usually forming a single trunk while shrubs have multiple branches at ground level. Lignotubers are present in 36 species and are an adaptation to fire. Leaves are tough, hard and prominently toothed or lobed. The inflorescences of *Banksia* are produced in dense spikes which contain hundreds or thousands of individual flowers. The arrangement of flowers within the spike is complex. A central woody core is covered by short hairy bracts. The flowers are grouped in pairs, and below each flower occurs a floral bract. A slightly larger common bract is found beneath this arrangement. This grouping of flowers and bracts are arranged in a double spiral pattern giving the appearance of vertical rows.

2.2 Commercial production and horticultural management

The production of *Banksia* for cutflowers began in South Australia over twenty five years ago (Barth, 1992). In 1992 there were 200 hectares of proteaceous crops in cultivation, 70 hectares of this being devoted to *Banksia*. The three major areas of *Banksia* production within South Australia are the south-east districts, the Blewitt Springs/Kangarilla area and the lower Fleurieu peninsula (Barth, 1992). *Banksia* inflorescences have been harvested from natural populations in Western Australia for both domestic and export use for the past thirty years (Webb, 1991) and in 1988 it was estimated that 45% of *Banksia* inflorescences exported were bush picked. In the same year over 190 hectares of *Banksia* in Western Australia were under cultivation, and since that time bush picking of several species has been restricted by legislation.

Nine major species are grown in South Australia and Western Australia, these being *Banksia ashbyi*, *Banksia baxteri*, *Banksia burdettii*, *Banksia coccinea*, *Banksia hookeriana*, *Banksia menziesii*, *Banksia prionotes*, *Banksia speciosa*, and *Banksia victoriae* (Webb, 1991; Barth, 1992). *Banksia* prefers deep, well drained, slightly acidic sand for optimal growth and inflorescence production (Elphick, 1985; George, 1987; Barth, 1989; Webb, 1991; Barth, 1992). Most plantations are grown from seed collected from natural populations and this inevitably creates variation among individuals within the plantation (Fuss and Sedgley, 1991c; Barth, 1992). Within row spacings vary from 2 to 3.5 m, with a between row spacing of between 3 and 5 m used for most species. This spacing allows 500-1250 plants per hectare to be grown (Webb, 1991; Barth, 1992).

Pruning of *Banksia* is necessary otherwise large scraggly bushes result which can be difficult to manage. The regularity and severity of pruning will determine the plant size and inflorescence yield. In South Australia the normal practice is to prune regularly and severely during the first three years of growth with subsequent pruning recommended during the

summer (Barth, 1992). It is believed that this practice may reduce plant size and flower yield, so an alternative approach has been tried by a grower in Western Australia (Webb, 1991). Pruning is started 12-18 months after planting, with initial pruning concentrating on pinching out main shoots and the removal of low growth. Subsequently trees are pruned after flowering every 2-3 years, using a hedge trimmer set to a predetermined height and width. The nutrient requirements of *Banksia* are still not well understood, but present practice is to use slow-release fertilisers or low phosphorous fertilisers on the soil surface. Irrigation or rainfall is essential for successful establishment of young plants and plants up to 3 years old have been shown to respond well to irrigation (Webb, 1991). The most important disease of *Banksia* is the fungus, *Phytophthora* spp. which affects the root system and crown. The single most important pest is the *Banksia* moth which lays its eggs on the developing inflorescence. When the larvae hatch, they burrow into the centre of the inflorescence and affected inflorescences may be severely damaged, thus preventing sale (Webb, 1991). *Banksia* is harvested fresh and marketed fresh, dried, sulphur treated, dyed and (Webb, 1991; Barth, 1992).

2.3 Species descriptions

Five different species of *Banksia* were used during this study. A brief description of each species follows, including distribution. growth habit and flowering time.

Banksia coccinea (Figure 2.1): Distribution in Western Australia is from Albany to the Stirling ranges and east to the Young River, often occurring with *Banksia baxteri*. The growth habit of this species is either a shrub or small tree to 8 m, without a lignotuber. Prolific germination after bushfires results in large populations of the same age. The leaves are pale green and leathery with toothed margins. The spectacular terminal inflorescence, which is often broader than long, has flowers arranged in vertical rows. The styles are

scarlet, with variants ranging from dark red to orange. The flowering period ranges from June to January. This species is distinct within the genus with no close relatives, and the relationship with other species in the genus is not well understood (George, 1987; Holliday and Watton, 1990).

Banksia hookeriana (Figure 2.2A): A restricted area between the Arrowsmith river and Eneabba is the only known location for this species and agriculture and mining development are thought responsible for habitat reduction. A bushy spreading shrub, 3 m tall and broad, without a lignotuber, the leaves are narrow-linear, up to 20 cm long, and are finely toothed. The conspicuous terminal inflorescence is rounded almost cylindrical. The perianths are covered in pale buff hairs, with orange styles resulting in a sharp contrast between open and unopened flowers. The main flowering period is between April and October (George, 1987; Holliday and Watton, 1990).

Banksia menziesii (Figure 2.2B): Usually occurring near the coast, from Murchinson River to Pinjarra. In the north of this range occurring with heaths and in the south can be found with jarrah and other tall banksias. A tree to 10 m, or shrub to 3 m, with lignotuber. The dull green leaves are tapered towards the stem, with small irregular teeth around the margins. Terminal inflorescences are cylindric but rounded at the top. The flowers are pale to deep pink, sometimes cream, rust or chocolate. The styles are yellow and provide and interesting display when contrasted to the unopened flowers. When the flowers fall a chequered cone is revealed. Flowering occurs mainly during the months of February to August (George, 1987; Holliday and Watton, 1990).

Banksia praemorsa (Figure 2.3A): This species has a restricted distribution on the south coast of Western Australia between Albany and Bald Island. It forms a dense shrub up to 4 m or small tree to 10 m, with no lignotuber. The dark green leaves are 20-60 mm long, with toothed margins and a blunt tip. Inflorescences occur on short lateral branches or terminally,

usually partly concealed within the foliage. Flowers are red-maroon when exposed, otherwise a pale greenish yellow colour. The flowering period is between August and November (George, 1987; Holliday and Watton, 1990).

Banksia prionotes (Figure 2.3B): The distribution extends along the coast from Shark Bay to Wagin with a number of inland populations. It forms a shrub or tree up to 10 m, without a lignotuber. The grey-green leaves are linear, tapered towards the stem, with regularly toothed margins. Well displayed terminal inflorescences, having cream flowers with orange limbs, provide a strong contrast between open and unopened flowers. February to August is the extent of the flowering period (George, 1987; Holliday and Watton, 1990).

2.4 Project Aims

Areas where research is lacking on the family Proteaceae include, floral initiation, population biology in relation to reproduction and an improvement in horticultural management of *Banksia*. Experiments to understand of the control of flowering using controlled environment chambers will be conducted using a combination of photoperiod and temperature treatments. The objective here is to enable better management derived from greater knowledge of the processes which trigger floral initiation. The population biology of both cultivated and natural populations will be explored. The level of genetic variation present in natural populations will provide more information on the available pool of variation, from which subsequent breeding will take place, and the level of diversity in cultivated populations will be compared with that present in natural populations. The dynamics of natural populations will be explored and the implications for conservation will be discussed. Finally several facets of reproductive biology including gene flow within populations and pollen competition will be explored. Molecular methods will be utilised to advance our current limited knowledge of these aspects in the genus *Banksia*.

Figure 2.1: A representative *Banksia coccinea* inflorescence, illustrating the characteristic deep red colour and the distinctive vertical rows of flowers.

Figure 2.2: A *Banksia hookeriana* and *Banksia menziesii* inflorescence.

2.2A A single *Banksia hookeriana* inflorescence, illustrating the cylindrical shape with a distinctively flatten top.

2.2B A *Banksia menziesii* plant showing a number of inflorescence at various stages of development. The inflorescence in the foreground, shows the striking contrast between the opened and unopened flowers.

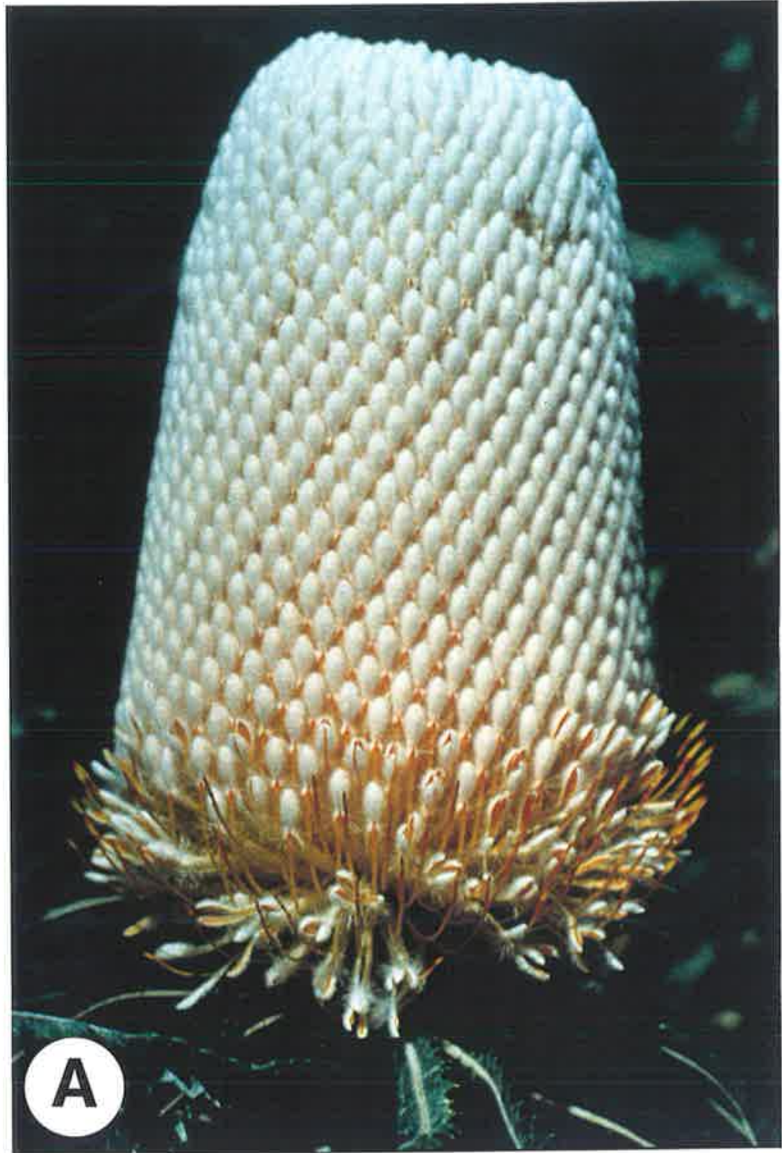
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Figure 2.3: A *Banksia praemorsa* infructescence and a *Banksia prionotes* inflorescence.

2.3A *Banksia praemorsa* plant showing leaf shape and whorled arrangmentof leaves. A infructescence with retained flowers is shown in the foreground.

2.3B A *Banksia prionotes* inflorescence, illustrating the contrast between the opened and unoped flowers.

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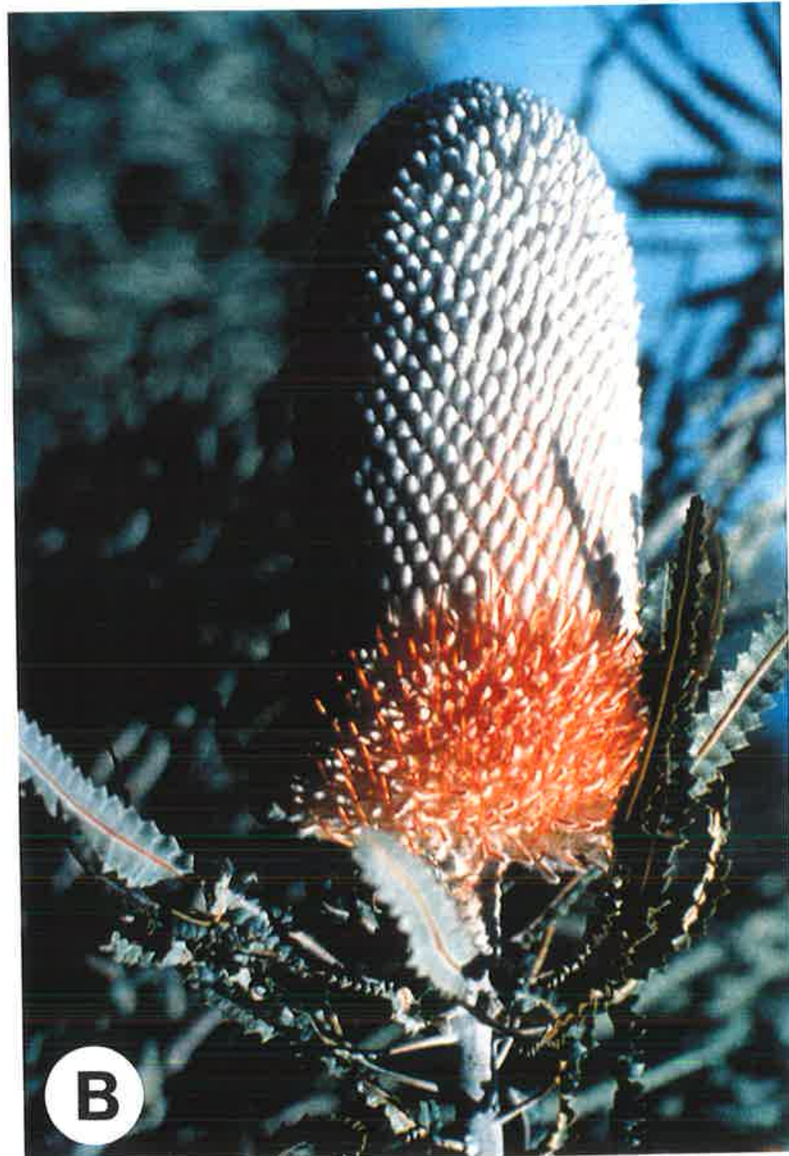
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Chapter Three: Floral initiation using controlled environment conditions.

3.1 Introduction

The initiation and development of flowers in many plant species is controlled by the environmental conditions under which that plant is grown (Trochoulias and Lahav, 1983; Moncur *et al*, 1985; Sedgley, 1985; Stephenson and Gallagher, 1986). Light, temperature and photoperiod are the most important parameters involved in the initiation and development processes. In recent years, work on some members of the Proteaceae has been conducted in South Africa and Israel mainly concentrating on the genus *Leucospermum* (Jacobs, 1983; Wallerstein, 1989b; Malan and Jacobs, 1990) and a general growth pattern description for this genus has been developed. Vegetative growth occurs in spring and summer, while reproductive development occurs in autumn, followed by inflorescence growth in winter with the flowers opening in early spring (Jacobs, 1983). The *Leucospermum* cultivar 'Red sunset' has been found to initiate flowers in response to short days (Malan and Jacobs, 1990), and *Leucospermum patersonii* requires a long day-short day treatment to flower (Wallerstein, 1989b). Shading or reducing light intensity during the reproductive phase of the *Leucospermum* cultivar "Red Sunset" results in lack of induction under short days (Napier and Jacobs, 1989).

Both temperature and light intensity have been found to play a role in the flowering response in other members of the Proteaceae (Storey, 1985; Napier and Jacobs, 1989). From observation on *Macadamia,* Storey (1985) suggests that temperature significantly affects floral initiation of this genus. In areas where the temperature varies only slightly, such as Trinidad, Panama and Honduras, *Macadamia*

trees flower and fruit sporadically throughout the year. In comparison, trees in the Hawaiian Islands are able to flower only below 800 m, as the temperatures above this elevation are too low.

Recent work in Australia on *Banksia coccinea* and *Banksia menziesii* has shown that the time of floral initiation and the morphological stages of floral development are similar in these two species. Floral initiation occurs in late spring, indicating that the plants may be responding to an increase in temperature and daylength. The period of floral development differs significantly between the two species, such that *Banksia menziesii* flowers from March to May but *Banksia coccinea* takes a further five months with peak flowering from August to October (Fuss and Sedgley, 1990). Apart from this work, the effect of climatic factors on Australian Proteaceae including *Banksia* has not yet been examined in detail. The developmental stages of an inflorescences of *Banksia coccinea* and *Banksia menziesii* under cultivated conditions are illustrated in Figures 3.1 and 3.2.

Control of flowering is particularly relevant to the floriculture industry. Understanding when vegetative growth is initiated allows pruning of plants to be done before floral initiation has occurred. Also information on how and when the inflorescence develops allows prediction of the time of harvest. Other aspects such as the optimal conditions for *Banksia* inflorescence development helps avoid abnormal growth. Thus an understanding of how environmental conditions affect plant development will benefit growers. Manipulation of the flowering process can also be beneficial to flower production. Many exotic species are manipulated using daylength control and 'pinching' to advance or delay flowering. Manipulation of *Banksia* has never been attempted, but work on related genera appears encouraging. Brits (1977) determined that removing the primary bud in *Leucospermum* releases the axillary buds

Figure 3.1: Floral developmental stages of *Banksia coccinea* under cultivated conditions at Blewitt Springs, South Australia.

3.1A. Immature inflorescence bud showing involucral bracts (arrow). Scale = 2 cm.

3.1B. Semi-mature inflorescence bud showing elongation of florets. Scale = 2 cm.

3.1C. Maturing inflorescence rows of florets clearly visible. Scale = 2 cm.

3.1D. Mature inflorescence with open florets (arrow). Scale = 2 cm.

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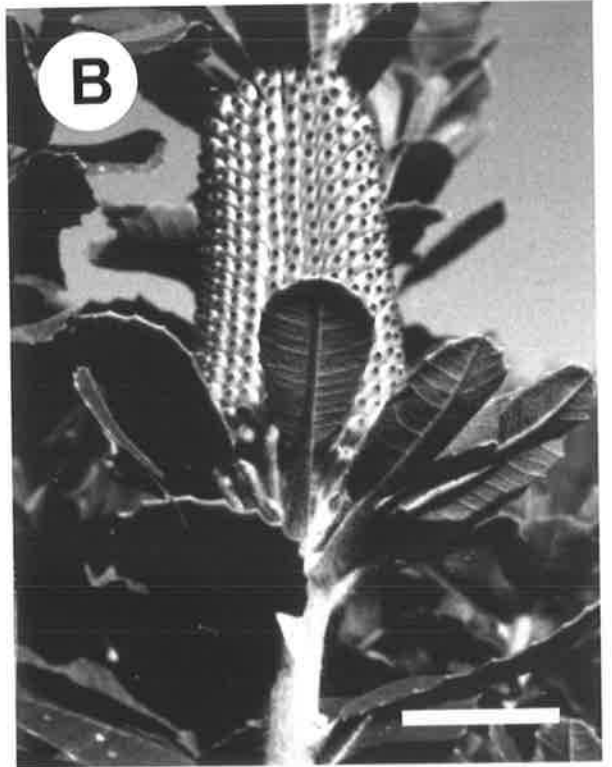
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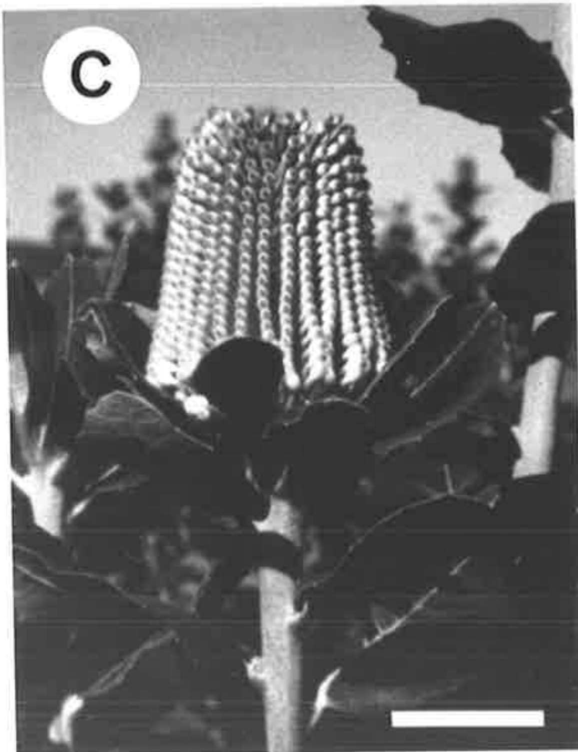
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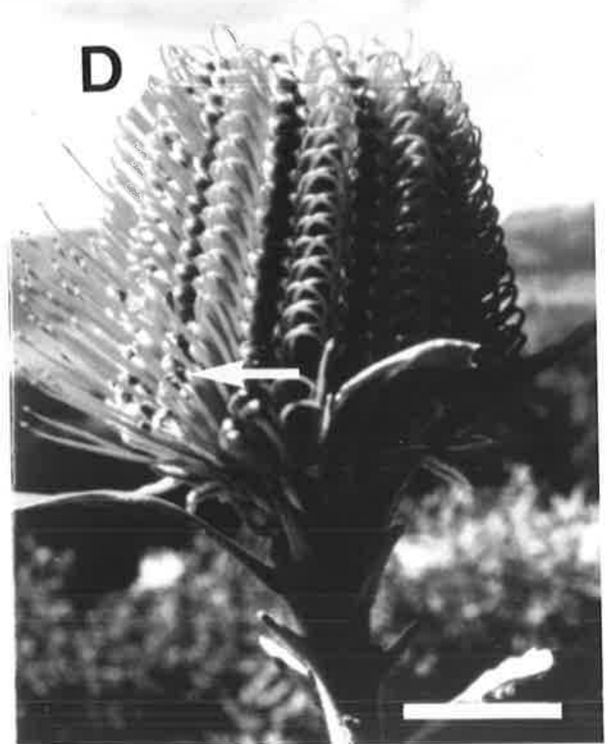
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Figure 3.2: Floral developmental stages of *Banksia hookeriana* under cultivated conditions at Blewitt Springs, South Australia.

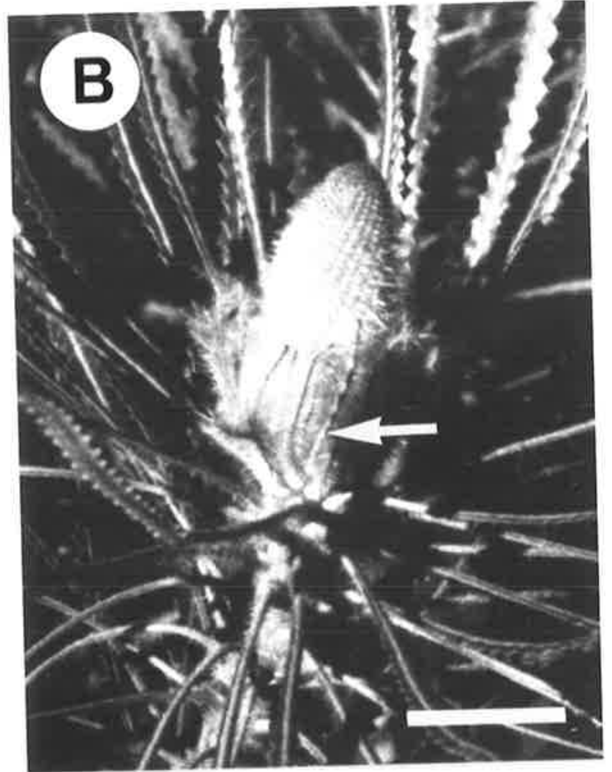
3.2A. Immature inflorescence bud. Scale = 2 cm.

3.2B. Semi-mature inflorescence bud, showing floret differentiation and involucral bracts (arrow). Scale = 2 cm.

3.2C. Maturing inflorescence florets clearly differentiated. Scale = 2 cm.

3.2D. Mature inflorescence with open florets (arrow). Scale = 4cm.

A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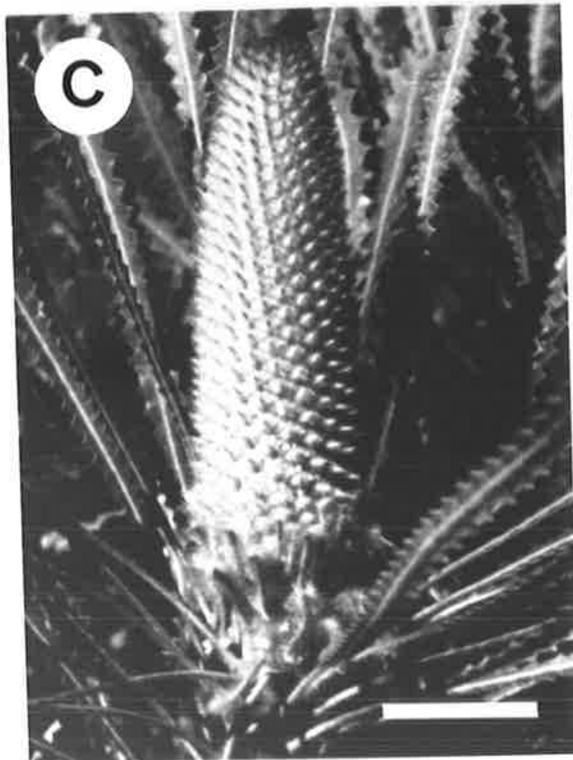
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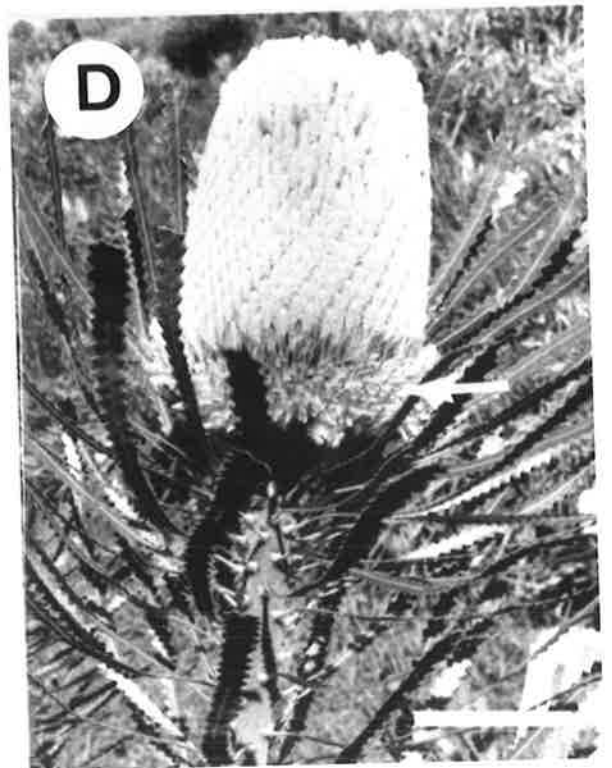
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to develop into inflorescences. This procedure delayed flowering by 4-8 weeks and removal of the primary bud of the *Leucospermum* cultivar 'Golden star' has been successful in delaying flowering time (Jacobs and Honeyborne, 1979). Moreover, multiple heads of King Protea attract premium prices on export markets.

3.2 Materials and Methods

3.2.1 Plant material

Plants of ***Banksia coccinea*** and ***Banksia hookeriana*** were raised from seed collected from wild populations in Western Australia. Plants were grown outside in the Claremont Orchard of the Waite Agricultural Research Institute (Table 3.1). The soil mixture contained equal proportions of sand, peat and perlite. Plants were four years old at the beginning of the experimental period with a pot size of 20 cm. A low phosphorous slow release fertiliser was applied during the experimental period.

3.2.2 Controlled environment experiments

The controlled environmental conditions used to investigated floral initiation are listed in Table 3.2. The photosynthetic photon flux density (PFD) during the controlled environment experiments ranged between 200-250 µmols m^{-2} s^{-1}. Light measurements were taken using a LICOR quantum sensor (LICOR Instruments, Nebraska). Two types of lights were used in the growth rooms at a ratio of 50:50. Four hundred watt metal halide lights (Model 400M/BU) from Venture lighting International, Ohio) with a spectrum of 350-750nm were used in conjunction with sodium lights (Model Sunlux, NHT360-lx from Eye, Iwasaki, Japan) with a spectrum of 400-700nm and a power output of 360 watts. Each September over the two years in which the experiments were run, thirty plants of each species were placed into each growth room (Figure 3.3).

Table 3.1: Environmental conditions in Claremont Orchard (1991 - 1993).

Month	*Temperature Maximum,°C*	*Temperature Minimum,°C*	*Rainfall (mm)*	*Daylength*	*Sunshine Hours*
		1991			
January	28.7	17.7	15.4	14.1	8.95
February	29.8	16.2	0.0	13.2	11.02
March	25.2	14.8	5.0	12.2	7.75
April	22.5	13.7	42.6	11.1	6.05
May	18.7	10.6	12.8	10.2	3.73
June	17.0	11.4	141.8	9.5	2.35
July	14.7	8.6	103.0	10.0	2.86
August	14.9	8.8	88.0	10.5	3.85
September	18.4	10.0	90.0	11.5	5.55
October	22.2	12.1	7.6	12.6	7.71
November	23.9	13.6	35.2	13.6	8.61
December	25.2	14.3	5.6	14.3	8.32
		1992			
January	24.6	13.9	1.4	14.1	8.49
February	28.7	17.4	14.0	13.2	8.11
March	25.3	15.8	89.2	12.2	6.89
April	22.1	13.8	44.4	11.1	5.66
May	17.2	10.6	95.2	10.2	3.95
June	14.8	8.9	84.8	9.5	2.55
July	14.9	8.9	72.4	10.0	3.64
August	14.5	7.7	162.6	10.5	3.78
September	14.8	8.1	141.4	11.5	4.44
October	20.2	11.9	90.2	12.6	5.01
November	20.6	11.9	94.4	13.6	4.29
December	24	15.6	88.2	14.3	5.94
		1993			
January	27.5	16.6	31.4	14.1	8.0
February	27.4	17.0	40.0	13.2	9.23
March	25.1	15.3	18.8	12.2	6.82
April	24.2	14.1	1.4	11.1	6.82
May	19.1	11.3	49.4	10.2	3.95
June	14.6	8.3	44.8	9.5	3.16
July	14.9	9.1	74.8	10.0	4.14
August	17.5	10.3	59.8	10.5	6.0
September	18.1	10.1	75.2	11.5	7.21
October	20.1	11.4	77.6	12.6	7.56
November	24.0	13.6	23.0	13.6	8.9
December	24.5	14.7	76.0	14.3	7.87

Table 3.2: Controlled environment conditions for *Banksia coccinea* and *Banksia hookeriana*.

Species	*Temperature, °C Day/Night*	*Daylength (Hours)*
B. coccinea	15/10	8
B. coccinea	15/10	16
B. coccinea	25/20	8
B. coccinea	25/20	16
B. hookeriana	15/10	8
B. hookeriana	15/10	16
B. hookeriana	25/20	8
B. hookeriana	25/20	16

Figure 3.3: Experimental plants and controlled environment room.

3.3A. *Banksia coccinea* under controlled environment conditions. *Banksia coccinea* plant under 8 hour daylength and 15/10°C temperature (left), 16 hour daylength and 15/10°C temperature (right).

3.3B. Controlled environment room showing the experimental setup using *Banksia coccinea* and *Banksia hookeriana* plants.

3.3C. *Banksia hookeriana* under controlled environment conditions. *Banksia hookeriana* plant under 16 hour daylength and 15/10°C temperature (left), 16 hour daylength and 25/20°C temperature (right).

A

B

C

Plants were allowed eight weeks to acclimatise to these conditions before measurements were taken. A total of twenty plants from both species remained outside throughout the experimental period so an estimate of flowering behaviour under Adelaide conditions could be established. Approximately 1% of the *Banksia coccinea* and 75% of the *Banksia hookeriana* plants had flowered prior to the start of the experiment. Plants which had not flowered the previous season were selected, as research has shown that flowering of *Banksia coccinea* and *Banksia menziesii* occurs primarily on two year old shoots (Fuss and Sedgley, 1992). The length of each shoot on each plant was recorded prior to the start of the experiment and growth was recorded monthly to the nearest 0.5 cm, from the base to the tip of the shoot. The presence of visible inflorescence buds was also recorded. After extension growth had ceased all terminal buds were removed, fixed in FPA50 and examined for vegetative or floral development using an Electroscan Environmental Scanning Electron Microscope (ESEM) (Figure 3.4). After fixing the samples were stored in 70% ethanol, but for viewing under the ESEM they were transferred to water.

3.2.3 Statistical analysis

As a consequence of the unequal number of shoots per replicate plant within treatments, a test of the correlation between the amount of growth and the number of shoots was performed for each treatment. No correlation was found. Shoots were separated into those which flushed in the first month of the experiment and those which remained unchanged. In the following month the shoots which were inactive in the previous month were further subdivided into flushed or unchanged. This procedure was carried out only for the three months in which flushing occurred. Binomial models were fitted to this data set using Genstat 5 to test for significant differences between treatments. The length of the new extension growth resulting from the flush which

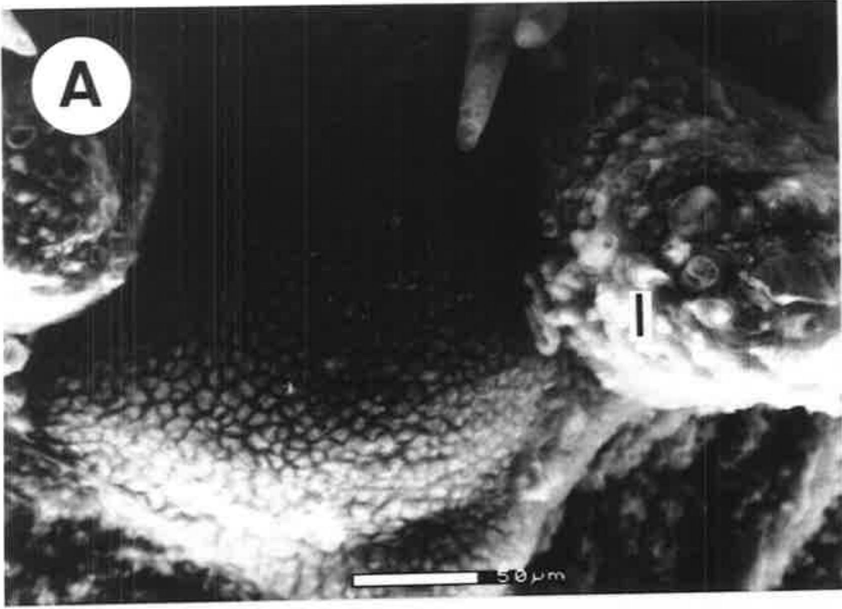
Figure 3.4: ESEM micrographs of floral development stages.

3.4A. ESEM micrograph of vegetative apex of ***Banksia coccinea***, producing leaf primordia (l). Scale 1.6 cm = 50 µm.

3.4B. ESEM micrograph of early floral apex of ***Banksia hookeriana***, showing involucral bract primordia. Scale 1.1 cm = 50 µm.

3.4C. ESEM micrograph of flora apex of ***Banksia hookeriana***, producing floral bract primordia (b). Scale 1.2 cm = 50 µm.

3.4D. ESEM micrograph of late floral apex of ***Banksia coccinea***, with individual floral bracts and hairs clearly visible. Two florets will differentiate in the axil of each floral bract. Scale 1.3 cm = 50 µm.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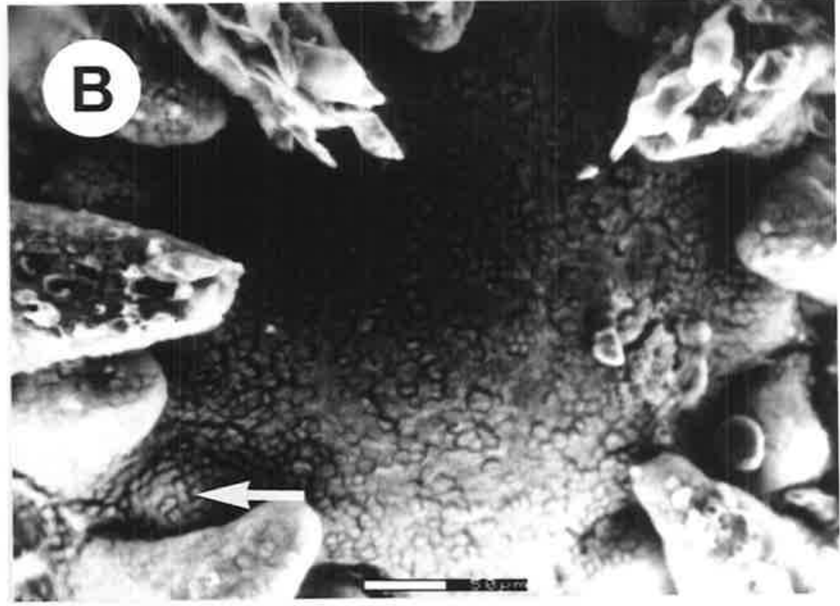
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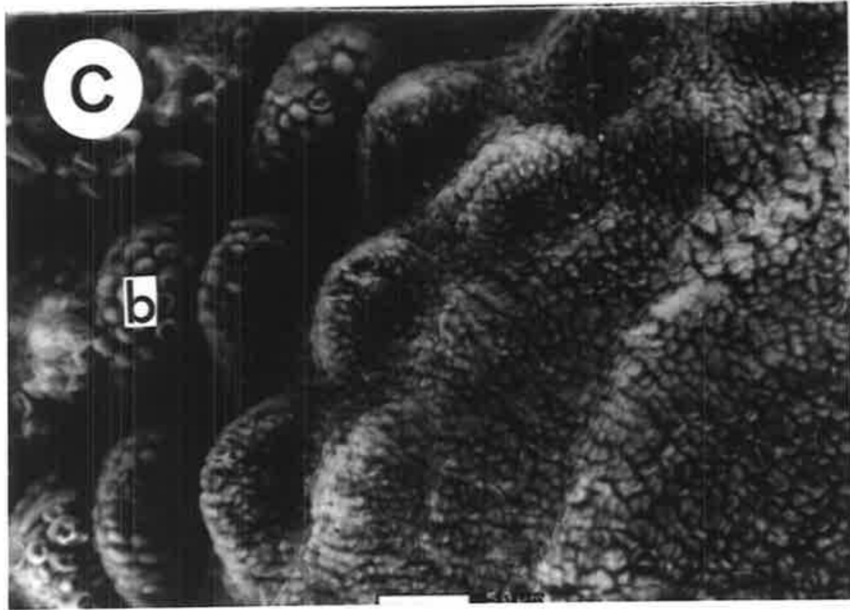
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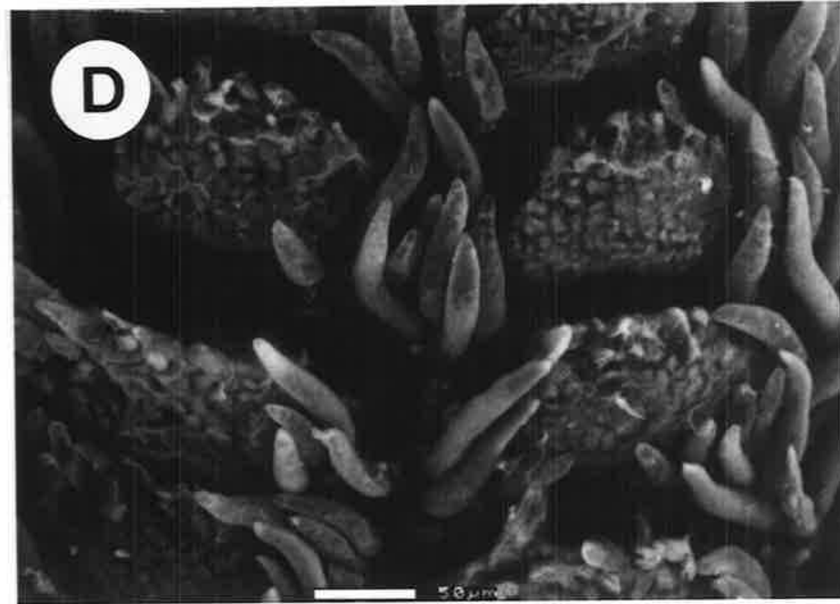
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occurred monthly was assessed by calculating the average growth per plant. Analysis of variance was performed for the months of November, December and January during which flushing was observed. The Schéffe statistic was employed to compare the groups of interest. For the ESEM data a binomial model was fitted using Genstat 5 to test the significance of differences between the treatments.

3.3 Results

3.3 1 Controlled environment experiments

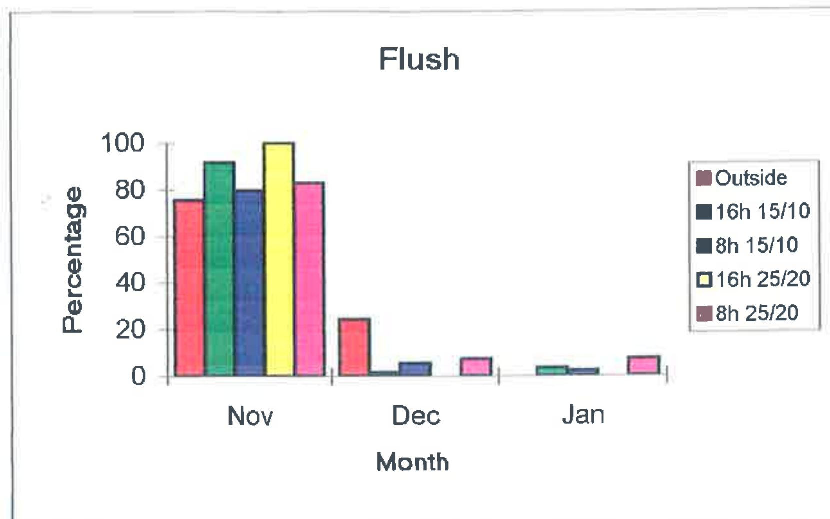
The percentage of shoots which flushed in the treatments located in Claremont Orchard was greater in *Banksia coccinea* than in *Banksia hookeriana*. The pattern of flushing was similar in the two species, with most shoots flushing in November (Figure 3.5A and 3.6A). In *Banksia coccinea* a similar pattern was observed in both the long and short day treatments at both temperatures(Figure 3.5A). The percentage of flush in November in the long day, low temperature (15/10°C) treatment was significantly greater than in the short day, low temperature treatment ($P<0.05$) but not significantly different from outside. At the higher temperature (25/20°C) all flushing in the 16 hour treatment occurred in the month of November. The 8 hour high temperature treatment showed the majority of flush in November also, but some flushing was still occurring until January (Figure 3.5A). Statistical analysis indicated a significant difference between these two treatments for November, December and January ($P<0.01$). For *Banksia hookeriana* in both long and short day treatments at 15/10°C only low levels of flush occurred in November and December (Figure 3.6A). There was no significant difference between these two treatments in these months but there was a significant difference between both treatments and the control ($P<0.01$) in November. Both the 16 hour and 8 hour treatments at the high temperature exhibited strong flush with the

Figure 3.5: Bar graphs illustrating the pattern of flush, vegetative growth and flowering of *Banksia coccinea* plants outside and in controlled environment conditions.

3.5A The patterns of flushing over a three month period for *Banksia coccinea* under outside and controlled environment conditions.

3.5B The patterns of average vegetative growth per plant (cm) over a three month period under outside as well as controlled environment conditions.

3.5C Flowering patterns as determined via ESEM examination over the three months of November, December and January for *Banksia coccinea* plants under outside and controlled environment conditions.



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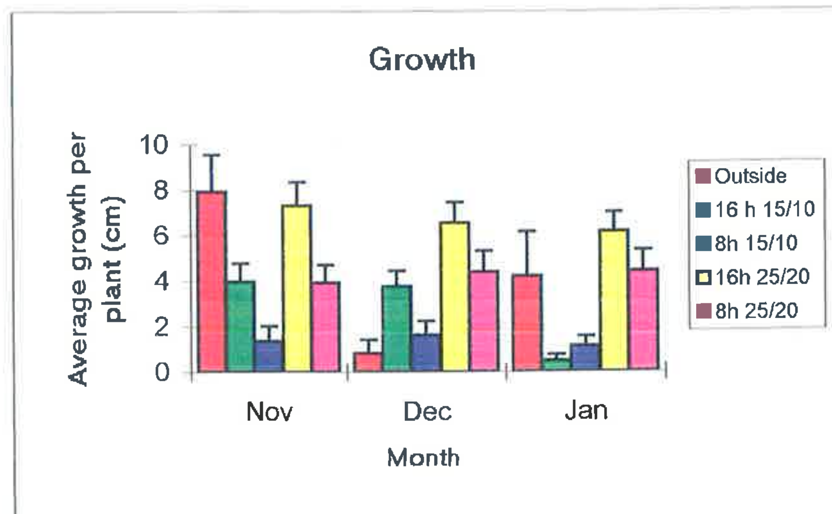

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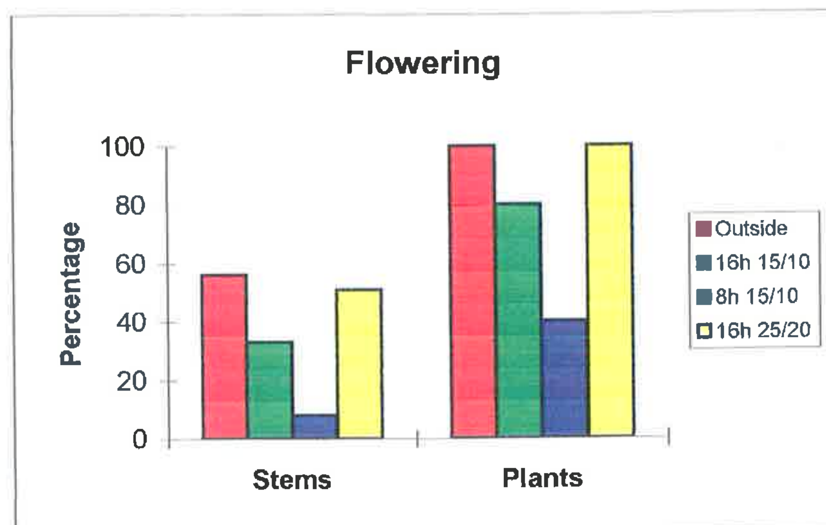

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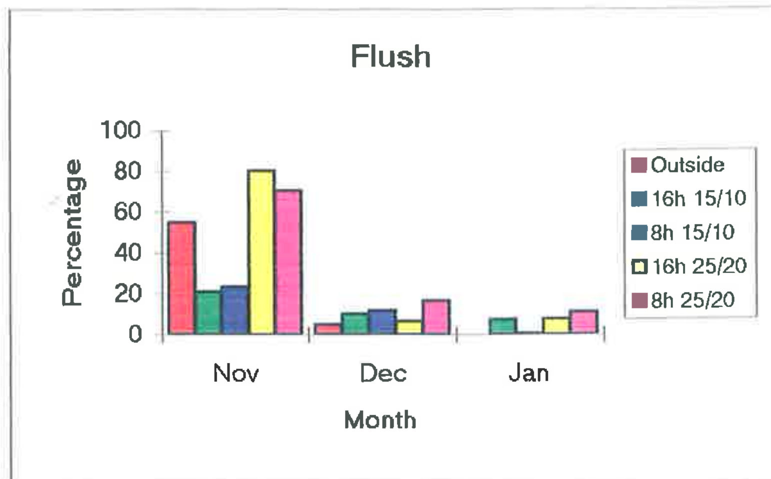
Figure 3.6: Bar graphs illustrating the pattern of flush, vegetative growth and flowering of *Banksia hookeriana* plants outside and in controlled environment conditions.

3.6A The patterns of flushing over a three month period for *Banksia hookeriana* under outside and controlled environment conditions.

3.6B The patterns of average vegetative growth per plant (cm) over a three month period under outside as well as controlled environment conditions, for *Bankisa hookeriana* plants.

3.6C Flowering patterns as determined via ESEM examination over the three months of November, December and January for *Banksia hookeriana* plants under outside and controlled environment conditions.



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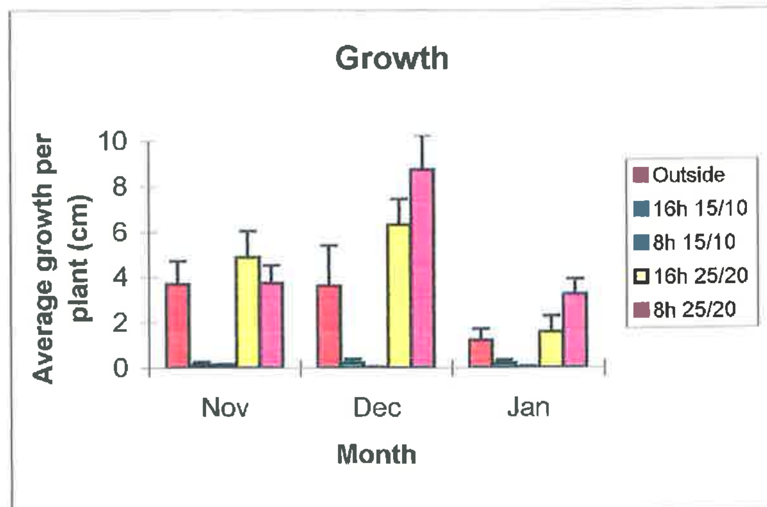

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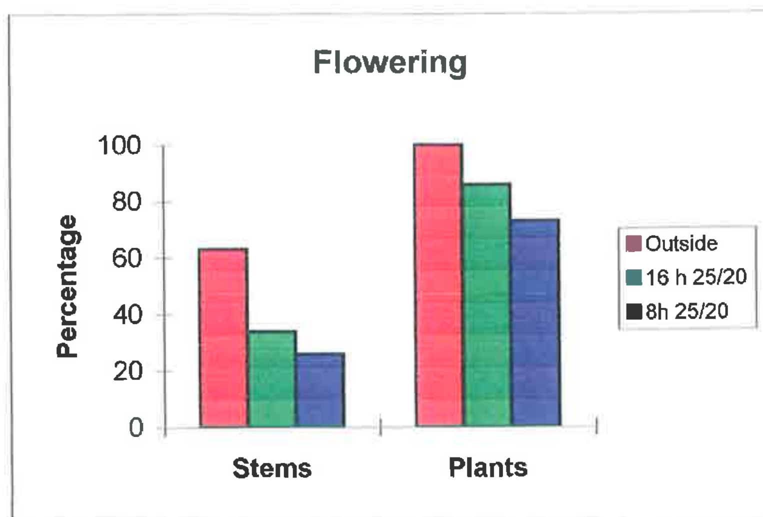

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majority in November, although flushing continued until January (Figure 3.6A). A comparison between these two treatments showed a significant difference in December ($P<0.05$).

Vegetative extension growth outside occurred during the growth flush between November and January in both species (Figure 3.5B and 3.6B). The maximum average growth per plant for *Banksia coccinea* outside was 8 cm, while for *Banksia hookeriana* outside it was 4 cm. *Banksia coccinea* plants under long day low temperature conditions showed extension growth in November and December, with a maximum average value of 4 cm, while the short day treatment at the same temperature produced a maximum average value of 1.5 cm (Figure 3.5B). A comparison between these two treatments showed a significant difference in December and January ($P<0.05$). *Banksia coccinea* plants at the long day high temperature treatment showed growth in all three months of over 6 cm per month. The short day high temperature treatment showed a similar pattern but less growth (Figure 3.5B) and a comparison between the two treatments showed a significant difference in January ($P<0.05$). In the daylength treatments applied to *Banksia hookeriana* at the low temperature the average extension growth per plant was below 0.5 cm (Figure 3.6B). Comparative analysis showed no significant difference between the treatments. When the temperature was 25/20°C, a significant increase in growth resulted. The maximum amount of growth for the 16 hour treatment was approximately 6 cm in November and January, while the 8 hour treatment peaked at 9 cm in January. A significant difference was shown between these treatments in the month of November ($P<0.05$).

The percentage of shoots and plants which flowered was higher for the outside treatments of both species at 60% of shoots and 100% of plants (Figure 3.5C and 3.6C). For *Banksia coccinea*, both long day treatments resulted in flowering and the

higher temperature treatment resulted in a similar level to the control. The low temperature short day treatment produced limited flowering (Figure 3.5C) and the high temperature short day treatment none. The short day treatments were significantly different from both the 16 hour and outside treatments, for both the percentage of plants flowering (P<0.01) and the percentage of shoots (P<0.001). For ***Banksia hookeriana***, only the high temperature long and short day treatments resulted in flowering. No flowering was observed for either daylength at the low temperature. There was no significant difference between treatments for the percentage of plants flowering, whereas for the percentage of shoots the control treatment was significantly different from both daylength treatments (P<0.001).

3.4 Discussion

This is the first study to examine floral initiation using controlled environment conditions with the genus *Banksia*. A link between vegetative growth and reproductive development has been established for both species. Floral initiation in ***Banksia coccinea*** appears to be influenced by daylength, while temperature appears to affect ***Banksia hookeriana***.

Vegetative flush and growth, for both species was highest in the November/ December period. For plants under outside conditions this was also the time when vegetative growth was optimal. Under controlled environment conditions, ***Banksia coccinea*** produced more flush and growth at 16 hours while for ***Banksia hookeriana*** this occurred under high temperature conditions regardless of daylength. Research using the family Proteaceae has indicated that a number of genera and species grow vegetatively at this time of year (Jacobs, 1983; Jacobs, 1985; Jacobs *et al*, 1986; Heinsohn and Pammenter, 1988; Faragher, 1989; Wallerstein, 1989a; Dupee and Goodwin, 1989a; Dupee and Goodwin,

1989b). These genera include *Leucospermum* (Jacobs, 1983; Jacobs, 1985; Jacobs *et al*, 1986), *Telopea* (Faragher, 1989; Dupee and Goodwin, 1989b), *Banksia* (Wallerstein, 1989a) and *Protea* (Heinsohn and Pammenter, 1988). Not all genera in the family Proteaceae produce vegetative growth in the spring /summer period. The growth pattern in *Macadamia* is such that vegetative flushes occur throughout the year and timing is dependent on location (Moncur *et al*, 1985; Storey, 1985).

A period of vegetative growth appears to be necessary before reproductive development commences. This has been documented numerous times in the family Proteaceae for genera such as *Leucospermum*, *Protea* and *Telopea* (Jacobs, 1983; Jacobs, 1985; Jacobs *et al*, 1986; Faragher, 1989; Wallerstein, 1989a; Dupee and Goodwin, 1989b; Malan and Brits, 1989; Malan and Jacobs, 1994). Wallerstein (1989a) states that vegetative and reproductive phases are separate but follow each other in sequence. In spring and summer vegetative growth takes place, and after cessation of shoot elongation macroscopic inflorescences are visible. The diameter and length of the shoot can also influence the commencement of reproductive development. Longer and thicker shoots of *Banksia coccinea* and *Banksia menziesii* have been shown to flower in their first year, whereas shoots which remain below a certain size do not produce flowers even after two years (Fuss and Sedgley, 1992). Gerber *et al* (1994) found that longer and thicker stems were most likely to produce flowers in *Protea* cv 'Ivy'. It was suggested that two seasons growth was necessary before flowering would occur in *Protea* cv 'Carnival'. Better quality flowers occurred on stems which were longer in *Leucospermum* (Napier *et al*, 1986). Not only is vegetative growth necessary before reproductive development will occur, but longer and thicker stems are more likely to be reproductive.

Under conditions of long daylength at both high and low temperature, *Banksia coccinea* plants produced more floral apices. This response was facultative; long days were

not an absolute requirement to produce inflorescences but floral production was enhanced. The trigger for floral induction may not necessarily be a response to the daylength conditions. A long day provides more light to plants than short days, hence the response recorded may be due to a higher level of assimilate accumulation. Observations on *Telopea speciosissima* indicate that assimilate supply may be critical to floral induction (Faragher, 1989). Floral initiation occurs at times of increasing light intensity and daylength. Fewer plants in natural stands produce inflorescences (0.5-1%) when compared to commercial plantations, where inflorescence production of mature plants is close to one hundred percent. Thin, weak shoots which do not flower and vegetative growth continuing on shoots which fail to initiate inflorescences, also indicates that assimilate accumulation is important. This is also true for *Banksia coccinea* indicating a role for the accumulation of assimilates in floral initiation. The exact nature of whether floral induction is promoted by assimilate accumulation or daylength needs to be further investigated using night break experiments and more detailed examination of the role light plays in induction.

Banksia hookeriana was found to show no clear daylength response under the conditions tested. At a temperature of 15/10°C vegetative flush was observed but little subsequent growth occurred. There was no microscopic evidence of floral initiation, all apices were found to be vegetative. Higher temperatures resulted in greater vegetative growth and floral apex production. The temperature effect in *Banksia hookeriana* appears to be absolute. It is postulated that below a certain temperature vegetative growth and floral induction will not occur. Two explanation are possible for this temperature response. Firstly a dormancy response may have been triggered by the low temperature treatment. Apices ceased growth due to the low temperature experienced and floral induction may not have occurred due to this dormant state. This response has been observed in *Banksia ashbyi* (Wallerstein, 1989a). Floral apices continued to develop during the winter months but if an

apex was not induced and remained vegetative a period of dormancy was observed due to the low temperatures. The second explanation for a temperature response is that temperature is playing a role in floral induction. *Macadamia integrifolia* requires a specific night temperature range to promote flowering (Stephenson and Gallagher, 1986). No statistically significant effect of night temperature was found on floral induction, but 20°C night temperature promoted more racemes. At 21°C inhibition of flowering was observed, whereas between 18-20°C flowering was promoted (Stephenson and Gallagher, 1986; Trochoulias and Lahav, 1983). Temperature has been found to affect floral induction in other plants (Zieslin and Gottesman, 1986; Roberts and Menary, 1989; King *et al*, 1992). Floral induction of *Chamelaucium uncinatum* exhibits an interaction of photoperiod and temperature such that temperature modifies the photoperiod response and slows floral development (Roberts and Menary, 1989). At low temperatures flowering could occur at either short or long daylengths whereas at high temperature only short days were inductive. Exposure to low temperature (< 15°C) was found to be essential for floral induction in two *Pimelea* species. Floral initiation occurred at low temperatures but floral development was enhanced by warmer temperatures (King *et al*, 1992). It would appear that *Banksia hookeriana* has an absolute requirement for a warm temperature to produce both vegetative growth and inflorescences. This is not surprising considering the natural habitat of *Banksia hookeriana* is a hot dry region of Western Australia. The extreme environmental conditions which prevail in this location may play an important role in floral induction in *Banksia hookeriana*.

Temperature and daylength have been found to play a role in the floral induction of two species in the genus *Banksia*. This information will assist in improving management practices on commercial plantations. Previous research (Fuss and Sedgley, 1990) and the information gained during this study suggest that the time of maximum vegetative growth

for *Banksia coccinea* is also the time when floral initiation occurs, and vegetative growth will produce inflorescences in the following year. The standard pruning practice is to prune after inflorescence production has ceased. Any pruning of shoots which have initiated in that year will result in a drop in production for the following year. By making growers aware of this phenomenon in *Banksia coccinea* a reduction in potential loss from pruning will be reduced. In addition, artificial extension of the light period by supplementary winter lighting may advance flowering of *Banksia coccinea* and so extend the flowering period. The requirements which *Banksia hookeriana* displays for warm temperatures during vegetative growth and floral induction has different management implications. The location of plantations has important consequences for growers. If the area proposed has conditions during the induction period, below 15/10°C *Banksia hookeriana* will fail to flower and this will result in financial loss.

3.5 Conclusions

Using controlled environmental conditions the parameters which induce floral initiation in two species of *Banksia* have been investigated. *Banksia coccinea* responses to an increase in daylength, while *Banksia hookeriana* response to increasing temperature. The mechanisms which have initiated these responses were discussed and further experimentation suggested. Floral initiation and development is a complex and intricate process, this investigation is one step in gaining a full understanding of the mechanisms which governs this fundamental process. The information gained from this study has enabled the current management practises to been evaluated and modified for these species to ensure maximum utilisation of floral resources.

Chapter Four: Genetic variation within and between cultivated and natural populations of *Banksia coccinea* and *Banksia menziesii*.

4.1 Introduction

Genetic variability is essential for the selection and breeding of new cultivars with novel traits. Morphological variation in cut flower quality both within and between cultivated *Banksia* populations in South Australia and Western Australia has been documented (Fuss and Sedgley 1991c), with parameters including bloom number, bloom quality, stem and inflorescence length and time of production showing high levels of variability. The extent of this variation is illustrated by a selection of inflorescences cultivated in South Australia (Figure 4.1). To extend this work, an examination of the level of genetic variation within and between cultivated populations in South Australia and natural populations in Western Australia is investigated using randomly amplified polymorphic DNA (RAPD) markers.

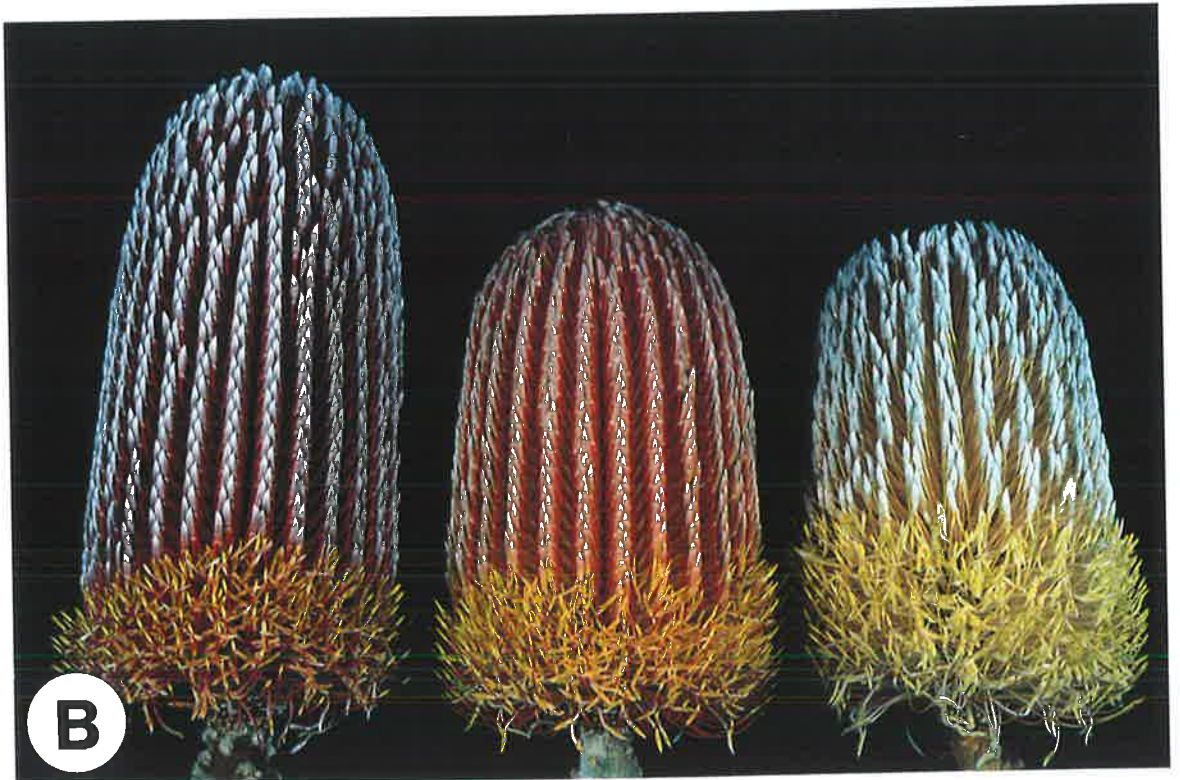
RAPD techniques have application to areas such as systematics, horticulture, floriculture, forestry and conservation biology. Expansion into these areas of biology have placed new demands on DNA extraction methodologies. Large numbers of samples must be collected, often from locations remote from laboratory facilities, and stored under conditions which preserve DNA integrity. A previously reported storage method (Thomson and Henry, 1993) was adapted for field collected material used for RAPD studies of genetic variability of *Banksia*. The benefits from this investigation are in the areas of cultivar selection and development, and in conservation of natural populations. Knowledge of the variability available in cultivated plantations in relation to that in natural populations will focus future collection from the wild in the most productive areas. South Australian cultivated populations were investigated in this study as the Australian *Banksia* selection and breeding

Figure 4.1: Floral variation of cultivated *Banksia coccinea* and *Banksia menziesii* under ~~cultivated~~ conditions at Blewitt Springs, South Australia.

4.1A Three *Banksia coccinea* inflorescences, colour variation from left to right, pink, red and orange inflorescences. Size variation also illustrated.

4.1B Three *Banksia menziesii* inflorescences, colour variation from left to right, bronze, maroon and yellow. Size variation also illustrated.

A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program is based in Adelaide, and most selection is conducted on local cultivated material. The information gained during this study will also facilitate the development of conservation strategies for the remaining natural populations of ***Banksia coccinea*** and ***Banksia menziesii*** in Western Australia.

4.2 Materials and Methods

4.2.1 Sampling

A survey was conducted of genetic variation of ***Banksia coccinea*** plants in seven cultivated populations in South Australia (SA) (CS1-7) and seven natural and one cultivated population in Western Australia (WA) (CW1-8) (Table 4.1, Figure 4.2) and of ***Banksia menziesii*** plants in five cultivated populations in SA (MS1-5) and in six natural populations in WA (MW1-6) (Table 4.2, Figure 4.3). The cultivated populations in SA were all close to Adelaide (Lat 35°S; Long 138°E) allowing collection of fresh leaf material. Ten mature leaves from between five and 19 plants per population were collected at random, and placed in plastic bags on ice, followed by storage at -20°C. In WA leaf material was collected in summer from the fourteen field locations. The leaves were placed directly into paper bags and allowed to dry naturally in cardboard boxes. The temperature within the boxes over the collection period ranged between 35°C and 40°C. The collection period lasted 2 weeks while covering a total distance of approximately 4000 km. After collection the boxes were airfreighted to Adelaide, SA. Upon return to the laboratory the leaves were stored at 2°C for the period of the study. At the time of the initial extraction *B. menziesii* leaf material was 35 days old while *B. coccinea* leaves were 42 days old and subsequent extractions were conducted up to 150 days after collection.

Table 4.1: *Banksia coccinea* populations in South Australia (35° 138°) and Western Australia (35° 118°).

Population Code	*Population Source*	*Year Planted*	*Population Size (approx)*	*Plants Sampled*	*Grid reference from 1:100 00 maps*
CS1	Waller	1980	100	5	Milang 6627 795 055
CS2	Waller	1983	100	10	Milang 6627 795 055
CS3	Keith	1976	300	13	Milang 6627 790 055
CS4	Devitt	1984	200	12	Milang 6627 805 060
CS5	Goodman	1984	300	19	Milang 6627 785 055
CS6	Barson	1981	100	10	Milang 6627 770 043
CS7	Branden	1981	100	12	Milang 6627 795 035
CW1	Redmond		12	8	Mount Barker 2428 476 371
CW2	Stirling Ranges		250	20	Borden 2529 854 070
CW3	Cape Richie		350	15	Chenye 2628 705 595
CW4	Chenye Beach		50	18	Many Peaks 2528 283 389
CW5	Two Peoples Bay		80	19	Many Peaks 2528 030 315
CW6	Hassell Highway		50	20	Pallinup 2629 820 475
CW7	Gull Rock		500	14	Mount Barker 2428 905 284
CW8	Mount Barker (cultivated)		225	19	Mount Barker 2428 788 728

Table 4.2: *Banksia menziesii* populations in South Australia (35° 138°) and Western Australia (31° 115°30′).

Population Code	*Population Source*	*Year Planted*	*Population Size (approx)*	*Plants Sampled*	*Grid reference from 1:100 00 maps*
MS1	Waller	1986	100	10	Milang 6627 795 055
MS2	Waller	1983	100	10	Milang 6627 795 055
MS3	Keith	1983	200	10	Milang 6627 790 055
MS4	Goodman	1984	100	10	Milang 6627 785 055
MS5	Barson	1981	100	10	Milang 6627 770 043
MW1	Kings Park and Botanic Garden		200	26	Perth 2034 630 890
MW2	Cataby		50	26	Wedge Island 1936 220 523
MW3	Gingin		2000	26	Perth 2034 060 025
MW4	Lake Indoon		1000	26	Wedge Island 1936 967 260
MW5	Yerina Springs		100	26	Hutt 1741 390 880
MW6	Ogilvie West		500	26	Hutt 1741 300 930

Figure 4.2: Location of populations of *Banksia coccinea* in Western Australia (Taylor and Hopper, 1988).

Population Codes: CW1, Redmond. CW2, Stirling Ranges. CW3, Cape Richie. CW4, Chenye Beach. CW5, Two Peoples Bay. CW6, Hassel Highway. CW7, Gull Rock. CW8, Mount Barker.

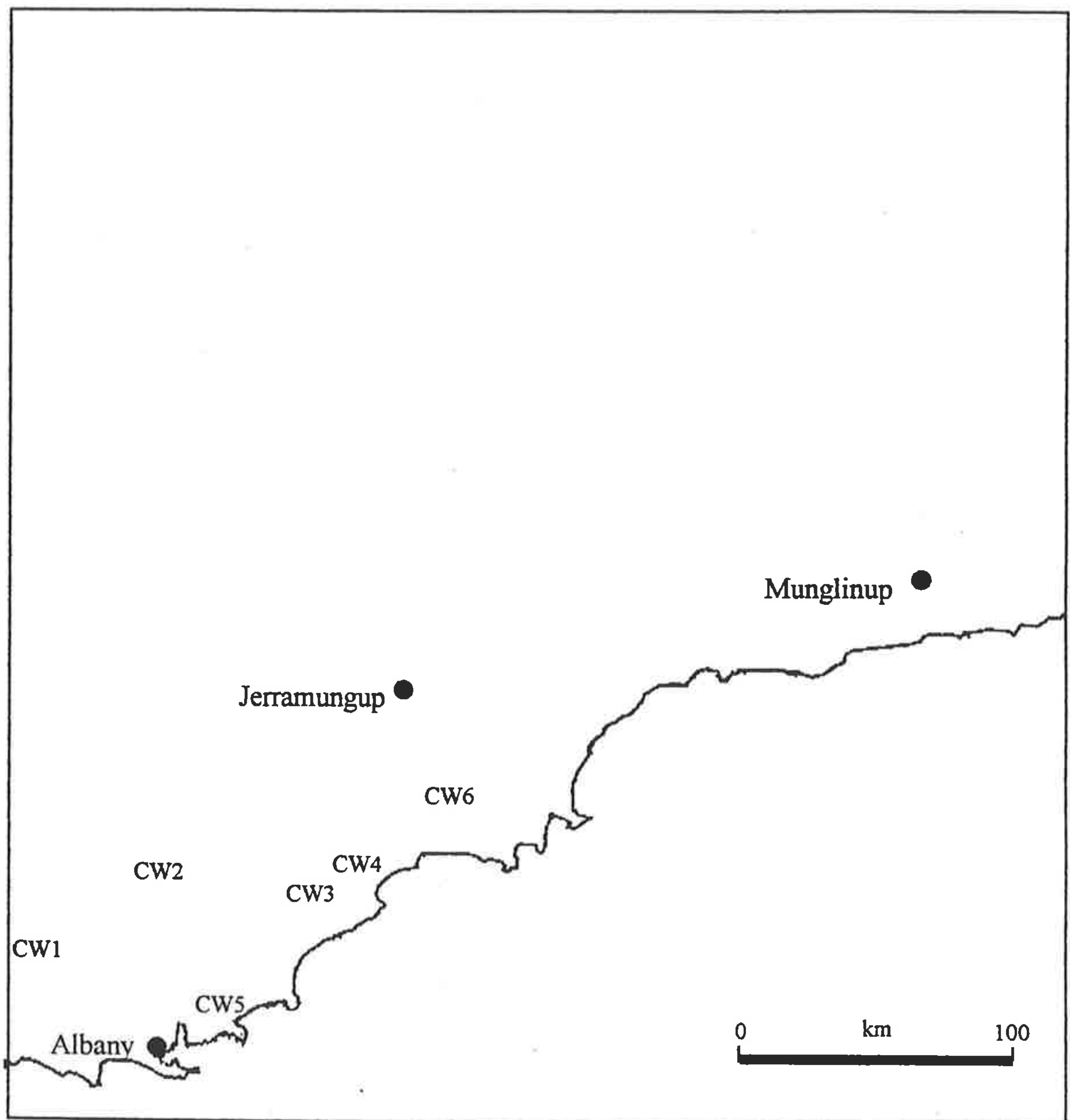
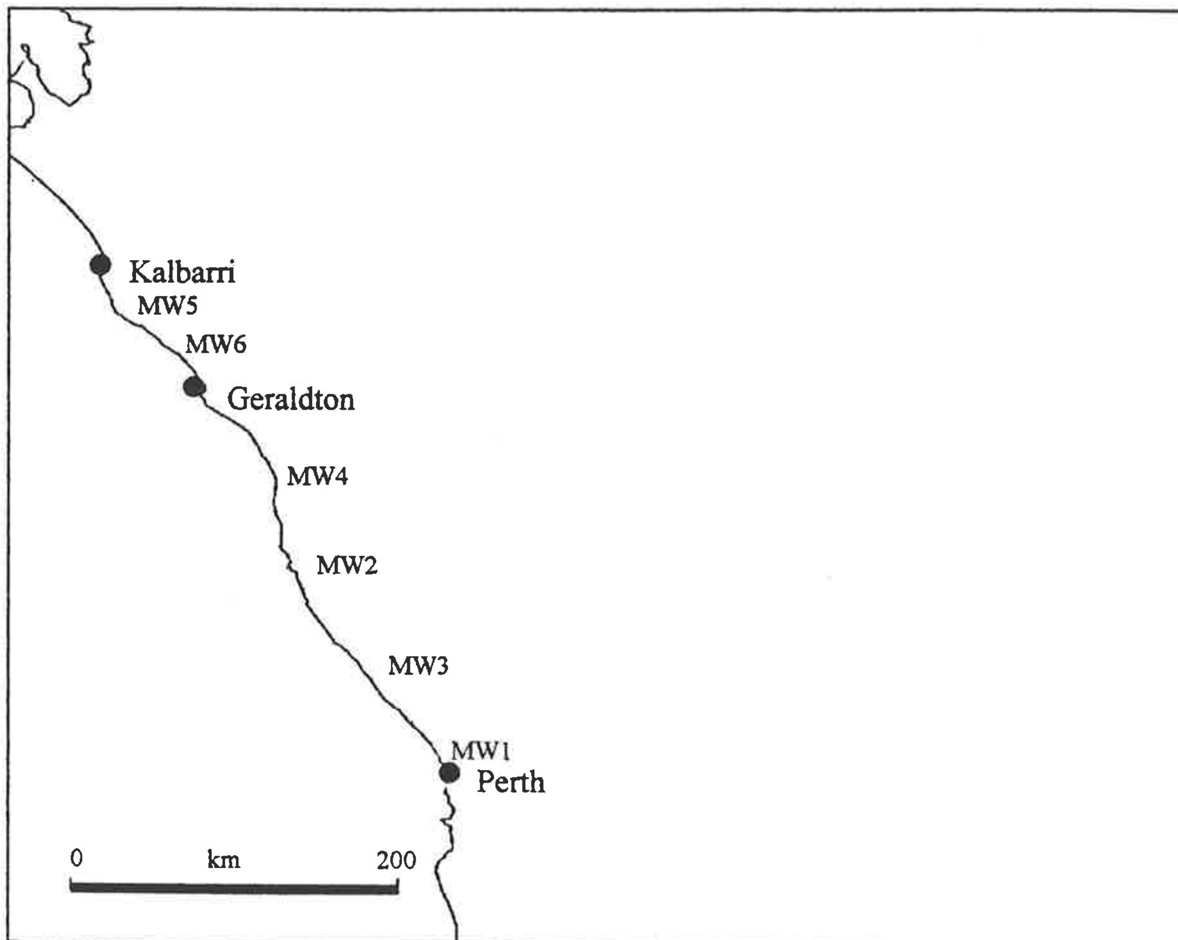

Munglinup
Jerramungup
CW6
CW2
CW4
CW3
CW1
CW5
Albany
0
km
100

Figure 4.3: Location of natural populations of *Banksia menziesii* in Western Australia (Taylor and Hopper, 1988).

Population Codes: MW1, Kings Parks and Botanic Garden. MW2, Cataby. MW3, Gingin. MW4, Lake Indoon. MW5, Yerina Springs. MW6, Ogilvie West.


Kalbarri
MW5
MW6
Geraldton
MW4
MW2
MW3
MW1
Perth
0
km
200

4.2.2 DNA methodology

DNA was isolated from mature tissue using a modified CTAB method (Doyle and Dickson, 1987; Doyle and Doyle, 1988). Additional purification of DNA extracted from leaf tissue entailed re-extracting the DNA with 200 ml phenol/chloroform/isoamyl alcohol and reprecipitation with 600 ml isopropanol. The pelleted DNA was resuspended in 100 ml of TE buffer (pH 8). Further treatment using a DNA purification kit (BresaClean, Adelaide, Australia) was required to remove polysaccharides from the DNA. The quality of the DNA was measured using a mini-fluorometer (Hoefer Scientific Instruments, San Fransisco, CA). Some of the DNA was restricted with the endonuclease *Hae*III to test purity. DNA was also subjected to PCR using the RAPD technique (Williams *et al*, 1990; Muralidharan and Wakeland, 1993).

4.2.2.1 Isolation of *Banksia* DNA from leaf tissue

CTAB isolation buffer (2% (w/v) CTAB, 1.4 M NaCl, 0.2% (v/v) 2-mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl (pH 8.0)) (7 ml) was added to a 10 ml centrifuge tube preheated to 60°C in a water bath. Between 1 and 1.5 grams of leaf tissue was ground to a powder in liquid nitrogen with a mortar and pestle. The powder was added directly to the preheated buffer and mixed thoroughly. The mixture was inverted several times and the sample incubated at 60°C for 30 minutes. This mixture was then extracted once with chloroform-isoamyl alcohol (24:1 v/v) and the phases were concentrated by centrifugation at 4000 rpm. The aqueous layer was removed and transferred to a clean centrifuge tube. Two to three volumes of cold isopropanol was added, gently inverting the tube several times to mix. The DNA usually appeared flocculent and was concentrated by centrifugation at 3000 rpm for 20 minutes. The pellet was washed with 70% ethanol overnight. A further centrifugation for 20 minutes at 3000 rpm produced another pellet. The supernatant was removed and the

pellet allowed to air dry at room temperature for 5 minutes. The pellet was resuspended in 100 µl of TE buffer (10 mM Tris-HCl (pH 7.4), 1 mM EDTA) and 3 µl of heat treated RNAase (10 mg/ ml). A bulk sample of DNA was prepared for each *Banksia coccinea* population comprising between 20 and 40 ng of DNA from each individual (Penner *et al*, 1993; Chaparro *et al*, 1994).

4.2.2.2 Polymerase Chain Reaction using random primers

A primer survey was conducted (Operon Technologies, Alameda CA), using a total of sixty primers from series A, B and D. Primers OPA03 (AGTCAGCCAC) and OPA07 (GAAACGGGTG) were selected for *Banksia coccinea* and primers OPD08 (GTGTGCCCCA) and OPB17 (AGGGAACGAG) for *Banksia menziesii* as these provided maximum polymorphisms. The 30 µl mixture (Table 4.3) was overlaid with one drop of paraffin oil and cycled through the following reaction profile using a Programmable Thermal Controller (MJ Research Inc, Watertown, Mass, USA): 94°C for 4 minutes, followed by 45 cycles of 94°C for 15 seconds, 36°C for 15 seconds, and 72°C for 30 seconds. This was followed by 72°C for 2 minutes (Muralidharan and Wakeland 1993).

4.2.2.3 Agarose gels

The PCR products (6 µl) from each reaction for both species were mixed with 4 µl of Ficoll Dye (x10, 0.25% bromophenol blue, 0.25% xylene cyanol, 25% Ficoll type 400) and checked on a 1% Promega molecular biology grade agarose gel using Tris-borate-EDTA buffer, run at 250 volts with a current of 150 mAmps for thirty minutes. The DNA was visualised under ultra violet light after staining for 15 minutes with ethidium bromide (concentration 200 µl [stock 10mg/ml] in 2 litres) and photographed (Polaroid Direct screen instant camera DS34, England; Polaroid Film 667, England). For assessment of *Banksia menziesii* PCR products Seakeam agarose was used. Agarose gels (2.5%) with 8mm combs,

Table 4.3: Contents of 30 μl reaction mixture for standard PCR reaction.

Solutions	*Volume (μl)*	*Final concentration*
25 mM $MgCl_2$	4	3.34 mM
10x buffer	3	x1
10 mM dNTPs	0.6	200 μM
T_4 Gene 32	0.2	
Taq polymerase	0.5 unit	5.5 units / μL
Primer	variable	0.25 μM
DNA	variable	20 - 40 ng
Water	variable	Add to 30 μl

run at 250 volts and 100 mAmps for 90 minutes satisfactorily separated ***Banksia menziesii*** PCR products. Product sizes ranged from 2000-100 bp.

4.2.2.4 Polyacrylamide gels

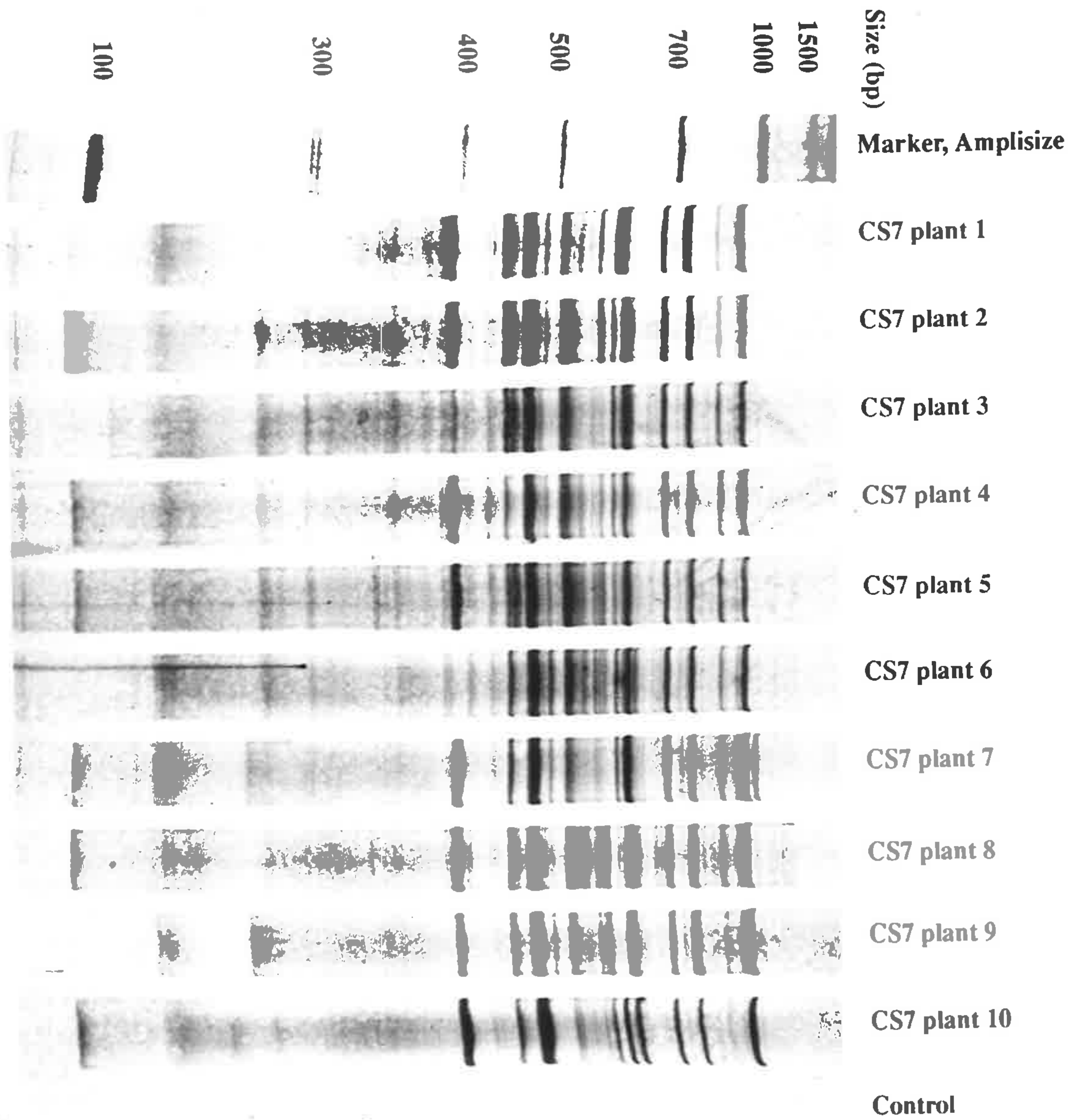
Polyacrylamide gels were used to increase band resolution for ***Banksia coccinea*** products (Bassam *et al*, 1991). A cross linkage of 19:1 bis-acrylamide was used with a final concentration of 4% acrylamide and 8 M urea. Equal volumes (12 µl) of PCR sample and loading dye (95% formamide, 10 mM EDTA, pH 8.0, 0.02% xylene cyanol FF, 0.02% bromophenol blue) were mixed, heated at 95°C for 5 minutes then immediately cooled on ice. The loaded gel was run at 20 mAmps for 1.5 hours in TBE buffer. Unbuffered 3 M sodium acetate, 20 ml, was added to the buffer, to ensure that high molecular weight bands travelled more slowly and the gel was run for a further 2 hours. Visualisation of the gel was carried out using a silver staining procedure (Bassam *et al*, 1991). A silver solution (0.2 g $AgNO_3$, 300 µl formaldehyde) was used to impregnate the gel and the absorbed silver was visualised using a developing solution (12 g Na_2CO_3, 600 µl formaldehyde, 800 µl Na_2S2O_3 (1 mg/ml)). The developing time was variable but did not exceed 5 minutes. The stained gel was fixed in 10% acetic acid, and transferred onto filter paper (Whatman Chromatography paper 3MMChr) and dried using a vacuum gel drier (BioRad, Hercules, CA, Model 583) at 80°C for 40 minutes. PCR products ranged in size from 2000-50 bp (Figure 4.4). Only clearly visible and reliable bands were scored. Reliability was assessed by repeating each gel twice, and comparing the bands.

4.2.3 Statistical analysis

For ***Banksia coccinea*** gels the distance of each band from the origin was measured using callipers (Mitutayo, Japan). Repeatable bands were numbered sequentially from the origin to facilitate comparisons between different gels. ***Banksia menziesii*** gels were scored

Figure 4.4: Polyacrylamide gel of *Banksia coccinea* with samples from Branden's population (CS7) in South Australia using primer OPA 07.

Lane 1: Marker (Amplisize,). Lanes 2-11: Population CS7, plants 1-10. Lane 12: Control, no DNA added.


Size (bp)
100
300
400
500
700
1000
1500
Marker, Amplisize
CS7 plant 1
CS7 plant 2
CS7 plant 3
CS7 plant 4
CS7 plant 5
CS7 plant 6
CS7 plant 7
CS7 plant 8
CS7 plant 9
CS7 plant 10
Control

with the aid of computer program (CREAM, Kem-en Tec, Sweden). Scanned images of each gel were entered via polaroid photographs using a desktop scanner (Hewlett Packard, Scanjet IIcx/T). Each lane of every gel was compared to a molecular weight standard lane and a list of the bands and their corresponding molecular weights was produced. Bands were numbered sequentially to facilitate comparison between different gels. The presence or absence of each band was recorded for each individual and given a value of 1 or 0 respectively. This information was used to calculate similarity matrices within and between populations using a Microsoft Excel macro program based on the formula $S = M_{ab}/N_t$. M_{ab} is the number of matches between two genotypes tested, including the bands which the two individuals share, and also the bands present in other individuals in the population but absent in the two individuals compared. N_t is the total number of bands identified, which remained constant. Genetic variation was calculated using the formula $V = 1\text{-}S$, where V is genetic variation and S is similarity (Lamboy, 1994; Rossetto *et al*, unpub.).

The data from each of the two primer sets were pooled, providing a single data set per species. Each individual within a population was compared with every other individual in that population in a pairwise fashion. This gave an estimate of the percentage similarity within a population. A condensed profile was constructed, for each *Banksia menziesii* population which was compared with other population profiles to generate a between population similarity value per population pair. For *Banksia coccinea* populations bulk DNA samples were used. The number of monomorphic and polymorphic bands per population was recorded, which allowed the overall percentage of polymorphism to be determined in each population.

Dendrograms were constructed using three different clustering methods based on the Numerical Taxonomy and Multivariate Analysis System (NTSYS-pc Version 1.80 Exeter Software, New York). These methods were the UPGMA, the single-linked, and the

complete-linked methods. Using NTSYS a comparison of these methods based on correlation coefficients and the Mantel statistic (Smouse, *et al* 1986) showed that dendrograms from all these methods were comparable. The dendrograms presented use the single-linked clustering method.

4.3 Results

4.3.1 DNA from leaf material of *Banksia* collected under field conditions

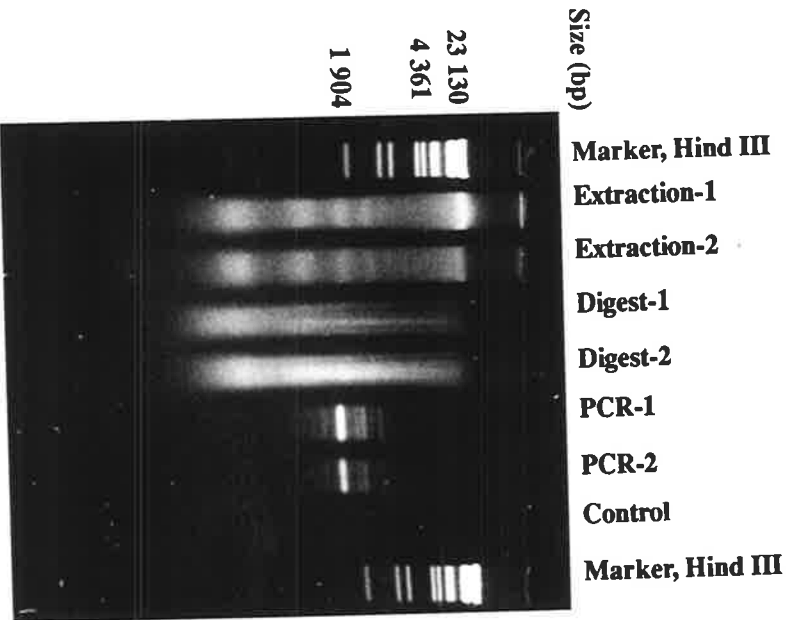
One hundred nanograms and one hundred and twenty nanograms of DNA was extracted from dried leaves of *Banksia menziesii* and *Banksia coccinea* respectively at 35 and 42 days, compared with eighty five nanograms and one hundred nanograms at 150 days. The *Hae*III digestion and RAPD fingerprint remained unchanged with time in storage (Figure 4.5). This result demonstrates that high quality DNA, which can be restricted and used for PCR can be extracted from field collected dried and stored leaf tissue

4.3.2 Similarity matrices within and between *Banksia coccinea* populations

The percentage of polymorphic bands in the SA populations ranged from 37.0% to 64.0% (Table 4.4), with the average level of polymorphism 48.5%. The total number of bands in the SA populations was 214 and in the WA populations 211. Polymorphic bands in the WA populations ranged from a very low 24.2% in CW1 up to 73.0%. The average level of polymorphism in the WA populations was 59.2%. The within population similarity values for the six SA populations of *Banksia coccinea* showed a range of between 70.1% and 93.5% (Table 4.5). The highest mean similarity was in populations CS7 (86.7%) and CS4 (86.0%). Population CS6 had the lowest mean percentage similarity (78.4%), whereas population CS3 had the greatest range of similarity values. Within population similarity matrices for the eight WA populations of *Banksia coccinea* ranged from 63.5% to 93.4%.

Figure 4.5: Gel electrophoresis of *Banksia coccinea* extracted DNA, restriction digest and RAPD PCR products.

Lane 1: Marker (λ phage restricted with *Hind*III/*Eco*RI). Lane 2: Total nucleic acids extracted 35 days after collection. Lane 3: Total nucleic acids extracted 150 days after collection. Lane 4: 35 day old DNA restricted with *Hae*III. Lane 5: 150 day old DNA restricted with *Hae*III. Lane 6: 35 day old DNA, RAPD banding pattern using primer OPA03. Lane 7: 150 day old DNA, RAPD banding pattern using OPA03. Lane 8: Control, containing no DNA. Lane 9: Marker (λ phage restricted with *Hind*III/*Eco*RI).



Size (bp)
23 130
4 361
1 904
Marker, Hind III
Extraction-1
Extraction-2
Digest-1
Digest-2
PCR-1
PCR-2
Control
Marker, Hind III

Table 4.4: Number of monomorphic and polymorphic bands detected with two primers in South Australian and Western Australian populations of *Banksia coccinea*.

Population Code	*Monomorphic bands*	*Polymorphic Bands*	*Percentage polymorphic bands*
CS1	132	82	38.3
CS2	106	108	50.5
CS3	77	137	64.0
CS4	125	89	41.6
CS5	112	102	47.7
CS6	85	129	60.3
CS7	135	79	37.0
CW1	160	51	24.2
CW2	90	121	57.3
CW3	79	132	62.6
CW4	57	154	73.0
CW5	73	138	65.4
CW6	69	142	67.3
CW7	64	147	69.7
CW8	96	115	54.5

Table 4.5: Within population percentage similarity for *Banksia coccinea* populations from South Australia and Western Australia.

Population	*Range*	*Mean (stdev)*
CS1	79.0 - 85.5	81.6 (0.02)
CS2	75.7 - 86.4	81.7 (0.03)
CS3	70.1 - 90.2	79.0 (0.05)
CS4	80.8 - 90.7	86.0 (0.02)
CS5	74.3 - 90.7	82.0 (0.04)
CS6	73.4 - 89.7	78.4 (0.04)
CS7	80.4 - 93.5	86.7 (0.04)
Mean		**82.2**
CW1	85.3 - 93.4	89.7 (0.02)
CW2	74.9 - 88.2	80.2 (0.03)
CW3	72.0 - 90.5	80.2 (0.04)
CW4	65.9 - 91.0	76.5 (0.06)
CW5	70.1 - 88.2	76.2 (0.04)
CW6	65.4 - 89.1	76.5 (0.05)
CW7	63.5 - 88.6	74.8 (0.05)
CW8	73.5 - 89.1	81.7 (0.04)
Mean		**79.5**

Population CW1 showed the highest mean similarity value (89.7%), while population CW7 had the lowest (74.8%). Overall the mean percentage similarity for the SA populations was higher than for the WA at 82.2% and 79.5% respectively.

The highest similarity value for between population comparisons was 96.0% between populations CS3 and CS5 (Table 4.6). Others with high between population similarity values included CS3 x CS7, CS2 x CS6 (92.9%), and CS2 x CS7, CS3 x CS6 (91.9%). The lowest similarity values between SA populations were CS4 x CS6 (85.9%) and CS1 x CS5 (84.8%). The lowest value within the WA populations was between populations CW4 x CW6 at 45.5%, whereas the highest similarity values between WA populations was 73.7% for populations CW1 x CW8 and 71.7% for CW3 x CW8. The lowest overall value was between population CS3 x CW4, and CS4 x CW4 at 39.4%, although all SA populations had low similarity to population CW4.

The mean within population genetic variation was approximately 0.2 for both SA and WA populations (Table 4.7). The mean variation between SA populations was 0.1, compared with 0.42 for between WA populations. Between SA and WA populations the mean genetic variation was 0.44.

A dendrogram was constructed for populations from SA and WA using between population similarity values (Figure 4.6). Comparison of clustering methods indicated that all three were comparable (Table 4.8), and single-linked clustering is presented. The dendrogram showed three clusters. One comprised the SA cultivated populations, all of which were closely related. The second cluster included the mainly inland WA populations of CW6 (Hassel Highway), CW2 (Stirling Ranges), CW1 (Redmond), CW8 (Mount Barker) and CW3 (Cape Richie), whereas the third, much looser cluster comprised the coastal WA populations of CW7 (Gull Rock), CW5 (Two Peoples Bay) and CW4 (Chenye Beach). The SA cluster was most closely related to the inland WA populations.

Table 4.6: Between population percentage similarity for *Banksia coccinea* populations from South Australia and Western Australia.

Population comparison	*Similarity*	*Population comparison*	*Similarity*	*Population comparison*	*Similarity*
CS1 x CS2	87.9	CS3 x CW5	61.6	CS7 x CW2	53.5
CS1 x CS3	88.9	CS3 x CW6	63.6	CS7 x CW3	62.6
CS1 x CS4	86.9	CS3 x CW7	50.5	CS7 x CW4	44.4
CS1 x CS5	84.8	CS3 x CW8	61.6	CS7 x CW5	60.6
CS1 x CS6	86.9	CS4 x CS5	89.9	CS7 x CW6	58.6
CS1 x CS7	85.9	CS4 x CS6	85.9	CS7 x CW7	53.5
CS1 x CW1	54.5	CS4 x CS7	88.9	CS7 x CW8	60.6
CS1 x CW2	57.6	CS4 x CW1	53.5	CW1 x CW2	64.6
CS1 x CW3	62.6	CS4 x CW2	54.5	CW1 x CW3	63.6
CS1 x CW4	42.4	CS4 x CW3	61.6	CW1 x CW4	51.5
CS1 x CW5	56.6	CS4 x CW4	39.4	CW1 x CW5	49.5
CS1 x CW6	58.6	CS4 x CW5	59.6	CW1 x CW6	65.7
CS1 x CW7	47.5	CS4 x CW6	57.6	CW1 x CW7	50.5
CS1 x CW8	58.6	CS4 x CW7	48.5	CW1 x CW8	73.7
CS2 x CS3	90.9	CS4 x CW8	61.6	CW2 x CW3	68.7
CS2 x CS4	86.9	CS5 x CS6	89.9	CW2 x CW4	56.6
CS2 x CS5	88.9	CS5 x CS7	90.9	CW2 x CW5	50.5
CS2 x CS6	92.9	CS5 x CW1	57.6	CW2 x CW6	70.7
CS2 x CS7	91.9	CS5 x CW2	56.6	CW2 x CW7	53.5
CS2 x CW1	60.6	CS5 x CW3	63.6	CW2 x CW8	66.7
CS2 x CW2	55.6	CS5 x CW4	41.4	CW3 x CW4	49.5
CS2 x CW3	66.7	CS5 x CW5	59.6	CW3 x CW5	53.5
CS2 x CW4	42.4	CS5 x CW6	61.6	CW3 x CW6	65.7
CS2 x CW5	60.6	CS5 x CW7	52.5	CW3 x CW7	50.5
CS2 x CW6	58.6	CS5 x CW8	61.6	CW3 x CW8	71.7
CS2 x CW7	49.5	CS6 x CS7	89.9	CW4 x CW5	55.6
CS2 x CW8	64.6	CS6 x CW1	59.6	CW4 x CW6	45.5
CS3 x CS4	89.9	CS6 x CW2	60.6	CW4 x CW7	58.6
CS3 x CS5	96.0	CS6 x CW3	63.6	CW4 x CW8	47.5
CS3 x CS6	91.9	CS6 x CW4	43.4	CW5 x CW6	47.5
CS3 x CS7	92.9	CS6 x CW5	61.6	CW5 x CW7	56.6
CS3 x CW1	57.6	CS6 x CW6	57.6	CW5 x CW8	53.5
CS3 x CW2	56.6	CS6 x CW7	48.5	CW6 x CW7	48.5
CS3 x CW3	65.7	CS6 x CW8	61.6	CW6 x CW8	67.7
CS3 x CW4	39.4	CS7 x CW1	56.6	CW7 x CW8	48.5

Table 4.7: Mean genetic variation within and between *Banksia coccinea* populations from South Australia and Western Australia.

Source of variation	*Genetic variation*
Within SA populations	0.18
Within WA populations	0.20
Between SA populations	0.10
Between WA populations	0.42
Between SA and WA populations	0.44

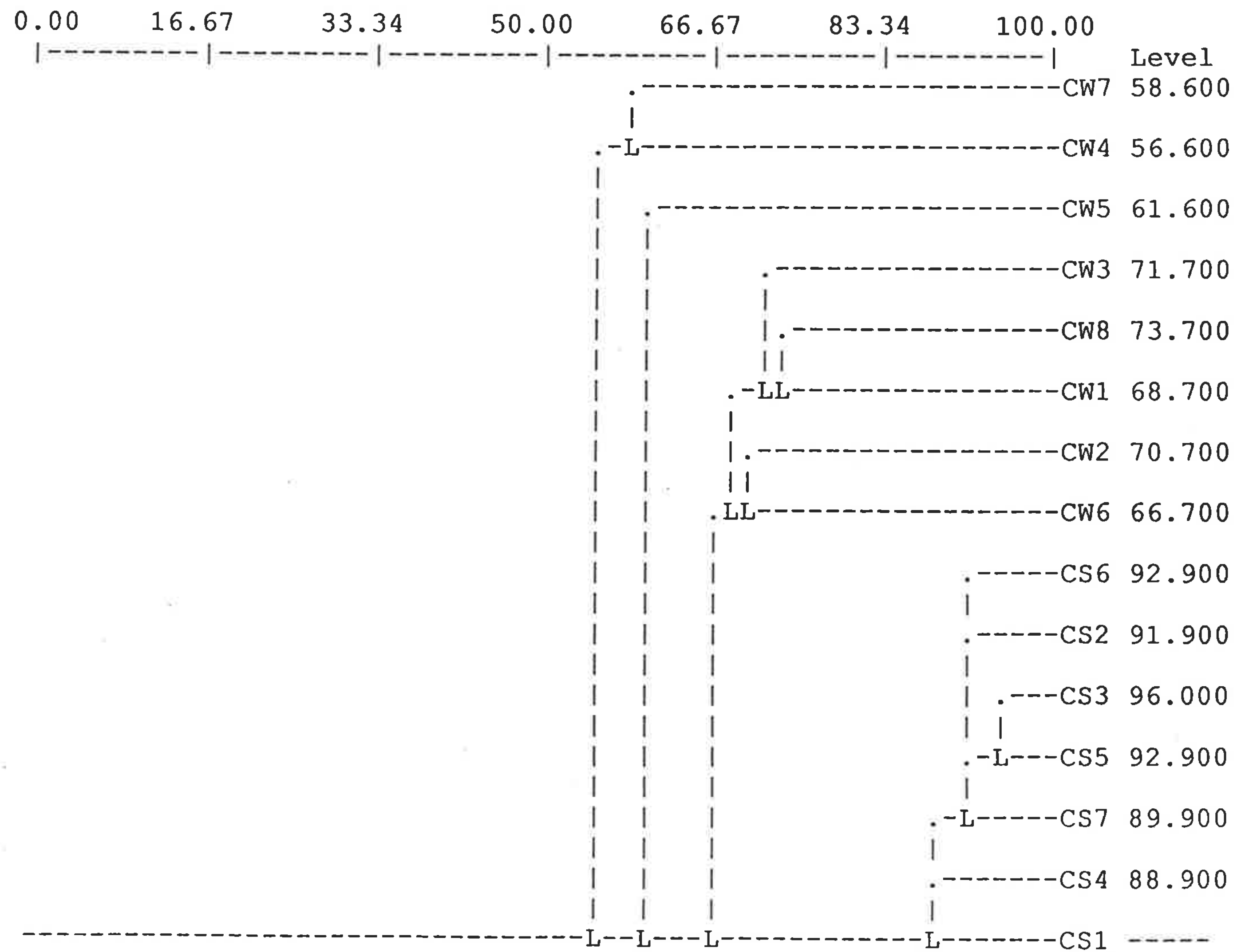
Table 4.8: Comparison of clustering methods using correlation coefficient (R) and the Mantel statistic (T) for *Banksia coccinea* RAPD data

Dendrogram clustering method	*Correlation coefficient (R)*	*Mantel statistic (T)**
UPGMA	0.967	5.886
Single-linked	0.964	5.529
Complete linked	0.956	6.287

* Values of the Mantel statistic above 3 are significant (Smouse *et al*, 1986)

Figure 4.6: Dendrogram of *Banksia coccinea* populations in South Australia and Western Australia using the single linked method.

Population Codes: South Australia CS1, Waller. CS2, Waller. CS3 Keith. CS4 Devitt. CS5 Goodman. CS6 Barson. CS7 Branden. Western Australia CW1 Redmond. CW2 Stirling Ranges. CW3 Cape Richie. CW4 Chenye Beach. CW5 Two Peoples Bay. CW6 Hassel Highway. CW7 Gull Rock. CW8 Mount Barker.


0.00
16.67
33.34
50.00
66.67
83.34
100.00
Level
CW7 58.600
CW4 56.600
CW5 61.600
CW3 71.700
CW8 73.700
CW1 68.700
CW2 70.700
CW6 66.700
CS6 92.900
CS2 91.900
CS3 96.000
CS5 92.900
CS7 89.900
CS4 88.900
CS1 -----

4.3.3 Similarity matrices within and between *Banksia menziesii* populations

The level of polymorphic bands in SA populations ranged from 62.6% in population MS1 to 83.8% in MS3 (Table 4.9). Populations in WA exhibited higher percentages of polymorphic bands, ranging from 78.8% to 89.9%. The average level of polymorphism in SA populations was 73.5% while in WA populations the average was 84.5%. Within population similarity values for the five SA populations of *Banksia menziesii* ranged from 54.5% to 80.8% (Table 4.10). The highest mean similarity value was in population MS5 (68.5%) and the lowest was in MS3 (65.7%). Similarity values within WA populations of *Banksia menziesii* ranged from 53.5% to 98.0%. Population MW6, had the lowest mean similarity value (68.8%), and MW4 (71.3%) the highest. Overall the mean percentage similarity for the SA populations was 67.4% compared with 70.0% for the WA populations.

The highest similarity between SA populations was MS3 x MS4 (91.8%), with the lowest MS1 x MS2 (65.9%) (Table 4.11). The highest similarity for WA populations was MW3 x MW5 (93.9%), with MW3 x MW6 (92.9%) and MW1 x MW6 (92.9%) at a comparable level. Populations MW2 x MW5 (78.8%) had the lowest similarity.

Within population genetic variation was comparable for the SA and WA populations at 0.33 and 0.30 respectively (Table 4.12). Between population variation was higher for SA (0.25) than WA populations (0.12). There was overall genetic variation of 0.21 between the SA and the WA populations.

A dendrogram was constructed using similarity values between populations from SA and WA (Figure 4.7). The three clustering methods were comparable (Table 4.13), and single-linked clustering is presented. The dendrogram showed a single cohesive cluster. In general the SA populations showed highest similarity to the WA populations of MW4

Table 4.9: Number of monomorphic and polymorphic bands detected with two primers in South Australian and Western Australian populations of *Banksia menziesii*.

Population Code	*Monomorphic bands*	*Polymorphic Bands*	*Percentage of polymorphic bands*
MS1	37	62	62.6
MS2	34	65	65.7
MS3	16	83	83.3
MS4	19	80	80.8
MS5	25	74	74.8
MW1	17	82	82.8
MW2	10	89	89.9
MW3	15	84	84.8
MW4	21	78	78.8
MW5	15	84	84.8
MW6	16	83	83.8

Table 4.10: Within population percentage similarity for *Banksia menziesii* populations from South Australia and Western Australia.

Population	*Range*	*Mean (stdev)*
MS1	63.6 - 68.7	67.5 (0.03)
MS2	60.6 - 76.8	67.5 (0.05)
MS3	54.5 - 76.8	65.7 (0.06)
MS4	56.6 - 76.8	67.5 (0.05)
MS5	61.6 - 80.8	68.5 (0.05)
Mean		**67.4**
MW1	55.6 - 98.0	70.8 (0.07)
MW2	55.6 - 86.9	69.3 (0.05)
MW3	55.6 - 90.9	70.2 (0.06)
MW4	58.6 - 90.9	71.3 (0.05)
MW5	56.6 - 85.9	69.8 (0.05)
MW6	53.5 - 81.8	68.6 (0.05)
Mean		**70.0**

Figure 4.7: Dendrogram of *Banksia menziesii* populations in South Australia and Western Australia using the single linked method.

Population Codes: South Australia MS1 Waller. MS2 Waller. MS3 Keith. MS4 Goodman. MS5 Barson. Western Australia MW1, Kings Parks and Botanic Garden. MW2, Cataby. MW3, Gingin. MW4, Lake Indoon. MW5, Yerina Springs. MW6, Ogilvie West.

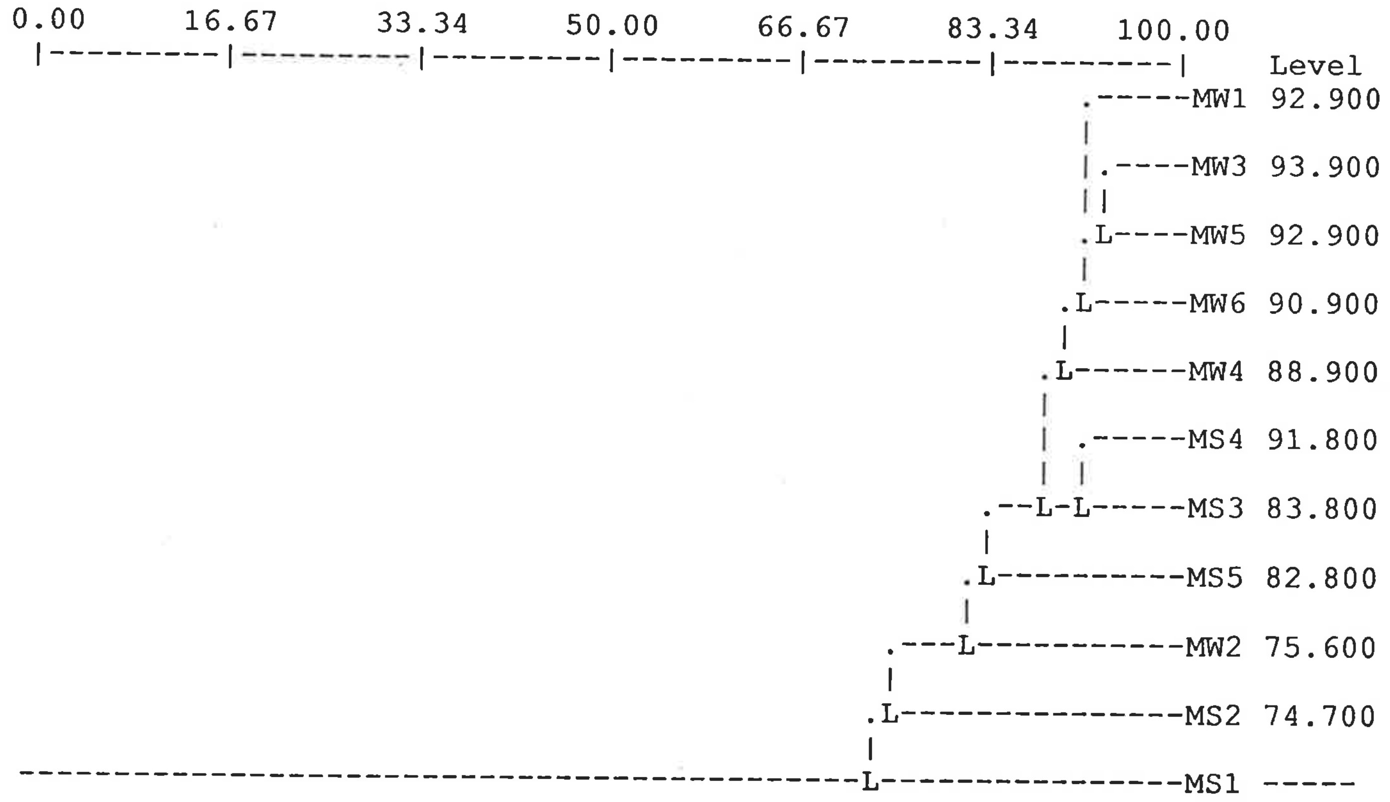

0.00
16.67
33.34
50.00
66.67
83.34
100.00
Level
MW1 92.900
MW3 93.900
MW5 92.900
MW6 90.900
MW4 88.900
MS4 91.800
MS3 83.800
MS5 82.800
MW2 75.600
MS2 74.700
MS1 -----

Table 4.11: Between population percentage similarity for *Banksia menziesii* populations from South Australia and Western Australia.

Population comparison	*Similarity*	*Population comparison*	*Similarity*
MS1 x MS2	65.9	MS4 x MW1	85.8
MS1 x MS4	73.9	MS4 x MW2	80.7
MS1 x MS5	68.7	MS4 x MW3	85.8
MS1 x MS3	69.7	MS4 x MW4	85.8
MS1 x MW1	71.7	MS4 x MW5	81.7
MS1 x MW2	68.7	MS4 x MW6	86.7
MS1 x MW3	71.7	MS5 x MW1	80.8
MS1 x MW4	71.7	MS5 x MW2	75.8
MS1 x MW5	67.7	MS5 x MW3	78.8
MS1 x MW6	74.7	MS5 x MW4	78.8
MS2 x MS3	73.5	MS5 x MW5	78.8
MS2 x MS4	73.4	MS5 x MW6	83.8
MS2 x MS5	70.5	MW1 x MW2	80.8
MS2 x MW1	75.5	MW1 x MW3	91.9
MS2 x MW2	70.9	MW1 x MW4	89.9
MS2 x MW3	73.5	MW1 x MW5	91.9
MS2 x MW4	69.5	MW1 x MW6	92.9
MS2 x MW5	69.5	MW2 x MW3	82.8
MS2 x MW6	74.5	MW2 x MW4	80.8
MS3 x MS4	91.8	MW2 x MW5	78.8
MS3 x MS5	80.8	MW2 x MW6	81.8
MS3 x MW1	87.8	MW3 x MW4	89.9
MS3 x MW2	78.8	MW3 x MW5	93.9
MS3 x MW3	87.9	MW3 x MW6	92.9
MS3 x MW4	87.9	MW4 x MW5	89.9
MS3 x MW5	85.9	MW4 x MW6	90.9
MS3 x MW6	88.9	MW5 x MW6	92.9
MS4 x MS5	76.7		

Table 4.12: Mean genetic variability within and between *Banksia menziesii* populations from South Australia and Western Australia.

Source of variation	*Percentage variation*
Within SA populations	0.33
Within WA populations	0.30
Between SA populations	0.25
Between WA populations	0.12
Between SA and WA populations	0.21

Table 4.13: Comparison of clustering methods using correlation coefficient (R) and the Mantel statistic (T) for *Banksia menziesii* RAPD data.

Dendrogram clustering method	*Correlation coefficient (R)*	*Mantel statistic (T)**
UPGMA	0.836	3.446
Single-linked	0.863	3.417
Complete-linked	0.801	3.416

* Values of the Mantel statistic above 3 are significant (Smouse *et al*, 1986).

(Lake Indoon), MW5 (Yerina Springs), MW3 (Gingin), MW1 (Kings Park) and MW6 (Ogilvie West), with less overall similarity to the MW2 (Cataby) population.

4.4 Discussion

There were low levels of genetic variation within SA populations of *Banksia coccinea* and variation between these populations was also low. This indicates that the SA populations were derived from similar seed sources, even though they were planted at different times. This is not entirely suprising given the limited number of seeds and plant suppliers. Within population variation for WA populations of *Banksia coccinea* is also low, but between population variation is four times that for the SA populations. This indicates that the full range of *Banksia coccinea* genetic variability is not represented in the SA cultivated populations. Sampling of a wider range of commercial populations would show whether this genetic variability can be accessed elsewhere in cultivation, or if further sampling from natural populations is required. In contrast, *Banksia menziesii* cultivated populations in SA have levels of variation comparable to the natural populations, and between population variation is higher in the cultivated than in the natural populations.

Genetic variation information from isozymes is available for natural populations of *Banksia cuneata* (Coates and Sokolwski, 1992) and *Banksia spinulosa* (Carthew, 1994), but previously only limited information has been available on the amount of genetic variation present in cultivated and natural populations of *Banksia coccinea* and *Banksia menziesii*. Commercial plantations of both species have been investigated for morphological variation by Fuss and Sedgley (1991c). Differences were identified between *Banksia coccinea* plantations, but some of this variation was attributed to climatic factors. The use of RAPDs eliminates the influence of environment, thus providing more reliable measures of genetic variation. Such estimates are invaluable so that effective breeding and selection can

be achieved and population relationships established (Chalmers *et al*, 1992; Soler *et al*, 1993; Wickneswari and Norwatti, 1993; Connolly *et al*, 1994; Stalker *et al*, 1994; Abo-elwafa *et al*, 1995; Karihaloo and Gottlieb, 1995). Previous selection of *Banksia coccinea* from SA cultivated populations has resulted in the registration of two distinct cultivars (Sedgley, 1995). The highest range of genetic variation occurs in population CS3 (Keith), which is the population from which the selections derived. Other highly variable *Banksia coccinea* populations are CS5 (Goodman) and CS6 (Barson). There has been less selection work conducted on *Banksia menziesii*, but a wide range of variation occurs in populations MS3 (Keith), MS4 (Goodman) and MS5 (Barson). A large range of genetic variation and high differentiation between individuals indicates a pool of available variability which may merit further selection and breeding for new cultivars, and using RAPDs to identify variation in cultivated populations may increase the probability of finding potentially unique selections. Further investigations of cultivated populations may lead to the identification of other genetically diverse locations.

The relationship between SA and WA populations indicates that the cultivated populations of *Banksia coccinea* are most likely derived from the inland populations with less representation from those at Two Peoples Bay, Gull Rock and Chenye Beach. Cluster analysis indicated that these natural populations show the least relationship to the SA cultivated populations tested and may provide unique selections not yet found in cultivation. In contrast, this study indicates that the genetic base for *Banksia menziesii* introduced into cultivation in SA is broad and reflects natural levels of variation. Nevertheless, the cultivated populations overall appear to be least closely related to MW2 (Cataby). Results indicate that the introduction of further plant material from WA populations is unnecessary.

When only the WA populations are examined, *Banksia coccinea* populations are less variable within than between, while *Banksia menziesii* populations are more variable within

than between. This will be considered in relation to population biology theory and conservation strategies for the two species. The aim of conservation is to preserve existing genetic variation and the potential of a species to evolve (Frankel 1982; Sampson, *et al* 1988; Hopper and Coates, 1990; Ellstrand and Elam, 1993). In the short term, the average fitness of a population is affected by the level of variation which alters how that population is able to cope with factors such as disease and herbivory (Chalmers *et al*, 1992) A population's capacity to evolve and adapt to environmental change in the longer term is also dependent on available variation (Frankel, 1982; Coates, 1988; Hopper and Coates, 1990; Sampson *et al*, 1994a). Many factors can affect the level of variation within and between populations, important among these are genetic drift and inbreeding, mating system, inbreeding depression, outbreeding depression and gene flow (Frankel, 1982; Hopper and Coates, 1990; Coates and Sokolowski, 1992; Coates, 1992).

Genetic drift is defined as a random change in allele frequencies between generations, due to only a sample of the alleles being transmitted from one generation to the next (Ellstrand and Elam, 1993). Inbreeding is mating between related individuals either by selfing or biparental inbreeding. Theoretically the effect of genetic drift and inbreeding is to alter genetic variation by decreasing variation within populations, while increasing the differentiation between (Hopper and Coates, 1990). Populations of *Agathis borneensis*, exhibit the variation patterns normally associated with genetic drift (Kitamura and Rahman, 1992), and this is cited as one of several factors contributing to genetic differentiation, along with a small fragmented population structure. Genetic drift is also proposed as the mechanism for genetic variation loss in *Nothofagus truncata* in New Zealand (Haase, 1992). Natural populations of *Banksia menziesii* exhibit higher variation within than between, which indicates that genetic drift and inbreeding are not affecting population structure. In contrast, the genetic variation levels calculated for *Banksia coccinea* follow the theoretical

expectations for genetic drift and inbreeding. Usually genetic drift, inbreeding and population size interact, such that the lower the size, the lower the corresponding variation. However, estimates of the sizes of ***Banksia coccinea*** populations, clearly indicate that size and levels of variation are not directly related in this species. When population size is not clearly involved in genetic drift historical factors affecting a species generally become more important.

An important historical factor affecting natural stands of ***Banksia*** is the presence of *Phytophthora cinnamomi* infection, which in recent years has devastated many species and populations (Cho, 1983; Grose, 1986; Robinson, unpub.). A direct consequence of this is reduction in population size, and the possibility of a bottleneck which results in the reduction of within population variation and differentiation between (Coates, 1992). Populations are generally able to cope with bottlenecks, although this depends on the severity of the decrease and the breeding system. Several examples are available which indicate that a reduction in population size does not necessarily result in reduced heterozygosity or genetic variation. These include populations of *Eucalyptus caesia* restricted to small granite outcrops, the rare grass wattle *Acacia anomala* and the geographically isolated *Stylidium coroniforme* (Moran and Hopper, 1983; Coates, 1988; Coates, 1992). A natural population of ***Banksia coccinea*** in WA, Redmond (CW1) has experienced a sharp decline in numbers due to *Phytophthora cinnamoni*, population size declining to twelve individuals. The level of polymorphic bands has dropped to 24.2%, while the mean within population similarity is 89.7%. This population has been unable to maintain a level of genetic variation comparable with others in WA. The decline in the number of individuals has resulted in a corresponding decline in genetic variation, polymorphism and presumably heterozygosity. When compared to all others, Redmond exhibits relatively low similarity indicating the majority of natural populations are genetically

distinct from it. The cultivated population at Mount Barker (CW8) shows the highest similarity with Redmond. Some species are able to exist in small populations, while maintaining genetic variation (Coates, 1992; Sampson *et al*, 1994a). The rare and geographically isolated species *Stylidium coroniforme* exhibits high levels of genetic variation despite evidence of repeated bottlenecks. Adaptive measures such as high post zygotic seed abortion, selection against inbreds and a population structure adapted to a bottleneck-flush cycle appear to be reasons for the maintenance of high genetic variation in these small populations (Coates, 1992). *Eucalyptus crucis,* located in isolated granite outcrops in south-WA, is also adapted to maintaining genetic variation (Sampson *et al*, 1988). Allozyme data confirmed that the mating system of this species is maintaining variation in small isolated populations by favouring heterozygotes.

The combination of genetic drift and inbreeding is able to influence fitness through inbreeding depression, or the reduction in fitness through an increase in homozygosity (Frankel, 1982; Coates, 1992; Ellstrand, 1992b). The mating system of a species may influence inbreeding depression (Sampson *et al*, 1994b) and the organisation of genetic variation. Theoretically an inbreeding mating system is less vulnerable to inbreeding depression due to a drop in the frequency of deleterious recessive alleles with increasing homozygosity, which are removed by selection (Ellstrand and Elam, 1993). It has been suggested that the relationship between inbreeding depression and mating system is not entirely straightforward. *Atherosperma moschatum* a widespread temperate rainforest species, occurring in Tasmania and south-eastern Australia, was recently investigated using isozymes. Most of the genetic variation was found to be within populations, with low differentiation between, despite the selfing breeding system (Sharpcott, 1994). In contrast, isozyme studies of *Eucalyptus caesia*, an outbreeding species, show low genetic variation within populations with marked differentiation between (Moran and Hopper, 1983). Many

difficulties arise when trying to predict the level of inbreeding depression, such as assessment of selfing rates, population size, and heterozygosity which are not necessarily associated with inbreeding depression in predictable ways (Ellstrand and Elam, 1993). The mating systems of *Banksia coccinea* and *Banksia menziesii* have been investigated by Fuss and Sedgley (1991a) and show partial self-incompatibility, like many other species in the genus (Carthew *et al*, 1988; Goldingay and Whelan, 1990; Ramsey and Vaughton, 1991). In view of this, the present patterns of variation which occur in natural populations of *Banksia coccinea* in WA, suggest that inbreeding depression may be playing a role in population structure.

Outbreeding depression is a reduction in fitness following hybridization within a species, that is, fitness is reduced by a breakdown in co-adaptation to local conditions. The effect of outbreeding depression will be severe for plant populations experiencing an increase in gene flow (Ellstrand, 1992b). Various parameters can increase gene flow including fragmentation of a continuous population which can result in an increase in immigrants, or an increase in immigrants arriving from distant sources. A disturbance which reduces population size, so that the fraction of seeds sired from immigrant pollen increases, with the expansion of a common subspecies, can result in an increase in genes exchanged. Despite sufficient pollination, outbreeding depression is manifested as a decline in seed set and progeny fitness. Due to this it is difficult to distinguish from inbreeding depression, and a knowledge of gene flow patterns are required to identify the cause of fitness reduction (Ellstrand, 1992b).

Members of the genus *Banksia* are a key nectar resource in many woodland communities for both bird and mammalian pollinators (Collins and Spice, 1986; Ramsey, 1988; Vaughton, 1990b; Wills, 1992; Carthew; 1993; Carthew, 1994). The accepted foraging behaviour of honeyeaters is to move between inflorescences within the same plant

and different plants promoting both geitonogamous and xenogamous pollination (Collins and Rebelo, 1987; Ramsey, 1988; Vaughton, 1990b). Ramsey, (1988) established that the degree of movement in *Banksia menziesii* communities between distant trees is related to the bird species. Smaller birds travelled to distant trees more often, perhaps as a result of interruptions by larger birds (Ramsey, 1988). In contrast, mammalian pollinators predominantly forage among inflorescences on the same plant or neighbouring plants, promoting geitonogamous pollination (Carpenter, 1978; Cunningham, 1991; Sampson *et al*, 1994a). Recently this accepted view of pollinator behaviour has been challenged (Carthew, 1994). The use of spool-and-line tracking in *Banksia spinulosa* woodland has established that marsupial foraging behaviour will transfer pollen as far as 59 metres, and that 70% of the pollinator movement is over five metres. Clearly further investigations are required to establish the contribution of marsupials to gene flow in *Banksia* communities. The loss of large numbers of plants due to disease or clearing may lead to pollinators becoming rare or locally extinct (Wills, 1992).

Gene flow, or the successful movement of genes via pollen or seeds within and between populations can affect genetic variation levels and population dynamics (Turner *et al*, 1982). Plant populations can be geographically isolated, but this does not necessarily lead to reproductive isolation (Ellstrand, 1992a; Ellstrand and Elam, 1993). Frequently small levels of gene flow among populations are high enough to counteract genetic drift. Based on theoretical expectations, gene flow rates are expected to increase as the recipient population size decreases (Ellstrand, 1992b; Ellstrand and Elam, 1993). The relative fraction of a fixed number of immigrants increases as the total population size decreases. Based on optimal foraging behaviour of pollinators, more time will be spent within large populations than small, effectively increasing interpopulation mating for small communities (Levin, 1981; Ellstrand and Elam, 1993). A reduction in one type of pollinator may result in

changes to the structure of gene flow within and between populations (Sampson *et al*, 1994a). Alterations in population structure such as fragmentation can also result in changes to pollinator behaviour (Ellstrand and Elam, 1993). The removal of inflorescences by bush picking decreases the available resource to pollinators and also reduces the potential seed production of a population, as in *Banksia hookeriana* (Witkowski *et al* 1994). A reduction in seed set due to decreased pollinator numbers or seed production will affect the overall number of alleles transmitted to the next generation.

Compounding these problems is the level of insect attack which has been observed in natural populations of *Banksia coccinea*. A number of the populations under investigation in this study, showed signs of extensive seed destruction by insects, and insect damage is a known cause for reduced seed set of *Banksia* (Scott, 1982; Vaughton, 1990a). Gene flow for natural populations of *Banksia coccinea* and *Banksia menziesii* were not investigated. However extensive clearing, reduction of plant and inflorescences numbers due to disease and bush-picking, and the resultant expectation of an alteration in pollinator behaviour are all likely to affect variation in *Banksia coccinea* populations.

Thus a number of factors are interacting in natural populations of *Banksia coccinea* to reduce variation within populations and increase differentiation between. Extensive clearing for agriculture in the 1960s and 70s, of approximately 50% of natural stands (Robinson, unpub.) and the severe damage caused by *Phytophthora cinnamomi* resulted in a large decline in plant numbers and in population fragmentation. Some may have successfully recovered from this, but other pressures were placed on these populations, such as the loss of inflorescence and seed material through bush picking, a probable reduction in pollinator numbers due to reduction of inflorescence numbers, fragmentation and additional disease and insect impact. Numerous positive steps to conserve the populations of *Banksia coccinea* still remaining have already taken place. Bush-picking of inflorescences from

plants on crown land has been banned (Barth, 1992; Baker, 1994; Morgan and Fuss, 1994), collection of seed material is now more restricted and *Phytophthora cinnamomi* disease control measures are in place. Unfortunately a new threat to *Banksia coccinea* populations has emerged in the last few years. Fungal diseases causing stem cankers are affecting populations which survived the *Phytophthora cinnamomi* outbreaks (Robinson, unpub.), and some with low levels of genetic variation may find it difficult to combat this new threat. All remaining populations of *Banksia coccinea* need to be protected and conserved to maintain the present level of genetic variation in WA. Further research investigating gene flow, pollinator types and behaviour and population dynamics is urgently needed to assess which processes are contributing to the reduction in genetic variation.

Natural populations of *Banksia menziesii* are large and widespread, and bush-picking has been more limited than in *Banksia coccinea*. These factors may have reduced the spread of *Phytophthora cinnamomi*, even though *Banksia menziesii* has been reported to be more susceptible that *Banksia coccinea* (Cho, 1983). Pollinator activity in *Banksia menziesii* communities in WA has been documented (Ramsey, 1988), indicating that honeyeaters such as the Western spinebill (*Acanthorhynchus supercillous*), Brown honeyeater (*Lichmera indistincta*), New Holland honeyeater (*Phylidonyris novaehollandiae*) and the Little wattlebird (*Anthochaera lunulata*) all commonly forage on *Banksia menziesii*. This range of pollinator types may be advantageous, reducing the possibility of local extinction of a single pollinator type. To conserve this species it will be necessary to maintain populations at their present level, by restricting clearing and further exploitation by bush pickers. The spread of *Phytophthora cinnamomi* or any other disease could easily affect the present population structure resulting in fragmentation and potential bottlenecks. Adequate monitoring of populations for disease, or reductions in seed set or genetic variation should ensure conservation. It is of interest to note that the highest within

population variability was detected at Lake Indoon, which is at the centre of the natural range of *Banksia menziesii*. In contrast, the lowest variability was found in the more northerly Ogilvie location.

RAPDs have detected genetic variation in *Banksia coccinea* and *Banksia menziesii* populations, providing information for selection and breeding, while assisting in the development of conservation strategies. This information was gained using two primers per species and between 5 and 26 individuals per population. Some published studies have used more primers while concentrating on fewer individuals (Lawson *et al*, 1994; Patwary *et al*, 1993; Tanhanpaa *et al*, 1993). In this study a large number of bands was generated using only two primers per species, particularly for *Banksia coccinea* where polyacrylamide gels were found to be more suitable than agarose for measuring maximum polymorphisms. The ideal number of individuals for measuring genetic variation has also received recent discussion (Grattapaglia *et al*, 1992; Waugh and Powell, 1992; Bassam and Benttey, 1994). For isozyme studies, a minimum number of twenty individuals per population has been recommended (Marshall and Brown, 1975), but recently it has been suggested that the number of individuals is less important than the number of populations (Pons and Petit, 1995). When examining optimal sampling strategies careful consideration must be given to the type of gel, the number of primers and bands, and also to the number of individuals and populations sampled. The use of RAPDs is now well-established in genetic variation studies, and is at least as effective as other molecular methods in detecting relationships between plant genotypes (N'Goran *et al*, 1994). An excellent example of the potential in active species conservation is the study by Rossetto *et al* (1995) on the rare and endangered species *Grevillea scapigera*. RAPD analysis was used to detect a small group of plants that captured maximum genetic variability for use in a recovery program for the species.

Thomson and Henry (1993) demonstrated that peach leaf tissue air-dried under laboratory conditions, was suitable for DNA extraction up to 122 days after collection. This study extends their work by demonstrating that leaf material for two *Banksia* species which had been collected under Australian summer field conditions can be used for DNA extractions up to 150 days after collection. Both young and mature leaves were removed from plants at the time of collection. With storage, mature leaves dried out and turned a pale green or brown colour, whereas the young leaves failed to dry as effectively as the mature leaves and developed mould. An alternative method of drying young or succulent leaves may be to separate each leaf between sheets of paper in a flower press.

4.5 Conclusions

The RAPD technique has been used to show that *Banksia coccinea* introductions into SA are closely related to one another, while *Banksia menziesii* introductions reflect the variation present in WA natural populations. This indicates that more introductions of *Banksia coccinea,* either from other cultivated populations or from the wild would increase the variation available for selection, but that for *Banksia menziesii* no further introductions are necessary. RAPDs have indicated that the genetic variation present in WA populations of *Banksia coccinea* is low, whereas there is less apparent threat to *Banksia menziesii* populations. Nevertheless conservation is needed for both species. The present level of genetic variation in *Banksia menziesii* populations needs to be maintained while *Banksia coccinea* requires close monitoring, active management and further research to preserve genetic variation levels. The relatively new RAPD technique has proved successful in providing information on genetic variation within and between cultivated and natural populations of *Banksia coccinea* and *Banksia menziesii*. This information can assist in targeted selection and in developing conservation strategies.

Chapter Five: Gene flow in small populations of *Banksia prionotes* and *Banksia praemorsa*

5.1 Introduction

Observations of the *Banksia* genus by many researchers has shown that a very small number of viable seeds is produced (Blake, 1971; Abbott, 1985a; Abbott, 1985b; Lamont *et al*, 1985). This trait is a serious drawback to a commercial floriculture species, where a large number of high quality plants is necessary for a successful breeding program. It may also be a factor in population or even species survival in the wild. Several *Banksia* species have been found to be self-incompatible to some degree (Paton and Turner, 1985; Collins and Spice, 1986; Whelan and Goldingay, 1986; Vaughton, 1988) and this mechanism combined with pollen tube competition, maternal and paternal selection and resource limitation may be at least partially responsible for the low seed-set observed.

In this and the following chapter, two different but interrelated types of competition are investigated. In this chapter the competition which occurs between different pollen donor plants in small populations is considered. Previous research on other plant species has indicated that some pollen donors appear to be favoured over others (Marshall and Ellstrand, 1986; Stanton *et al*, 1986; Thomson, 1989; Snow and Spira, 1991b). This indicates that there is competition between pollen donors, which in turn may affect seed quality. The possibility that some males in a breeding population have a competitive advantage may facilitate the production of high quality cultivars in plant breeding, or may result in a shift in population characteristics in the wild. This will be examined in relation to the proportion of seeds produced by each pollen donor following natural pollen flow in small populations.

5.2 Material and methods

5.2.1 Plant material

The populations of eleven *Banksia prionotes* and eight *Banksia praemorsa* plants were located in a *Banksia* collection at the Happy Valley Reservoir Reserve of SA Water, situated to the south of Adelaide (Map Reference: Milang 6627 792 165). A representative inflorescence, infructescence, seed and leaf of each species is illustrated (Figure 5.1). The collection was isolated from other *Banksia* plantings, with the closest populations of *Banksia prionotes* and *Banksia praemorsa* located at a distance of 25 km. The population included plants of a number of *Banksia* species. The location of each *Banksia prionotes* and *Banksia praemorsa* plant was noted and interplant distances measured (Figures 5.2 and 5.3). When the majority of plants in the populations were in bloom between five and fifteen inflorescences were labelled on two plants of each of *Banksia prionotes* (plants 223 and 244; 6 inflorecences) and *Banksia praemorsa* (plants 213 and 214; 10 inflorecences). Potential *Banksia prionotes* pollen donors for plant 223 and 244 were plant numbers 11, 12, 27, 86, 218, 223, 224, 225, 232, 237, 244. Potential *Banksia praemorsa* pollen donors for plants 213 and 214 were plant numbers 1, 64, 70, 104, 213, 214, 235 and 376. Once the open pollinated infructescences had developed, the number of follicles was recorded and the seeds collected (Table 5.1). DNA was extracted from the seeds and the RAPD technique was used to generate a banding pattern for each individual plant in the two populations and for each offspring of the four mother plants.

Figure 5.1: *Banksia prionotes* and *Banksia praemorsa* inflorescence, infructescence, seed and leaf.

5.1A A *Banksia prionotes* inflorescence is approximately 7-15 cm in length, covered with white unopened florets, opened florets are orange in colour resulting in a two toned inflorescence. The infructescences of *Banksia prionotes* does not retain any florets and the follicles are clearly visible. This species has a wedged shaped seed with a curved wing. The leaves of *Banksia prionotes* are long typically 15-27 cm and are toothed at the margins (George, 1987).

5.1B A *Banksia praemorsa* inflorescence is approximately 10-27 cm in length, covered in pale greenish yellow florets which turn maroon as the inflorescences matures. The infructescence of *Banksia praemorsa* retains the florets after the follicles are produced. Two seeds are usually produced in each follicle, with the seed having a notched wing. The individual leaves of

Banksia praemorsa are relatively small land are flat with a wedged shape (George, 1987).

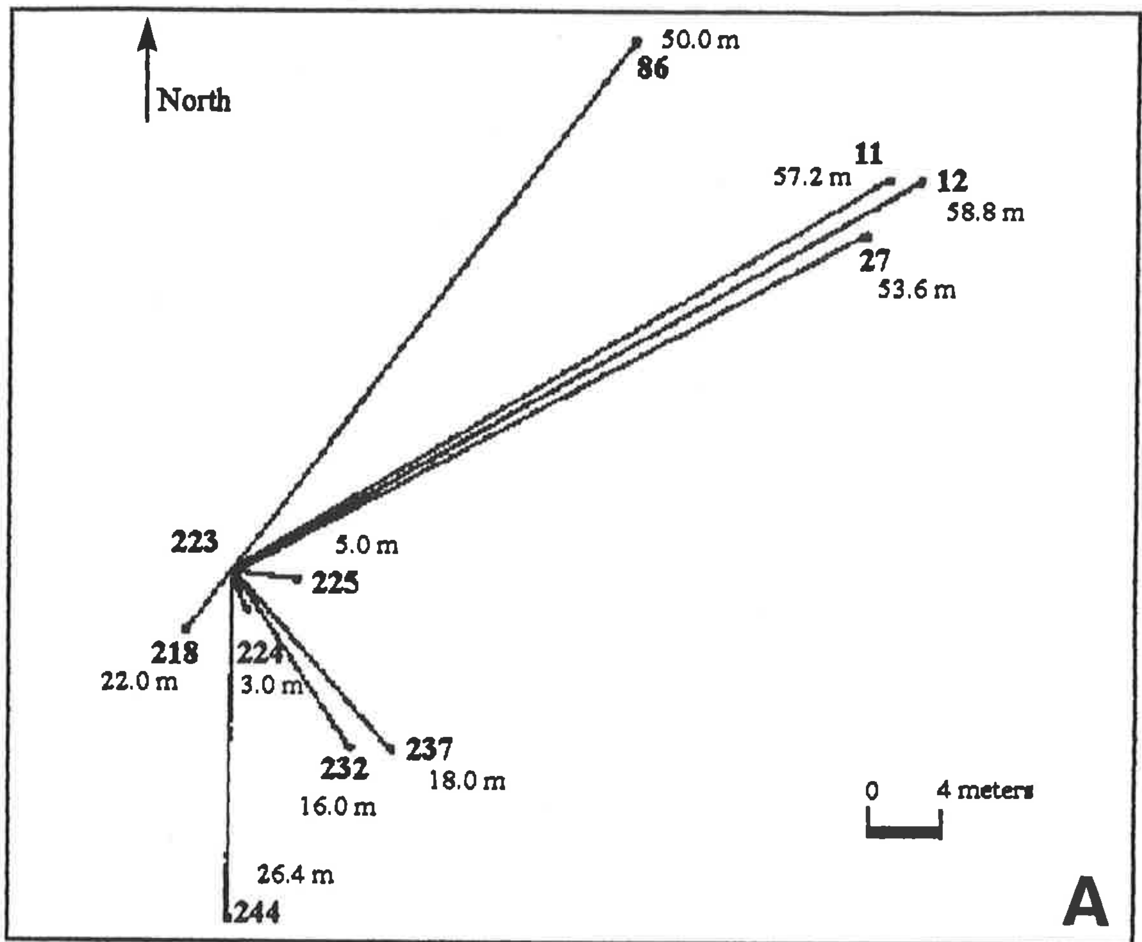
A

B

Figure 5.2: Location of all plants of *Banksia prionotes* used at the Happy Valley population, clearly identified by numbers. Plant Codes: Mothers: 223, 244. Potential pollen donors: 11, 12, 27, 86, 218, 223, 224, 225, 232, 237, 244.

5.2A Mother 223, direction and distance (~~meters~~ metres) from mother 223 to all potential pollen donors. Scale 1cm = 4meters.

5.2B Mother 244, direction and distance (~~meters~~ metres) from mother 244 to all potential pollen donors. Scale 1cm = 4 meters.


North
50.0 m
86
11
57.2 m
12
58.8 m
27
53.6 m
223
5.0 m
225
218
22.0 m
224
3.0 m
237
18.0 m
232
16.0 m
0
4 meters
26.4 m
244
A

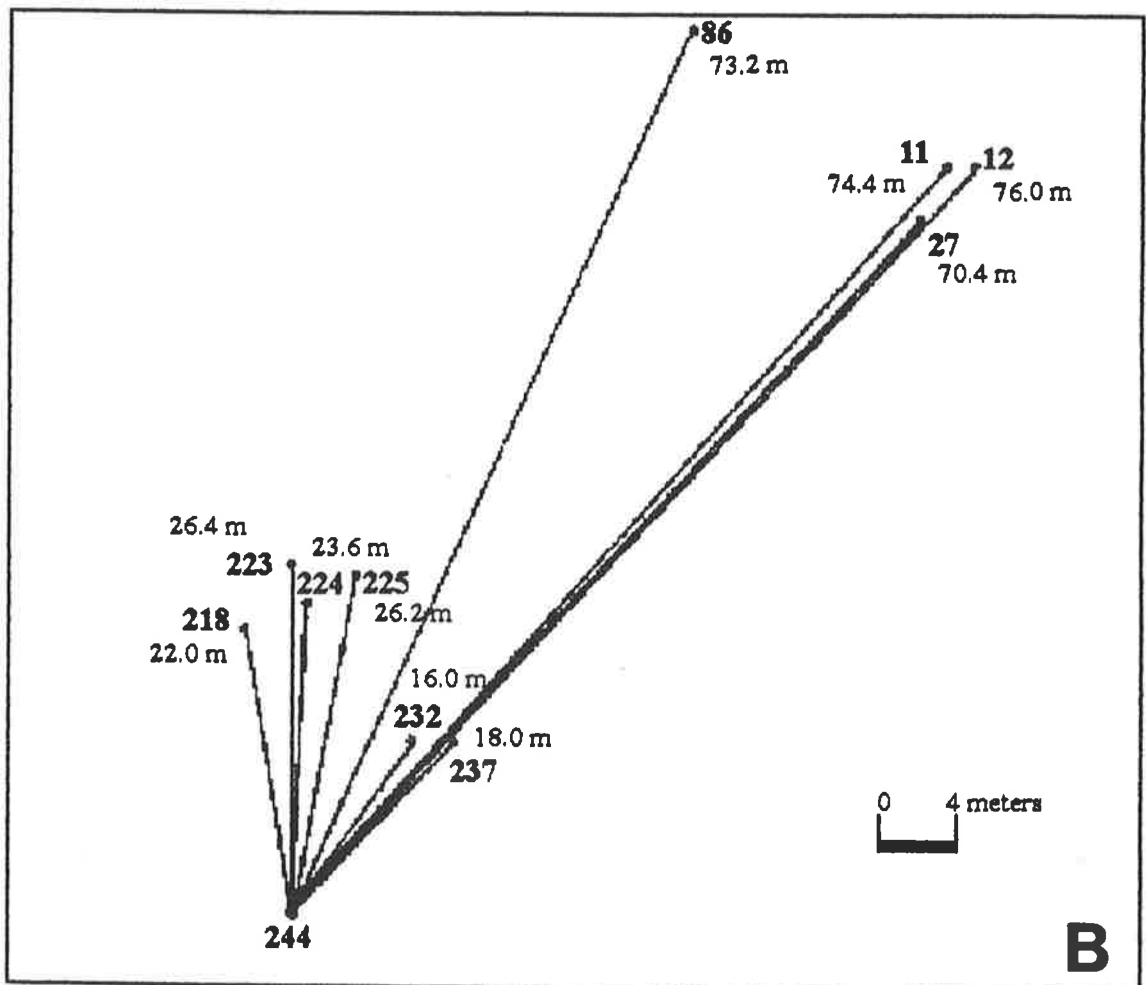
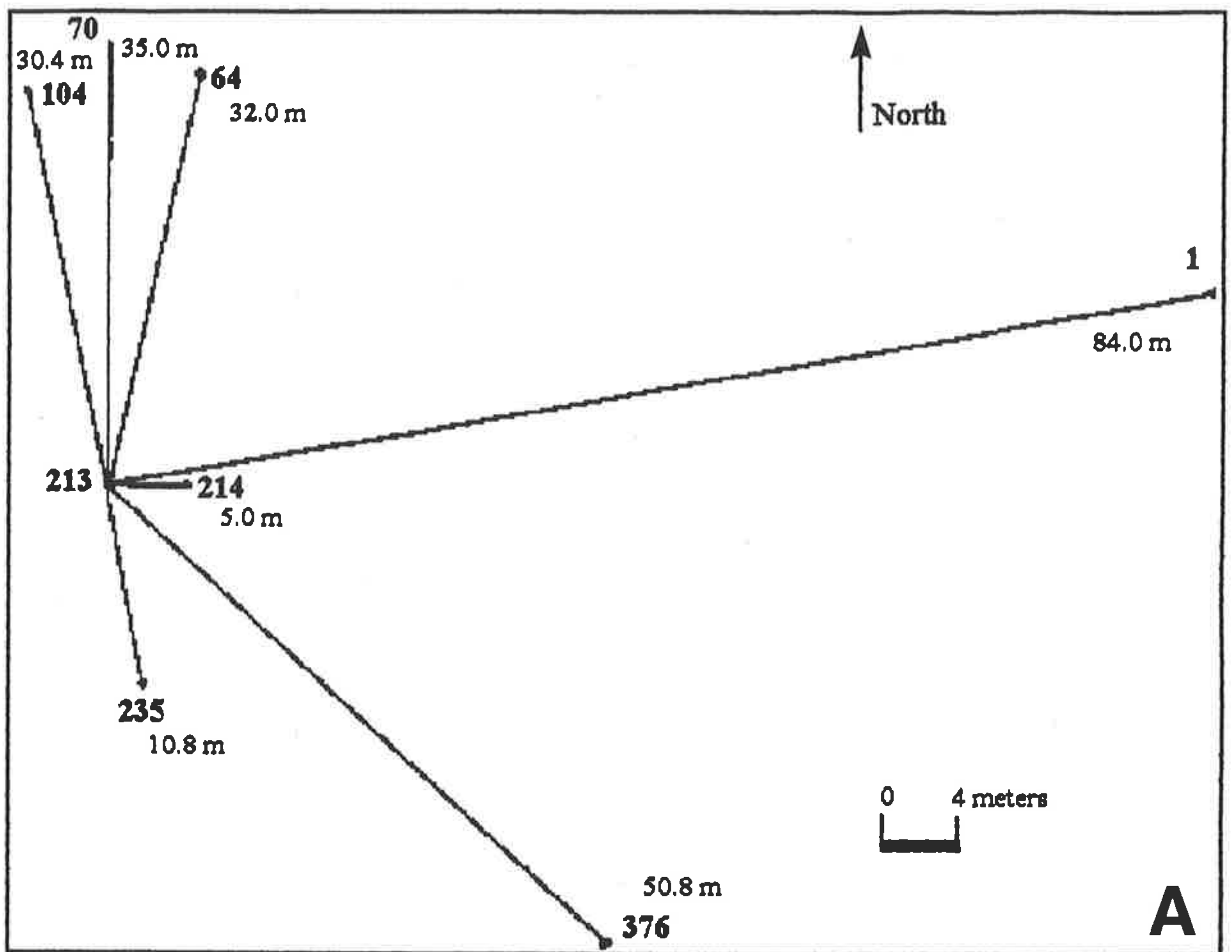

86
73.2 m
11
74.4 m
12
76.0 m
27
70.4 m
26.4 m
223
23.6 m
224
225
26.2 m
218
22.0 m
16.0 m
232
18.0 m
237
0
4 meters
244
B

Figure 5.3: Location of all plants of *Banksia praemorsa* used at the Happy Valley population, clearly identified by numbers. Plant Codes: Mothers: 213 and 214. Potential pollen donors: 1, 64, 70, 104, 213, 214, 235, 376.

5.3A Mother 213, direction and distance (~~meters~~ metres) from mother 213 to all potential pollen donors. Scale 1cm = 4 meters.

5.3B Mother 214, direction and distance (~~meters~~ metres) from mother 214 to all potential pollen donors. Scale 1cm = 4 meters.


70
35.0 m
30.4 m
104
64
32.0 m
North
1
84.0 m
213
214
5.0 m
235
10.8 m
0
4 meters
50.8 m
376
A

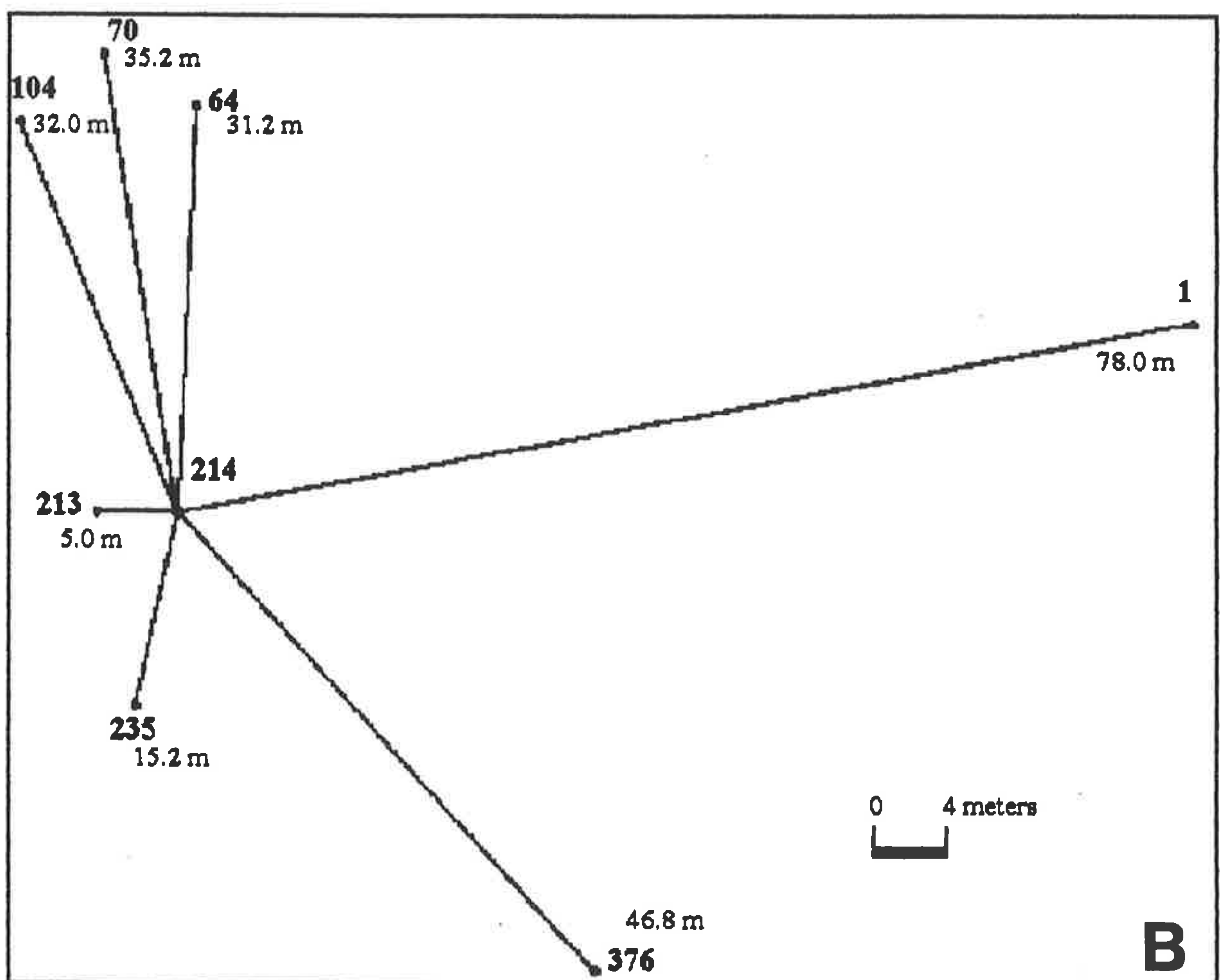

70
35.2 m
104
32.0 m
64
31.2 m
1
78.0 m
214
213
5.0 m
235
15.2 m
0
4 meters
46.8 m
376
B

Table 5.1: Open pollinated seed numbers per infructescence analysed for *Banksia prionotes*, plants 223 and 244 and *Banksia praemorsa* plants 213 and 214.

Infructescence number	*Mother plant*			
	Banksia prionotes		*Banksia praemorsa*	
	223	**244**	**213**	**214**
1	2	6	10	11
2	7	6	20	6
3	5	5	8	0
4	2	1	12	11
5	11	8	11	20
6	5	2	0	20
7			11	10
8			11	10
9			11	0
10			10	10
Total	32	28	104	98

5.2.2 Flower visitors

A survey of potential pollinators of the *Banksia prionotes* and *Banksia praemorsa* plants was conducted. Flower visitors were observed for a period of two hours per session and the type of visitor, the time spent foraging and the visitor's movement between plants was recorded. This was repeated four times during the peak flowering period of each species, such that both diurnal and nocturnal visitors were observed.

5.2.3 Isolation of DNA from seed material

A single seed was placed in a 1.5 ml tube, liquid nitrogen was added and the seed crushed to a powder. 600 µl DNA extraction buffer (4% sarkosyl, 100 mM Tris-HCL, 100 mM NaCl, 10 mM EDTA, (pH 8.5)) was added and mixed well. A further 600 µl of phenol/chloroform (25% phenol, 24% chloroform, 1% iso-amyl-alcohol) was added and mixed thoroughly for five minutes. The samples were placed on ice until all samples were extracted. All samples were centrifuged at high speed for 2-3 minutes and the upper aqueous layer was transferred to a new tube. The extraction was repeated once with phenol/chloroform. 75 µl of 3M sodium acetate and 600 µl isopropanol were added. The samples were mixed by inversion and the DNA allowed to precipitate at room temperature for 1 minute. Samples were centrifuged for 10 minutes and the supernatant carefully decanted. 1 ml of 70% ethanol was added and the sample gently vortexed. The sample was centrifuged for 2-3 minutes and the supernatant was removed. The wash with 70% ethanol was repeated, and the final pellet was air dried ensuring that all ethanol was removed. The pellet was resuspended in TE buffer (10 mM Tris-HCL (pH 7.4), 1 mM EDTA) overnight at 4°C.

5.2.4 Random amplified polymorphic DNA analysis

A primer survey using Operon Technologies primer kits was conducted to generate unique RAPD banding patterns for each maternal plant and for each potential pollen donor. Eighty primers were surveyed for each species, of which 30% produced polymorphic bands for *Banksia prionotes*, and 15% for *Banksia praemorsa*. Two primers were chosen per species, which gave maximum numbers of bands specific to the potential fathers (Table 5.2). Primers OPC 16 (CACACTCCAG) and OPD 03 (GTCGCCGTCA) were chosen for *Banksia prionotes* and OPA 11 (CAATCGCCGT) and OPA 19 (CAAACGTCGG) were chosen for *Banksia praemorsa*. RAPD banding patterns of the mother and potential fathers were compared with those for each seed.

5.2.5 Agarose gels

Examination of the banding patterns of the RAPD reactions was carried out using agarose gels (Figure 5.4). Ficoll dye (0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll (Type 400; Pharmacia) in water) was added (3 ml) to the 30 µl RAPD reaction. 20 µl of this mixture was analysed on a 2.5% SeaKeam agarose gel using Tris-borate-EDTA buffer, run at 150 mAmps for a period ranging from 60 to 90 minutes. The DNA was visualised under ultra violet light after staining for 15 minutes with ethidium bromide (200 ml per 1L of 1 mg/ml stock solution) and photographed (Polaroid, Direct screen instant camera DS34, England; Polaroid film 667, England).

Table 5.2: Summary of bands for *Banksia prionotes* and *Banksia praemorsa* mothers, fathers and offspring.

Mother and father combinations				
	Number of bands (Father code)			
	Banksia prionotes		*Banksia praemorsa*	
	223	244	213	214
Number of unique mother bands	9 (11)	8 (11)	9 (1)	5 (1)
Number of unique father bands	10 (11)	3 (11)	9 (1)	8 (1)
Number of unique mother bands	9 (12)	8 (12)	8 (64)	3 (64)
Number of unique father bands	11 (12)	4 (12)	9 (64)	6 (64)
Number of unique mother bands	6 (27)	7 (27)	4 (70)	0 (70)
Number of unique father bands	12 (27)	8 (27)	11 (70)	8 (70)
Number of unique mother bands	6 (86)	8 (86)	8 (104)	4 (104)
Number of unique father bands	10 (86)	5 (86)	8 (104)	5 (104)
Number of unique mother bands	5 (218)	8 (218)	7 (214)	7 (213)
Number of unique father bands	12 (218)	8 (218)	8 (214)	10 (213)
Number of unique mother bands	6 (224)	7 (223)	9 (235)	4 (235)
Number of unique father bands	11 (224)	6 (223)	8 (235)	6 (235)
Number of unique mother bands	6 (225)	7 (224)	8 (376)	4 (376)
Number of unique father bands	9 (225)	4 (224)	11 (376)	5 (376)
Number of unique mother bands	6 (232)	7 (225)		
Number of unique father bands	8 (232)	4 (225)		
Number of unique mother bands	11 (237)	13 (232)		
Number of unique father bands	9 (237)	4 (232)		
Number of unique mother bands	4 (244)	0 (237)		
Number of unique father bands	9 (244)	0 (237)		

Mother, fathers and offspring				
	Number of bands			
Number of bands unique to mother	0	0	1	2
Number of bands unique to mother and at least one offspring	0	0	1	0
Number of bands unique to fathers	0	0	0	0
Number of bands unique to fathers and at least one offspring	24	14	21	21
Number of bands unique to mother and at least one father	0	0	0	0
Number of bands unique to mother and all fathers	2	2	6	5
Number of bands common to all individuals	15	19	11	13
Number of bands unique to offspring	0	0	5	8
Total number of bands	41	35	45	49

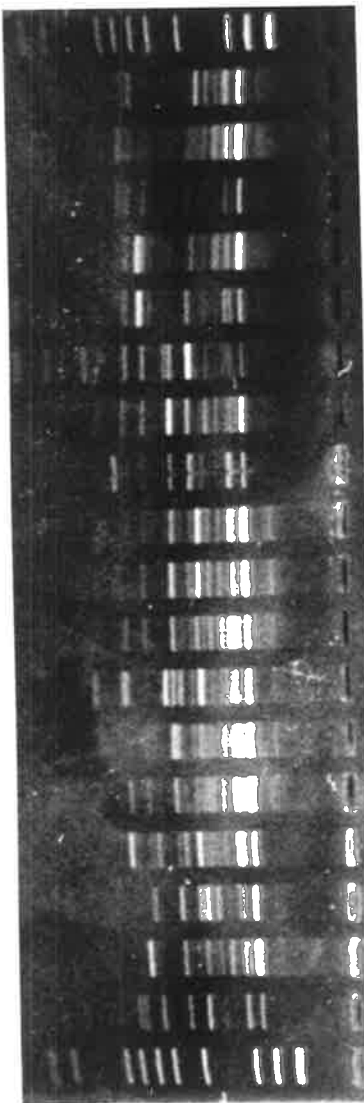
Figure 5.4: Agarose gel illustrating the RAPD banding pattern for all potential pollen donors of *Banksia prionotes* and a selection of offspring of mother 244.

5.4A The gel (2.5% TBE) shows twelve potential fathers of *Banksia prionotes* offspring using primer OPD 03. One of the potential fathers failed to flower in 1991 and was subsequently removed from the analysis.

5.4B The RAPD banding patterns of a selection of offspring produced by mother 244 are illustrated, using primer OPD 03.

Size (bp)

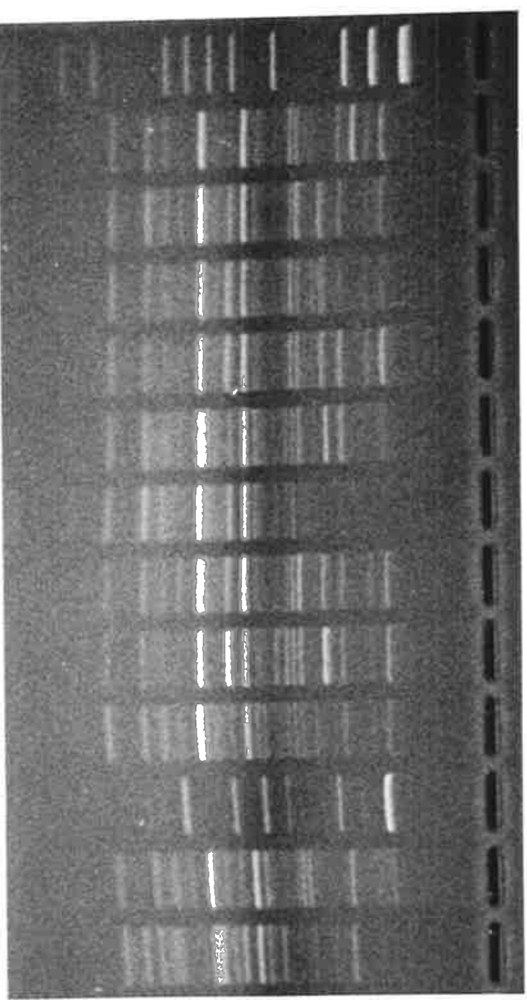
2645
1198
676
517
350



Marker, pGEM
214 offspring 73
214 offspring 74
214 offspring 75
214 offspring 76
214 offspring 77
214 offspring 78
214 offspring 79
214 offspring 80
214 offspring 81
214 offspring 82
214 offspring 83
214 offspring 84
214 offspring 85
214 offspring 86
214 offspring 87
214 offspring 88
214 offspring 89
214 offspring 90
Marker, pGEM

Size (bp)

2645
1605
1198
676
517
350
222
179



Marker, pGEM
Plant 11
Plant 12
Plant 27
Plant 86
Plant 218
Plant 223
Plant 224
Plant 225
Plant 232
Plant 237
Plant 244
Plant 407

5.2.6 Statistical analysis

RAPD bands for all agarose gels were scored as described in Chapter 4, using CREAM software. Similarity matrices were generated for each of the four mothers, their offspring and all possible pollen donors, and the means compared using an independent t-test. The difference between the mean is hypothesised to be zero, and deviation from this assumption generates a significant difference. In addition paternal bands were identified in the offspring patterns and the number and proportion of each paternal band calculated.

5.3 Results

For *Banksia prionotes*, RAPD banding patterns were generated for 32 and 28 seeds of the two mother plants 223 and 244 respectively. For *Banksia praemorsa* banding patterns were generated for 104 and 98 seeds of the two mother plants 213 and 214 respectively. An example of the RAPD banding patterns for the potential fathers and a selection of offspring of each species is presented (Figures 5.4 and 5.5).

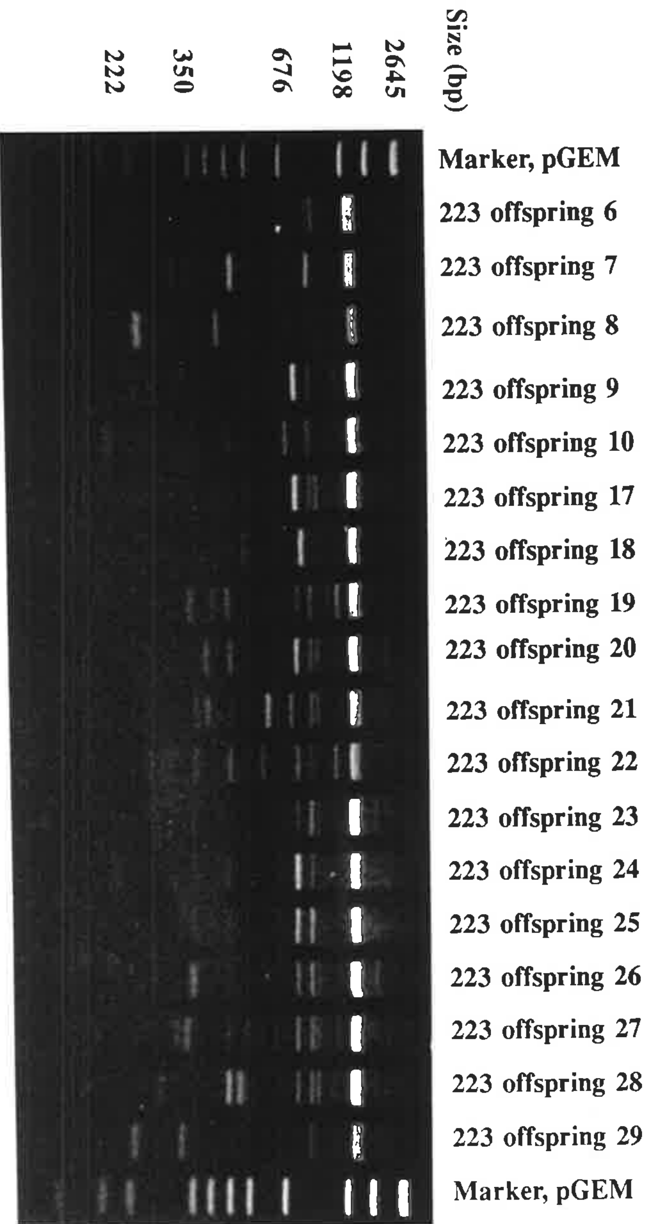
Three criteria were used to assign paternity. These were: (1) percentage similarity; (2) the proportion of bands specific to each potential father; (3) paternal specific bands (Table 5.3).

(1) Percentage similarity. The highest percentage similarity between each offspring and potential father was used to assign paternity. The mean highest value was compared with the mean similarity to the mother using and independent t-test (Table 5.4). Overall the mean similarity between parents ranged from 0.69 to 0.78. Offspring mean similarity values were lower ranging from 0.66-0.68 for *Banksia prionotes* and 0.72-0.74 for *Banksia prionotes*. The overall level of similarity between all parents and offspring ranged from 0.56-0.62. Comparison of mean similarity between father and offspring and mother and offspring using the independent t-test demonstrated a significant difference in all cases.

Figure 5.5 Agarose gel illustrating the RAPD banding patterns for all potential pollen donors for *Banksia praemorsa* and a selection of offspring of mother 213.

5.5A This gel (2.5% TBE) shows the banding patterns of sixteen potential fathers of *Banksia praemorsa* offspring using the primer OPA 11. Eight of these sixteen potential fathers failed to flower in 1991 and were subsequently disregarded during analysis.

5.5B The RAPD patterns of a selection of the offspring produced by mother 213, generated with primer OPA 11.


222
350
676
1198
2645
Size (bp)
Marker, pGEM
223 offspring 6
223 offspring 7
223 offspring 8
223 offspring 9
223 offspring 10
223 offspring 17
223 offspring 18
223 offspring 19
223 offspring 20
223 offspring 21
223 offspring 22
223 offspring 23
223 offspring 24
223 offspring 25
223 offspring 26
223 offspring 27
223 offspring 28
223 offspring 29
Marker, pGEM

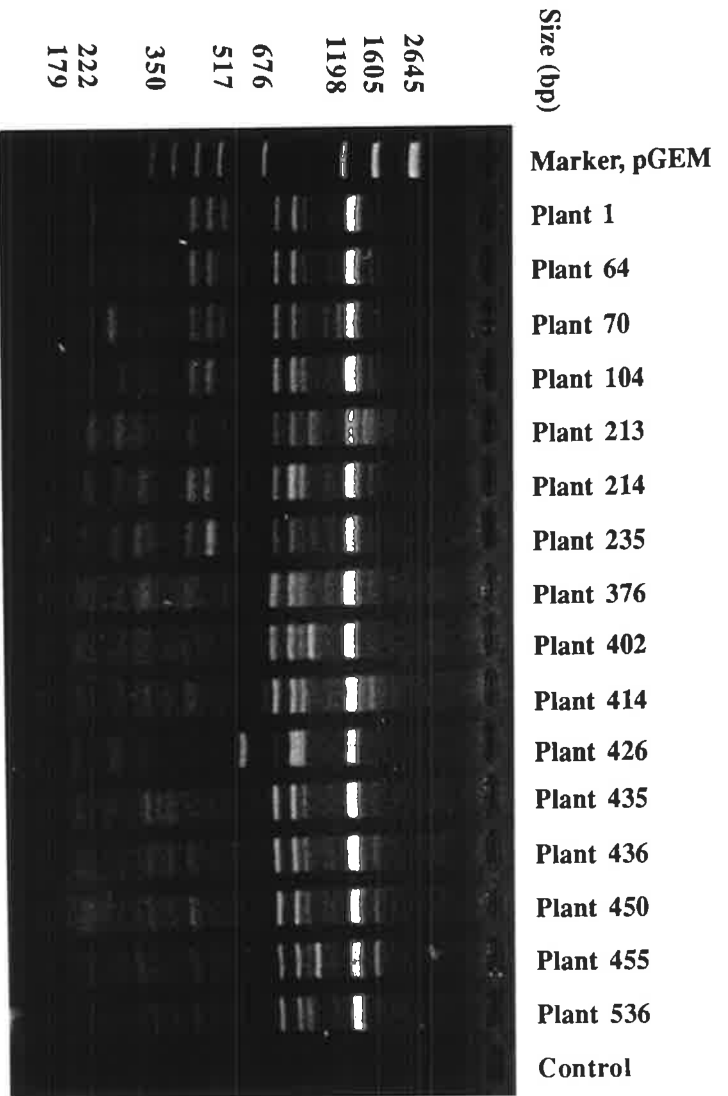

179
222
350
517
676
1198
1605
2645
Size (bp)
Marker, pGEM
Plant 1
Plant 64
Plant 70
Plant 104
Plant 213
Plant 214
Plant 235
Plant 376
Plant 402
Plant 414
Plant 426
Plant 435
Plant 436
Plant 450
Plant 455
Plant 536
Control

Table 5.3: Number of paternal specific bands for *Banksia prionotes* and *Banksia praemorsa.*

Banksia prionotes			*Banksia praemorsa*		
Fathers	Mothers		Fathers	Mothers	
	223	**244**		**213**	**214**
11	0	0	1	2	2
12	0	1	64	0	1
27	1	1	70	1	1
86	0	0	104	0	0
218	0	0	213	-	1
223	-	0	214	0	-
224	0	0	235	0	0
225	0	0	376	1	1
232	0	1			
237	2	0			
244	0	-			

Table 5.4: Percentage similarity of bands between parents and offspring of *Banksia prionotes* and *Banksia praemorsa.*

	Banksia prionotes		*Banksia praemorsa*	
	223	**244**	**213**	**214**
Mean similarity between all parents (range)	0.69 (0.49-0.85)	0.71 (0.46-0.86)	0.77 (0.58-0.87)	0.78 (0.59-0.85)
Mean similarity between all offspring (range)	0.66 (0.39-0.93)	0.68 (0.46-0.97)	0.72 (0.44-0.91)	0.74 (0.55-0.98)
Mean similarity between all parents and all offspring (range)	0.56 (0.39-0.76)	0.57 (0.37-0.80)	0.62 (0.40-0.80)	0.62 (0.43-0.82)
Mean similarity between offspring and fathers (SE)	0.66 (0.001)	0.68 (0.12)	0.69 (0.005)	0.69 (0.005)
Mean similarity between offspring and mother (SE)	0.60 (0.012)	0.55 (0.012)	0.58 (0.005)	0.67 (0.005)
t value (df) father vs mother	4.14 (62)	7.43 (54)	15.7 (206)	2.98 (194)
Probability	P>0.05	P>0.05	P>0.05	P>0.05

This indicates that the mean similarity between father and offspring is higher than between mother and offspring and that all mothers were predominantly outcrossed. The highest similarity value identified a single father for 31% of the 223 offspring (Table 5.5), 39% of the 244 offspring (Table 5.6), 58% of the 213 offspring (Table 5.7) and 44% of the 214 offspring (Table 5.8). For the remainder the highest similarity value was shared by more than one potential father.

(2) Proportion of bands specific to each potential father. Band differences were generated between each parental combination except *Banksia prionotes* plants 244 and 237 (Table 5.2). There were up to 12 (*Banksia prionotes*) and 11 (*Banksia praemorsa*) paternal specific bands per parental combination, most of which were shared with other parental combinations. For each offspring the proportion of bands specific to each potential father was calculated, and the highest proportion used to assign paternity. This identified a single father for 34% of the 223 offspring (Table 5.5), 29% of the 244 offspring (Table 5.6), 81% of the 213 offspring (Table 5.7) and 51% of the 214 offspring (Table 5.8). For the remainder the highest proportion of parental bands was shared by more than one potential father, which generally included one of the potential fathers identified by the highest similarity value.

(3) Paternal specific bands. Although most paternal bands were shared between potential fathers, a few were specific to only one (Table 5.3). These were used to assign paternity in 19% of the 223 offspring (Table 5.5), 25% of the 244 offspring (Table 5.6), 12% of the 213 offspring (Table 5.7) and 28% of the 214 offspring (Table 5.8).

A combination of these three criteria identified the father of all of the 223 offspring 93% of the 244 offspring, 96% of the 213 offspring and 95% of the 214 offspring (Table 5.9).

Table 5.5: Possible fathers of offspring of *Banksia prionotes* plant 223 based on similarity percentage, proportion of paternal bands and paternal specific bands.

Offspring	*Highest similarity*	*Highest proportion of paternal bands*	*Paternal specific bands*	*Father*
1	218	11/86/218	-	218
2	86/223	86	-	86
3	11/27	11/27	27	27
4	11	11/86	-	11
5	12/86/223	86	-	86
6	12/232	232	-	232
7	218	86/218/232	-	218
8	218	86/218/232	-	218
9	223/225/232	225/237	-	225
10	12/232	232/237	-	232
11	12/237	224/237	237	237
12	12/86/225	225/237	-	225
13	12/237	237	237	237
14	12	12	-	12
15	223/232	232/237	-	232
16	225/237	232/237	237	237
17	27/86/223	27/86	27	27
18	11	11/27/232	-	11
19	86	27/86	-	86
20	86/223	86	-	86
21	86/223	86	-	86
22	225/244	225/237	-	225
23	218	27/218	-	218
24	86/223	86	-	86
25	27/86/223	27	27	27
26	86	86	-	86
27	86/223	11/86	-	86
28	11	11/86/237	-	11
29	12/86	86/225/237	-	86
30	223/224	224/237	-	224
31	224/225	225	-	225
32	223/224	11/224	-	224

Table 5.6: Possible fathers of offspring of *Banksia prionotes* plant 244 based on similarity percentage, proportion of paternal bands and paternal specific bands.

Offspring	*Highest similarity*	*Highset proportion of paternal bands*	*Paternal specific bands*	*Father*
1	224	224	-	224
2	11/232	11/224	-	11
3	225	86/225	-	225
4	12	12/224	12	12
5	218	11/218	-	218
6	218/224	224	-	224
7	218	218/224	-	218
8	218/223	27/223/224	-	223
9	27/223/225	12/27/224	27	27
10	225	225/232	-	225
11	11/218	218/232	-	218
12	223	223/224/232	-	223
13	218	218/232	-	218
14	12/218	12	12	12
15	225	11/225	-	225
16	27/244	27/232	27	27
17	225/232	12/232	-	232
18	225/232	232	-	232
19	225	12/224/225	-	225
20	218/225	12/218	-	218
21	224/244	12/244	-	244
22	218	218/232	-	218
23	224	12/224	-	224
24	12	12	12	12
25	223/224	223/224	-	223/224
26	12/218	12	12	12
27	12/224	12	-	12
28	12/224	12	12	12

Table 5.7: Possible fathers of offspring of *Banksia praemorsa* plant 213 based on similarity percentage, proportion of paternal bands and paternal specific bands.

Offspring	*Highest similarity*	*Highest proportion of paternal bands*	*Paternal specific band*	*Father*	*Offspring*	*Highest similarity*	*Highest proportion of paternal bands*	*Paternal specific band*	*Father*
1	104/376	376	-	376	36	64/104/214	214	-	214
2	104/235	104	-	104	37	64/235	64	-	64
3	1	1	1	1	38	64	64	-	64
4	104	104	-	104	39	64/104	64	-	64
5	104	104	-	104	40	1	1	1	1
6	104	104	-	104	41	1/104	1	1	1
7	104/235	104	-	104	42	214/376	214	-	214
8	104	104	-	104	43	1	1	1	1
9	104/376	104	-	104	44	214/235	235	-	235
10	235	235	-	235	45	104/214	104	-	104
11	64/104/214	64	-	64	46	104/214	104/214	-	104/214
12	214	214	-	214	47	235	235	-	235
13	104	104	-	104	48	104	104	-	104
14	104/213	104	-	104	49	104	104	-	104
15	1/214	214	-	214	50	104/235	104	-	104
16	213/214	214	-	214	51	235	235	-	235
17	64/104	104	-	104	52	214	214	-	214
18	104	104	-	104	53	104	104	-	104
19	104	70/104/376	-	104	54	64	64/214	-	64
20	104	104	-	104	55	235	235	-	235
21	104	1/104/376	-	104	56	213/235	235	-	235
22	104	104	-	104	57	214/235	214	-	214
23	214	214	-	214	58	104/214	104	-	104
24	214/376	214	-	214	59	104	104	-	104
25	214	214	-	214	60	64/104	104	-	104
26	104	104	-	104	61	235	70/235	-	235
27	104	104	-	104	62	214/235	214	-	214
28	104	104	-	104	63	104/235	104	-	104
29	1/104/214	214	-	214	64	214	214	-	214
30	64/214	64	-	64	65	214	214	-	214
31	1	1	1	1	66	214	214	-	214
32	1/214/376	1	1	1	67	214	214	-	214
33	1/104	104	-	104	68	235	235	-	235
34	104	104	-	104	69	104	104	-	104
35	214	214	-	214	70	214	214	-	214

71	214	214	-	214	88	214	214	-	214
72	104/214	104/214	-	104/214	89	104	104	-	104
73	64/104/214	104/214	-	104/214	90	64	64/214	-	64
74	235	70/235	-	235	91	104	104/376	-	104
75	214	214	-	214	92	1	1/235	-	1
76	64/104	104	-	104	93	64/104	64	-	64
77	235	235	-	235	94	235	235	-	235
78	104/214/376	104	-	104	95	1/214	1	1	1
79	104/376	104	-	104	96	104/214	104/214	-	104/214
80	1/213	1	1	1	97	235	70/235	-	235
81	1/104	1	1	1	98	64/104/214/376	104	-	104
82	235	235	-	235	99	214	104/214	-	214
83	104/213	104	-	104	100	1/104/214	1/104	1	1
84	104	104	-	104	101	214	214	-	214
85	235/376	104/376	376	376	102	235	1/235	-	235
86	1/235	70/235	-	235	103	214	70/214	-	214
87	214	214	-	214	104	1	1/104	1	1

Table 5.8: Possible fathers of offspring of *Banksia praemorsa* plant 214 based on similarity percentage, proportion of paternal bands and paternal specific bands.

Offspring	*Highest similarity*	*Highest proportion of paternal bands*	*Paternal specific bands*	*Father*	*Offspring*	*Highest similarity*	*Highest proportion of paternal bands*	*Paternal specific bands*	*Father*
1	376	70/376	376	376	36	214/235	1/235	-	235
2	104/214	104	-	104	37	70/214	70	70	70
3	214/235	70/235	-	235	38	104	104/376	-	104
4	376	376	376	376	39	104/214	104/376	-	104
5	1/214	1	1	1	40	64/376	376	-	376
6	1/214	1	1	1	41	104/214	104/376	-	104
7	104	104/376	-	104	42	104	104	-	104
8	376	376	376	376	43	64/104	64	-	64
9	104/376	104/376	-	104/376	44	214/235	213/235	-	235
10	64/214	64	64	64	45	64	64	-	64
11	104	104	-	104	46	1/214	1	1	1
12	104	104	-	104	47	1/214	1/70	-	1
13	1/214	1	1	1	48	376	376	376	376
14	235	235	-	235	49	104/214	104/376	-	104
15	235	213/235	-	235	50	104	104	-	104
16	376	376	-	376	51	376	376	-	376
17	235	235	-	235	52	104	104/376	-	104
18	214	-	-	214	53	1/214	1	1	1
19	214/235	235	-	235	54	214	-	-	214
20	104/214/376	104/376	-	104/376	55	104/214	104/376	-	104
21	213/214	213	213	213	56	104/214/376	104/376	-	104/376
22	104	1/104	-	104	57	104	1/104	-	104
23	104	104	-	104	58	104/376	376	-	376
24	104/376	104/376	376	376	59	214/235	70/235	-	235
25	104/376	104/376	-	104/376	60	104	104/376	-	104
26	104	104	-	104	61	104/214	1/104	-	104
27	376	70/376	-	376	62	64	64	-	64
28	64/376	64	-	64	63	104	1/104	-	104
29	376	376	-	376	64	104/214	70/104	-	104
30	64/235	64	-	64	65	104/376	104	-	104
31	104/214	104/213	-	104	66	235	235	-	235
32	64/214	64	64	64	67	214	-	-	214
33	104/214	1/104	-	104	68	214	-	-	214
34	1/214	1/213	-	1	69	64/214	64	-	64

35	64/214	64	64	64	70	104/214/376	104/376	-	104/376
71	376	1/376	-	376	85	1	1	1	1
72	214	-	-	214	86	104/214	104/376	-	104
73	376	376	-	376	87	1/214	1	1	1
74	214/235	213/235	-	235	88	1/214	1	1	1
75	70/214	70	70	70	89	214/376	1/376	-	376
76	376	1/376	-	376	90	1	1	1	1
77	376	376	-	376	91	1/376	1	1	1
78	376	376	-	376	92	214	-	-	214
79	235	64/235	-	235	93	1/214	1	1	1
80	1/214	1	-	1	94	104/214	70/104	-	104
81	64/104	64	64	64	95	1	1/70	1	1
82	70/104/214	70	70	70	96	214/235	235	-	235
83	104	104/376	-	104	97	1/214	1	1	1
84	1	1	1	1	98	1/104	104	-	104

Table 5.9: Fathers of offspring of *Banksia prionotes* plants 223 and 244, and of *Banksia praemorsa* plants 213 and 214.

Father	*Offspring*			
	Number (possible)	Percentage (possible)	Number (possible)	Percentage (possible)
		Banksia prionotes		
	223		**244**	
11	3	9.4	1	3.6
12	1	3.1	6	21.4
27	3	9.4	2	7.1
86	9	28.1	0	0
218	4	12.5	6	21.4
223	0	0	2 (1)	7.1 (3.6)
224	2	6.2	4 (1)	14.3 (3.6)
225	4	12.5	4	14.3
232	3	9.4	2	7.1
237	3	9.4	*0	0
244	0	0	0	0
		Banksia praemorsa		
	213		**214**	
1	12	11.5	17	17.3
64	8	7.7	10	10.2
70	0	0	3	3.1
104	38 (4)	36.5 (3.8)	27 (5)	27.5 (5.1)
213	0	0	1	1.0
214	25 (4)	24.0 (3.8)	6	6.1
235	15	14.4	12	12.2
376	2	1.9	17 (5)	17.3 (5.1)

* No paternal specific bands

Two types of bird visitors were observed foraging on both *Banksia prionotes* and *Banksia praemorsa* inflorescences. These were the Little wattlebird (*Anthochaera lunulata*) and the New Holland honeyeater (*Phylidonyris novaehollandiae*) (Figure 5.6). Small numbers of honeybees (*Apis mellifera*) were observed, with no between inflorescence or between plant movement. Thirty honeybees were observed on *Banksia prionotes* inflorescences, with ten observed on *Banksia praemorsa*. No visitors were observed during nocturnal hours. For both types of bird visitors, behavioural patterns were consistent on both species (Table 5.10). Little wattlebirds spent time foraging, calling and chasing New Holland honeyeaters, while the New Holland honeyeaters foraged for shorter periods whenever possible. Interplant movement was observed for both Little wattlebirds and New Holland honeyeaters (Table 5.11).

Figure 5.6: Pollinator types observed foraging on *Banksia prionotes*.

5.6A New Holland Honeyeater (*Phylidonyris novaehollandiae*) perched on a inflorescence of *Banksia prionotes*, clearly showing yellow wing patch.

5.6B Little Wattlebird (*Anthochaera lunulata*) located among *Banksia prionotes* inflorescences.

5.6C Little Wattlebird foraging on a *Banksia prionotes* inflorescence. The mode of foraging is clearly visible with the bird inserting it's beak at the front of the newly opened florets.

A

B

C

Table 5.10: Flower visitors to *Banksia prionotes* and *Banksia praemorsa*. Mean data of 4 x 2 hour observations.

	Banksia prionotes		*Banksia praemorsa*	
	Little wattlebird	New Holland honeyeater	Little wattlebird	New Holland honeyeater
Total number of visits	83	26	54	27
Number foraging	61	25	52	25
Mean foraging time (sec)	2.6 ±0.39	0.8 ±0.13	2.5 ±0.33	0.8 ±0.14
Number calling	25	0	15	0
Mean calling time (min)	1.2 ±0.10	-	0.96 ±0.17	-
Number chasing	13	0	18	0
Number moving between plants	13	5	5	0

Table 5.11: Inter-plant movements of flower visitors to *Banksia prionotes* and *Banksia praemorsa*. Mean data of 4 x 2 hour observations.

Plant Code	*Total number of inflorescences*	*Little wattlebird*	*New Holland honeyeater*
		Plant code (number of inter-plant movements observed)	
		Banksia prionotes	
11	10	-	12 (2)
12	10	-	-
27	55	86 (2)	-
86	50	-	-
218	40	224 (1)	-
223	40	224 (3)	232 (2)
224	60	244 (2)	-
225	20	-	-
232	30	-	244 (2)
237	20	-	-
244	130	218 (5)	-
		Banksia praemorsa	
64	30	-	-
70	30	214 (2)	-
104	55	-	-
214	60	104 (3)	-

5.4 Discussion

Pollen flow investigations based on similarity values and paternal bands demonstrated that non-random mating was occurring in the two species of *Banksia*. From the paternity information and the map of the populations, the approximate distance and direction of pollinator movements were assessed. The mother plants of *Banksia prionotes* were located ~~26.5~~ 26.4m apart, plant 223 was centrally located adjacent to three other plants with mother 244 more isolated from the rest of the population. The main successful pollen movement was from plant 86, 50 m away for mother 223, with the more closely located plants 218 and 225 also providing successful pollen. For plant 244 the main successful pollen flow was from plant 12 at a distance of 76 m, with successful pollen also from plants 224 (23.6 m) and 225 (26.2m). There were no selfs of either plant , and 244 set no seed with 86, despite its high success with 223. *Banksia praemorsa* plants 213 and 214 were adjacent to each other and pollen from plant 104 (30.4 and 32 m) was most successful for both. There were six selfs of 214 but none of 213.

The development of molecular techniques has encouraged an increase in several areas of research, one of which is the determination of paternity (Achmann *et al*, 1992; Hadrys *et al*, 1992; Hadrys et al, 1993; Apostol *et al*, 1993; Tegelstrom and Hoggren, 1994; Von Segesser *et al*, 1994; Pepin, *et al*, 1995). A disadvantage of using the RAPD technique is the inability to detect heterozygosity, as the DNA fragments show dominant inheritance (Lynch, 1988; Hadrys *et al*, 1992). This restricts analysis of data to non-genetic based methods or requires adaptation of existing formulae to account for the limitation. Lewis and Snow (1992) have calculated the average exclusion probability for both dominant and co-dominant markers, and not suprisingly co-dominant perform better than dominant markers. They suggest that 50 loci should be scored for RAPD analysis of paternity exclusion. Consequently most researchers have chosen to either use similarity based

assessments or inference of paternity through paternal band sharing. A further disadvantage of RAPDs that has been identified by some workers is the incidence of non-inherited bands (Hadrys *et al*, 1993; Morell *et al*, unpub.; Newbury and Ford-Lloyd, 1993). These have been variously described as amplification artefacts (Hadrys *et al*, 1993) or as resulting from competition for amplification sites (Newbury and Ford-Lloyd, 1993). In an outcrossing population genetic crossing over and recombination would be expected to produce sequence differences which may result in new amplification sites, particularly in hybrid progeny (Newbury and Ford-Lloyd, 1993). In this study 41 and 35 loci were scored for *Banksia prionotes* plants 223 and 244, none of which was a non-parental inherited band. For *Banksia praemorsa* 45 and 49 loci were scored for plants 23 and 214, five and eight of which were new bands unique to the offspring. This study has shown that the RAPD approach is successful for paternity analysis of *Banksia*. The most important requirement is large numbers of band differences between the mother plant and potential fathers. This was achieved for all plants in this study except plants 244 and 237, so the lack of identification of plant 237 as a father of 244 seed may be spurious. Despite large numbers of paternal bands, most were shared and few were specific to one particular father. More father specific bands would increase the efficiency of paternity assignment.

Both the Little wattlebird and the New Holland honeyeater were regular visitors to the flowers of both species, and inter-plant movement was common. This suggests that both species were transferring pollen, although observations were limited and no capturing or swabbing of birds for pollen was attempted. Bird pollination has been reported in some other *Banksia* species (Paton and Turner, 1985; Collins and Spice, 1986; Ramsey, 1989; Vaughton, 1990b) and individuals were observed to thrust their bills deep into the flowers bringing the head in contact with the pollen presenter. The birds were also observed to forage sequentially on adjacent flowers on the inflorescence, and this may explain the

groups of adjacent seeds with the same father detected in the analysis. Thus, pollen transfer may be expected to be a random event depending on which plant the bird visited immediately prior to visiting the designated mother plant. This is clearly not the case, as all potential father plants in the population were in bloom, yet some fathers were not represented at all in the seed population. It is also interesting to note that for *Banksia prionotes* different fathers were successful with the two mothers, both of which were located long distances away. *Banksia prionotes* is also reported to be highly self incompatible (Collins and Spice, 1986), so the lack of selfs in the offspring is in keeping with this observation.

The interaction between the pollen donor and recipient has been found to affect competition of a particular pollen type (Aizen *et al*, 1990: Jonhston, 1993; Martin and Lee, 1993). *Dianthus chinensis* self pollen grew slower than cross pollen when compared between flowers, but when compared within flowers the differences were greater, indicating that an interaction was occurring (Aizen *et al*, 1990). The effect of pollen source in *Cassisa fasciculata* was also found to be affected by the maternal plant (Martin and Lee, 1993). These studies indicate that self and cross pollen are interacting with each other and also with the pollen recipient. Selective abortion of a particular seed type can also provided an insight into the result of competition between self and cross pollen. Several recent studies on Australian species have clearly indicated that self pollinated seeds are selectively aborted (Whelan and Ayre, 1992; Hariss and Whelan, 1993; Vaughton and Carthew, 1993). *Grevillea barklyana* is a self-compatible species with relatively low fruit set averaging less than 5% (Hariss and Whelan, 1993). In two separate studies outcrossed fruits were selectively matured on a single inflorescence when half the inflorescence was crossed and the other half selfed (Whelan and Ayre, 1992; Hariss and Whelan, 1993). Interestingly when *Grevillea barklyana* was studied using isozymes at the population level, three of the four

populations exhibited almost complete selfing (Ayre *et al*, 1994). A similar single inflorescence protocol was used to show that *Banksia spinuolsa* also selectively matures outcrossed seeds (Vaughton and Carthew, 1993). On a single inflorescence with both self and cross pollination, selfed seed set was reduced by 63% compared with cross seed set. The authors suggest that self seeds are produced but are aborted in preference to outcrossed progeny. In addition the outcrossing rate in the study population was determined on a number of siblings. Complete outcrossing was indicated, which was consistent with the conclusion that self seeds were selective aborted. All these studies indicate that caution is needed when examining the competition occurring between different pollen types. Numerous factors including small sample size, the techniques used and whether individuals or populations are examined can affect the experimental outcome. Another equally important consideration is the interaction which occurs between the pollen donor and the recipient.

Random fertilisation, or the likelihood that all pollen sources in a population have an equal chance to fertilise is a central premise of population genetic theory (Snow and Spira, 1991a). The validity of this assumption has been tested by various investigations (Bertin, 1985; Marshall and Ellstrand, 1986; Snow and Spira, 1991a; Snow and Spira, 1991b; Marshall, 1991; Polanco *et al*, 1994; Snow, 1994; Marshall and Fuller, 1994). Non-random mating has been demonstrated in a wide variety of species, including *Campsis radicans* (Bertin, 1985), *Hibiscus moscheutos* (Snow and Spira, 1991a; Snow and Spira, 1991b) and the crop species *Secale cereale* (Polanco *et al*, 1994), with most extensive information available on the wild radish *Raphanus sativus* (Marshall and Ellstrand, 1986; Marshall, 1991; Marshall and Fuller, 1994). Male-male competition in wild radish increased fruit-set in multiple-sired compared to single-sired fruit, and the total seed weight of multiple-sired was higher than single-sired fruits (Marshall and Ellstrand, 1986). The consistency of a

particular donor, whether mixed with one or six other donors, has also been demonstrated in this species (Marshall, 1991). Most studies on *Raphanus sativus* have been glasshouse experiments, but recently Marshall and Fuller (1994) demonstrated that non-random mating is possible under field conditions, although the ranking of donor success varied in the two environments.

This study has established that non-random mating occurs in two species of *Banksia* in isolated populations but what are the possible causes and implications of non-random mating ? The occurrence of mixed pollen is an obvious requirement and many researchers have found that mixed pollen loads are common in nature (Bertin, 1985; Marshall and Ellstrand, 1986; Snow and Spira, 1991b; Snow, 1994). Adaptations which encourage or enhance the occurrence of mixed pollen loads will facilitate non-random mating. Once a mixture of pollen is present on the stigma additional factors become involved. The number and type of pollen may affect the ability to initiate germination (Marshall, 1991; Richardson and Stephenson, 1992), or once pollen tubes have been initiated competition between different types of pollen may occur (Snow and Spira, 1991b; Plitmann, 1993; Snow, 1994). Another possibility is that the maternal plant may have a mechanism to sort between pollen grains and pollen tubes. Some pollen types may be more efficient at fertilisation, or once a seed is produced, particular embryos may garner resources more effectively (Marshall, 1991; Richardson and Stephenson, 1991).

Non-random mating can be split into two categories; non-random fertilisation and non-random abortion (Snow and Spira, 1991a). A combination of male-male competition and female choice are active in selective fertilisation, while selective abortion involves female choice. The events which occur pre- and post-pollination are important when considering how non-random mating is occurring in any population (Stephenson and Bertin, 1983; Marshall and Ellstrand, 1986). Pre-pollination factors such as phenology, flower

number and arrangement, proximate attraction, and flower reward have been identified as important (Stephenson and Bertin, 1983; Stanton *et al*, 1986; Devlin *et al*, 1992). ***Banksia*** typically displays thousands of florets in the form of an inflorescence with sequential opening of these florets over time (George, 1987). This mode of presentation of pollen and nectar resources has an impact on pre-pollination aspects of non-random mating. Sequential opening of *Banksia* stamens increases the availability of high quality pollen over a long period of time, which is thought to increase a male's competitive advantage (Stephenson and Bertin, 1983). The arrangement of florets in an inflorescence is also thought to enhance male competition, as a large number of florets, and hence nectaries increases the number of pollinator visits and hence the movement of pollen. If mixed pollen loads occur and the number of viable pollen grains on the stigma is larger than the number of ovules, there is the possibility that male competition will occur. Pollen germination and pollen tube growth are considered to be strongly influenced by male attributes, and therefore can be distinguished from female choice. Many studies have indicated that different pollen types have different germinating abilities and that there is a difference in the rate and length of pollen tube growth from different donors (Marshall and Ellstrand, 1986; Thomson, 1989; Marshall, 1991; Cruzan, 1993; Plitmann, 1993). Pollen tube growth in *Banksia* has been investigated in relation to breeding systems and hybridisation (Fuss and Sedgley, 1991a; Fuss and Sedgley, 1991b) but this is the first study on *Banksia* which indicates that different pollen donors have varying success when fertilising seeds.

A plant is under selection pressure to allow only the highest quality pollen to sire the seeds, provided pollen donors differ in genetic quality and reproduction is not pollen limited (Mulcahy and Mulcahy, 1975; Snow, 1994). Female choice can operate in numerous ways, influencing the type of pollen deposited on a stigma, choosing which pollen fertilises ovules, restricting which ovules develop into mature seeds and which of the juvenile fruit reach

maturity (Stephenson and Bertin, 1983; Marshall and Ellstrand, 1986; Snow, 1994). Outcrossing rates in many *Banksia* species have been reported to be high (Scott, 1980; Carthew *et al*, 1988) despite these species showing some level of self-compatibility. Recently Vaughton and Carthew (1993) reported that crossed progeny are favoured over self progeny on a single inflorescence basis for *Banksia spinulosa*. These two pieces of information combined suggest that selective abortion may be occurring in *Banksia*. Many aspects of pollination, fertilisation and seed maturation in *Banksia* suggest that non-random mating, by a combination of male-male competition and female choice, may be common in this genus. Further investigations are required to clarify the extent and heritability of male-male competition, and if selective abortion is consistent within populations over several years (Bertin, 1985; Snow and Spira, 1991b).

The direction and distance that pollen moves within and between populations has been studied in many different ways over many years (Levin and Kerster, 1974; Schaal, 1980; Snow, 1982; Thomson *et al*, 1986; Goldenberg, 1987; Thomson and Thomson, 1989: Rasmussen and Brodsgaard, 1992; Nilsson *et al*, 1992). Normally the movement of pollen is determined using indirect methods such as pollinator flight distances (Schaal, 1980; Rasmussen and Brodsgaard, 1992) or pollen analogues (Thomson *et al*, 1986; Campbell, 1991). In recent years various new techniques have been developed to investigate the movement of pollen (Stanton, 1986; Campbell, 1991; Nilsson *et al*, 1992). Using both genetic markers (Campbell, 1991) and statistical probabilities (Stanton, 1986), isozymes have been used to track pollen flow via paternity. The use of microtags has been recently been tried on the orchid species *Aerangis ellisii* (Nilsson *et al*, 1992), indicating infrequent pollen movement. The study of indirect pollen flow has been shown to be inadequate (Klinkhamer *et al*, 1994) and these new methods of monitoring pollen flow are promising. Another approach is using the RAPD technique. RAPDs can be used to determine the

paternity of offspring and if the positions of specific plants are recorded, the distance and direction of pollen movement can be tracked. This approach enables only the pollen which has successfully fathered progeny to be identified. The maximum distance over which pollen flow occurred at the Happy Valley population was approximately 76 meters for *Banksia prionotes* and 84 meters for *Banksia praemorsa*. Over 50% of the progeny produced by plants 213 and 214 occurred as a result of pollen flow of 35 meters or less. For *Banksia prionotes* up to 60 meters movement of pollen from plants 11 and 12 proved to be common but over 60% of the pollen movement to plant 244 was from between 20 and 26 meters. The distance between plants does not dictate pollen flow, as plants close together do not necessarily exchange pollen, as shown by plants 244 and 223.

5.5 Conclusions

The RAPD technique has been used to examine pollen competition, paternal success and pollen movement in two *Banksia* species. Non-random mating was found to be occurring in *Banksia prionotes* and *Banksia praemorsa* in small isolated populations.

Chapter 6: Competition between self and cross pollen in *Banksia menziesii.*

6.1 Introduction

The mechanisms of self-incompatibility and pollen tube competition, may operate to reduce selfing and increase the vigour of offspring. Investigations of the *Banksia* genus have shown complete or partial self-incompatibility to be widespread (Carthew *et al*, 1988; Goldingay and Whelan, 1990; Fuss and Sedgley, 1991a). Pollen tube competition, however, has not been widely studied in the genus. Such experiments have been carried out on *Banksia spinulosa* over two flowering seasons. A small number of the inflorescences which were pollinated produced seeds, and from isozyme data the conclusion was drawn that outcrossed pollen was selectively favoured over selfed pollen (Carthew *et al*, 1988). Studies with other genera have found that pollen competition is dependent on both the genotype of the microgametopyte and the genotype of the pistil (Winsor *et al*, 1987; Snow and Spira, 1991b), and intense pollen competition has been found to result in more vigorous seed germination, growth and survival of seedlings (Mulcahy and Mulcahy, 1975; Winsor *et al*, 1987). Research in other genera has determined ambiguous results relating to the performance of outcrossed pollen (Thomson, 1989; Aizen *et al*, 1990; Snow and Spira, 1991a). In *Hibiscus moscheutos* self pollen tubes grew more slowly than outcrossed tubes in some crosses, but faster in others (Snow and Spira, 1991a).

The direct competition between self and cross pollen on the stigma is examined here using *Banksia menziesii*. Direct pollen competition is important when considering the reasons behind low seed set, and experimentation in this area may facilitate our understanding of the pollination process.

6.2 Materials and Methods

6.2.1 Plant material

Plantation grown *Banksia menziesii* plants at Blewitt Springs were used to investigate pollen competition.

6.2.2 Pollinations

Experiments were carried out on *Banksia menziesii* plants in their peak flowering period and were conducted in two years on different sets of plants. In both years three pairs of plants were each assigned to a family and six experimental inflorescences were randomly chosen and bagged prior to anthesis on the three maternal plants. All other inflorescences on the plants were removed as they opened by pickers over the season. All flowers on one inflorescence were selfed, while all on another inflorescence were crossed with pollen from the paternal parent. Two inflorescences were treated by first applying self pollen to all flowers then applying cross pollen, and in the final two inflorescences, this process was reversed. Pollen was transferred by removing a style with fresh pollen from the donor inflorescence which was used to deposit pollen in the recipient stigmatic groove (Fuss and Sedgley, 1991b). The pollination treatment was continued until all flowers on the inflorescence had opened, and all flowers had been pollinated. The delay between application of the self and cross pollen types was minimal, one type being applied immediately after the other. Pollination of all flowers on an inflorescence took up to 10 days as they opened progressively, and for this reason self pollen removal prior to pollination was not attempted. Once the infructescence had developed, the number of follicles was counted and the seeds removed. The DNA was extracted (Chapters 4, 5) from all the seeds, and from leaves of the mothers and pollen donors and the RAPD method used to generate banding patterns.

6.2.3 Randomly amplified polymorphic DNA analysis

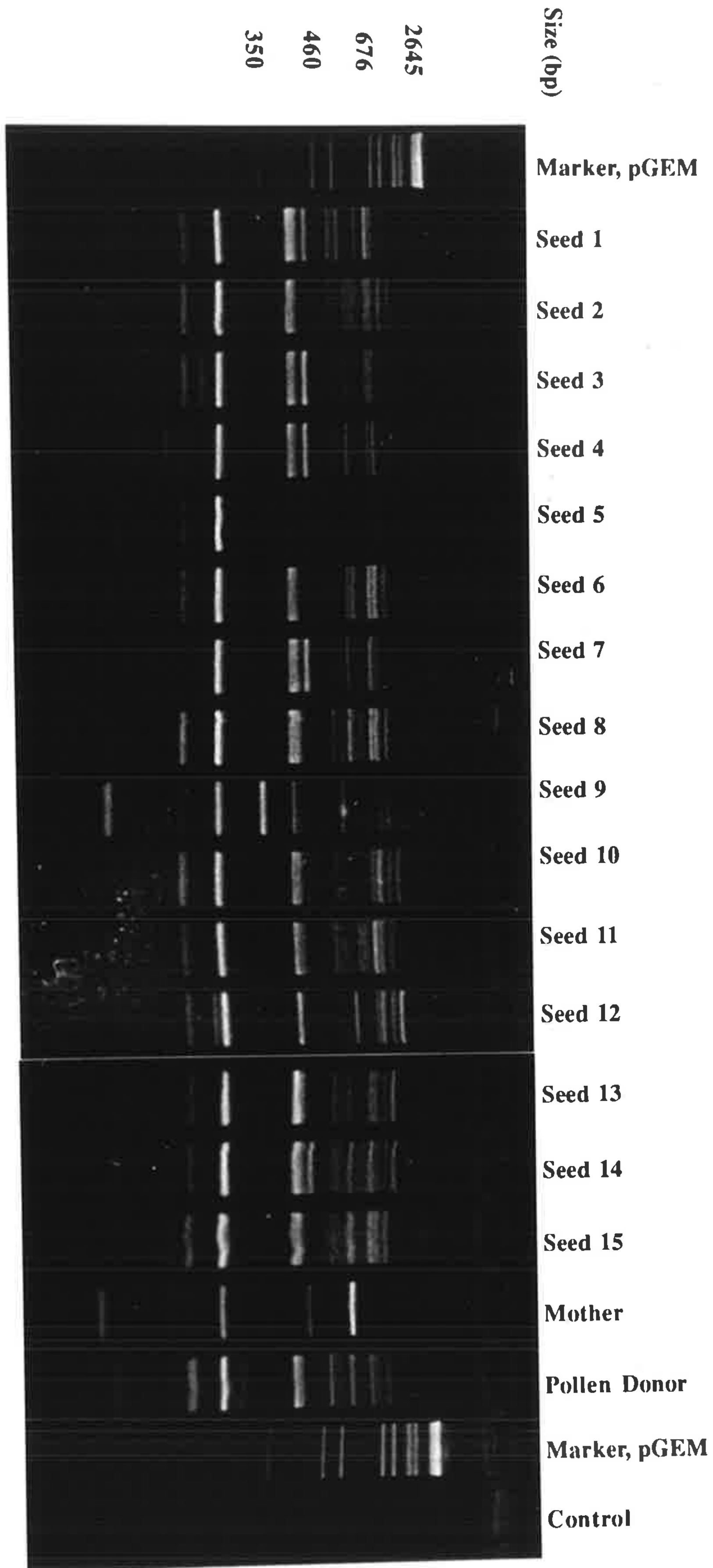
A primer survey was conducted on each parental pair to identify unique band(s) distinguishing each set of parents. Operon Technologies primer kits A, B, C and D were tried. Once the presence of unique bands was established, RAPD analysis using the chosen primer was run on the offspring seed DNA. The two primers OPC 13 (AAGCCTCGTC) and OPC 14 (TGCGTGCTTG) were used independently for Family A and the results combined, while for Families B and D primers OPC 13 and OPB 16 (TTTGCCCGGA) were used respectively. For Family E primer OPB 08 (GTCCACACGG) was used, while primer OPB 03 (CATCCCCCTG) was used for Family F. The offspring RAPD banding patterns were compared to those of the parents (Figure 6.1).

6.2.4 Analysis and interpretation

The bands from all families with seeds were identified as maternal, paternal and offspring bands. Similarity matrices were generated using Excel software, as described in Chapter 4. All offspring from each family were compared with the mother and father following removal of all shared maternal and paternal bands, as well as unique offspring bands and bands shared by all individuals. The mean percentage similarity of the offspring to the maternal and paternal parent was generated from the similarity matrices, and to test the difference between the means an independent t-test was performed for each treatment. The number of paternal bands for each offspring in each of the families was determined and where possible paternal bands were used to assign crossed status.

Figure 6.1: An agarose gel illustrating the mother, pollen donor and offspring of Family A.

Lane 1: Marker, pGEM. Lane 2 - 9, Cross/self treatment. Lane 10 -16, Cross treatment. Lane 17, Mother. Lane 18, Pollen donor. Lane 19, Marker, pGEM. Lane 20, Control, containing no DNA.


Size (bp)
2645
676
460
350
Marker, pGEM
Seed 1
Seed 2
Seed 3
Seed 4
Seed 5
Seed 6
Seed 7
Seed 8
Seed 9
Seed 10
Seed 11
Seed 12
Seed 13
Seed 14
Seed 15
Mother
Pollen Donor
Marker, pGEM
Control

6.3 Results

In total 233 seeds were produced for five families over a two year period; Family C failed to produce any seeds (Table 6.1). Family E produced the highest number of seeds, followed by Families A and F, and more seeds were produced in the second than the first year. Seed numbers were low and the standard errors indicated no significant difference between treatments. Difficulties were encountered in DNA extraction (Table 6.2) and in PCR analysis, such that banding patterns were obtained for only 93 of the original 233 seeds (Table 6.3). Only Families A and E had representatives of each treatment. An example of an agarose gel is presented in Figure 6.1, illustrating parents and offspring from Family A. There were 24 band differences between mother and father in Family A. Of these 14 were unique to the father and 10 to the mother. A total of 62 bands were generated for the mother, father and offspring of Family A (Table 6.4), and twenty three bands were present only in the offspring. For Family B there were only three band difference between mother and father and a total of 12 bands were produced (Table 6.5). The number of unique offspring bands for Family D was 8 out of a total of 22 bands (Table 6.6). One band was unique to the mother while the father had 5 unique bands. For Family E there were four band differences between the mother and father, with one band unique to the father. The offspring had 16 bands which were unique out of a total of 27 bands (Table 6.7). For Family F a total of 34 bands were generated, with 6 differences between mother and father (Table 6.8). A single unique band was present in the mother while the father had 5 unique bands.

The percentage similarity to the mother and father for each of the four treatments in Family A were compared using a independent t-test, and all were found to be non significant (Table 6.9), indicating that the similarity to the father and mother was indistinguishable. The comparison of the mean percentage similarity for Family B also produced non significant results (Table 6.10).

Table 6.1: Number of seeds collected from infructescences of *Banksia menziesii* from hand pollinations conducted at Blewitt Springs in 1992 and 1993.

Family Code		*Number of seeds per treatment*			
		Self	Self/cross	Cross/self	Cross
1992	A	13	28	11	11
	B	0	7	0	7
	C	0	0	0	0
	Mean	**4.3**	**11.7**	**3.7**	**6.0**
1993	D	1	8	0	8
	E	28	23	26	12
	F	0	24	20	6
	Mean	**9.7**	**18.3**	**15.3**	**8.7**
Overall Mean ± SE		7.0 ± 4.7	15.0 ± 4.7	9.5 ± 4.7	7.3 ± 1.7

Table 6.2: Number of *Banksia menziesii* seeds from which DNA was extracted.

Family Code		*Number of seeds per treatment*			
		Self	Self/cross	Cross/self	Cross
1992	A	10	12	8	13
	B	0	6	0	4
	C	0	0	0	0
	Mean	**3.3**	**6.0**	**2.7**	**5.6**
1993	D	1	6	0	7
	E	5	9	18	6
	F	0	11	12	5
	Mean	**2.0**	**8.7**	**10.0**	**6.0**

Table 6.3: Number of *Banksia menziesii* seeds for which RAPD banding patterns were obtained.

Family Code		*Number of seeds per treatment*			
		Self	Self/cross	Cross/self	Cross
1992	A	6	7	8	7
	B	0	2	0	2
	C	0	0	0	0
	Mean	**2.0**	**3.0**	**2.7**	**3.0**
1993	D	1	6	0	3
	E	5	9	10	6
	F	0	7	11	3
	Mean	**2.0**	**7.3**	**7.0**	**4.0**

Table 6.4: Summary of bands in Family A.

	Number of bands
Mother and Father	
Number of band differences between mother and father	24
Number of bands unique to mother	10
Number of bands unique to father	14
Mother, Father and Offspring	
Number of bands common to all individuals	1
Number of bands unique to mother and at least one offspring	10
Number of bands unique to mother	0
Number of bands unique to father and at least one offspring	14
Number of bands unique to father	0
Number of bands unique to mother and father	0
Number of bands common to mother and father	14
Number of bands unique to offspring	23
Total number of bands	62

Table 6.5: Summary of bands in Family B.

	Number of bands
Mother and Father	
Number of band differences between mother and father	3
Number of bands unique to mother	2
Number of bands unique to father	1
Mother, Father and Offspring	
Number of bands common to all individuals	2
Number of bands unique to mother and at least one offspring	2
Number of bands unique to mother	0
Number of bands unique to father and at least one offspring	1
Number of bands unique to father	0
Number of bands unique to mother and father	0
Number of bands common to mother and father	5
Number of bands unique to offspring	2
Total number of bands	12

Table 6.6: Summary of bands in Family D.

	Number of bands
Mother and Father	
Number of band differences between mother and father	6
Number of bands unique to mother	1
Number of bands unique to father	5
Mother, Father and Offspring	
Number of bands common to all individuals	1
Number of bands unique to mother and at least one offspring	2
Number of bands unique to mother	0
Number of bands unique to father and at least one offspring	2
Number of bands unique to father	2
Number of bands unique to mother and father	0
Number of bands common to mother and father	7
Number of bands unique to offspring	8
Total number of bands	22

Table 6.7: Summary of bands in Family E.

	Number of bands
Mother and Father	
Number of band differences between mother and father	4
Number of bands unique to mother	3
Number of bands unique to father	1
Mother, Father and Offspring	
Number of bands common to all individuals	1
Number of bands unique to mother and at least one offspring	3
Number of bands unique to mother	0
Number of bands unique to father and at least one offspring	1
Number of bands unique to father	0
Number of bands unique to mother and father	0
Number of bands common to mother and father	6
Number of bands unique to offspring	16
Total number of bands	27

Table 6.8: Summary of bands in Family F.

	Number of bands
Mother and Father	
Number of band differences between mother and father	6
Number of bands unique to mother	1
Number of bands unique to father	5
Mother, Father and Offspring	
Number of bands common to all individuals	0
Number of bands unique to mother and at least one offspring	1
Number of bands unique to mother	1
Number of bands unique to father and at least one offspring	5
Number of bands unique to father	0
Number of bands unique to mother and father	0
Number of bands common to mother and father	13
Number of bands unique to offspring	14
Total number of bands	34

Table 6.9: Mean percentage similarities of offspring to maternal and paternal parents for Family A.

	Percentage similarity to		*t-test (df)*
	Mother	Father	
Mother	100	0	-
Father	0	100	-
Self	45.5	54.5	-1.03 (10)
Self/cross	31.9	68.1	-7.96 (12)
Cross/self	45.7	54.3	-2.14 (14)
Cross	26.9	73.1	-6.92 (14)

df - degrees of freedom

Table 6.10: Mean percentage similarities of offspring to maternal and paternal parents for Family B.

	Percentage similarity to		*t-test (df)*
	Mother	Father	
Mother	100	0	-
Father	0	100	-
Self/cross	33.3	66.6	-0.71 (2)
Cross	66.6	33.3	-0.71 (2)

df - degrees of freedom

All treatments for Family D were non significant, except the cross treatment (P>0.05), but the similarity was higher to the mother than to the father (Table 6.11). For Family E (Table 6.12) the mean percentage similarity values were all non significant. The cross treatment for Family F (Table 6.13) was significant (P>0.05) with all other treatments non significant.

All Family A offspring had at least one of the 14 paternal bands, including all of the selfs (Table 6.14). For Family B the single father-specific band was present in three of the four seeds produced. Paternal bands were present in all of the self/cross seeds produced for Family D, with a single paternal band in the self. For Family E a single father-specific band was present in one of the self/cross, and two of the cross/self and one cross, but none of the selfed offspring (Table 6.14). For Family F paternal bands were present in seven of the nine selfs and two of the crossed seeds.

Table 6.11: Mean percentage similarities of offspring to maternal and paternal parents for Family D.

	Percentage similarity to		*t-test (df)*
	Mother	Father	
Mother	100	0	-
Father	0	100	-
Self	83.3	16.7	-
Self/cross	76.7	23.3	4.44 (8)
Cross	66.7	33.3	65535 (4) *

df - degrees of freedom
* P>0.05

Table 6.12: Mean percentage similarities of offspring to maternal and paternal parents for Family E.

	Percentage similarity to		*t-test (df)*
	Mother	Father	
Mother	100	0	-
Father	0	100	-
Self	40	60	-2.31 (8)
Self/cross	47.2	52.8	-2.14 (16)
Cross/self	45	55	-1.13 (18)
Cross	37.5	62.5	-1.65 (10)

df - degrees of freedom

Table 6.13: Mean percentage similarities of offspring to maternal and paternal parents for Family F.

	Percentage similarity to		*t-test (df)*
	Mother	Father	
Mother	100	0	-
Father	0	100	-
Self/cross	42.8	57.2	-0.63 (12)
Cross/self	36.3	63.6	-3.30 (20)
Cross	28.6	71.4	65535 (4) *

df - degrees of freedom
* P>0.05

Table 6.14: Number of paternal bands in Families A, B, D, E and F.

	Family A		*Family B*		*Family D*		*Family E*		*Family F*	
Offspring number	Treatment	Number of paternal bands	Treatment	Number of paternal bands	Treatment	Number of paternal bands	Treatment	Number of paternal bands	Treatment	Number of paternal bands
1	Self	9	S/C	1	Self	1	Self	0	Self	1
2	Self	10	S/C	1	S/C	1	Self	0	Self	1
3	Self	6	Cross	1	S/C	2	Self	0	Self	1
4	Self	7	Cross	0	S/C	1	Self	0	Self	1
5	Self	6			S/C	1	Self	0	Self	1
6	Self	1			S/C	1	S/C	0	Self	1
7	S/C	11			Cross	0	S/C	0	Self	1
8	S/C	7			Cross	0	S/C	0	Self	0
9	S/C	7			Cross	0	S/C	1	Self	0
10	S/C	7					S/C	0	C/S	0
11	S/C	8					S/C	0	C/S	0
12	S/C	6					S/C	0	C/S	0
13	S/C	7					S/C	0	C/S	0
14	C/S	3					S/C	0	C/S	0
15	C/S	8					C/S	0	C/S	0
16	C/S	6					C/S	0	C/S	0
17	C/S	6					C/S	1	C/S	0
18	C/S	6					C/S	1	C/S	0
19	C/S	7					C/S	0	Cross	0
20	C/S	6					C/S	0	Cross	1
21	C/S	7					C/S	0	Cross	1
22	Cross	6					C/S	0		
23	Cross	7					C/S	0		
24	Cross	11					C/S	0		
25	Cross	10					Cross	0		
26	Cross	10					Cross	0		
27	Cross	11					Cross	0		
28	Cross	12					Cross	1		
29	Cross	10					Cross	0		
30							Cross	0		

6.4 Discussion

The low number of seeds produced per treatment and the fact that only two families produced seeds in all of the four treatments reduced the likelihood of assigning seeds to self or crossed categories. To combat this difficulty in future experiments the initial number of pollinations needs to be substantially increased, including both the number of inflorescences per family as well as the total number of families. The number of plants and inflorescences available for this experiment were limited, as each experimental inflorescence represented an economic loss to the grower. It was hoped that commercially-managed plants would give higher seed set than those in the wild or in minimally-cultivated collections, so as to overcome the low set problems of wild plants encountered by Carthew *et al* (1988). This was not the case, and pollination of more flowers in either cultivated or wild stands will be required to further investigate this question. The failure to remove self pollen prior to the application of cross pollen has resulted in ambiguity with regard to the seeds produced. There is the potential that the seeds from the cross treatment may be either crossed or selfed and as a result comparison with other potentially crossed offspring is confused. Despite the technical difficulties, in future experiments of this nature removal of self pollen is recommended.

In addition RAPD methology difficulties confounded clear conclusions for the pollen competition experiments. This is the first time the RAPD technique has been used to investigate competition between cross and self pollen and primers were chosen on the basis of differences between the mother and pollen donor with the assumption that paternal-specific bands would identify crossed offspring. Two factors confounded this assumption, paternal bands appearing in the selfed individuals and unique offspring bands. In Family A, all of the paternal bands which could have been used to trace paternity occurred in at least one of the selfed individuals. This is most likely the result of co-migrating bands, which can

be resolved by including an additional step of hybridisation in the experimental protocol to clearly discriminate the paternal bands (Chalmers *et al*, 1992; Orozco-Castillo *et al*, 1994). Only in Family E was a paternal band which did not occur in the selfs identified. The large numbers of unique offspring bands are of particular concern. Many authors have reported a low number of non-inherited bands in PCR analysis (Morell *et al*, 1995; Hadrys *et al*, 1993; Newbury and Ford-Lloyd, 1993). These have been explained by competition between primers for amplifiable sites, with different competitive advantages in different genomic backgrounds. This is especially likely in F1 hybrids where specific amplifiable sequences from the backgrounds of the mother and father are likely causing intense competition (Newbury and Ford-Lloyd, 1993). Nevertheless, the large number of unique offspring bands recorded in this experiment has not been previously reported and is of concern. Such large numbers were not observed in previous experiments (Chapter 4, 5), and further work with different primers is needed to fully assess the use of RAPD analysis in pollen competition research. Primer choice should be carefully considered, with the whole family screened for each primer to produce a minimum of unique offspring bands, and paternal bands chosen which can not be confused with those occurring in the selfs. It is also advisable to select primers, or combinations of primers, which produce more father specific bands than were generated in this study.

A new approach is required as conflicting evidence has characterised the area of pollen competition over the last few years, particularly where cross and self pollinations are concerned. Many studies on agricultural crops have suggested that cross pollen is favoured over self pollination, although in *Brassica oleracea* (Brussels sprout), the number of cross pollen tubes is reduced by the presence of self pollen (Ockendon and Currah, 1977). In non-agricultural species, conflicting results have also emerged. In some studies the measurement of pollen tube growth rate has demonstrated that self pollen grows slower (Aizen *et al*,

1990; Whelan and Ayre, 1992; Hariss and Whelan, 1993) or has an increased rate of attrition compared to cross pollen (Cruzan, 1989) but other studies have not found this (Hessing, 1986; Fenster and Sork, 1988; Snow and Spira, 1991a). With further experimentation and refinement of techniques RAPDs may provide new insight into this expanding area of reproductive biology research.

6.5 Conclusions

The competition between self and cross pollen on a single stigma was not clearly resolved during this study and further experimentation is required to clarify the problem. Improvement in experimental technique such as increasing the number of pollinations, pollen removal prior to pollination and improved primer choice are suggested.

Chapter Seven: General Discussion.

The research conducted during this study has two applications, the improvement of floriculture based on *Banksia* spp. in relation to horticultural management and selection, while enhancing the understanding of population ecology and conservation of this uniquely Australian genus.

The *Banksia* genus has long been recognised as having floricultural potential, but must be commercially viable. The production of consistent high quality blooms from registered cultivars with predictable requirements is a necessity to sustain a viable industry (Tranter, 1989; Pegrum, 1990). At the present time *Banksia* blooms are either bush-picked or produced on plantations grown from seed-derived material. These methods of production are inadequate if a sustainable export industry is to be maintained. One of the first steps to reliable flower production involves determination of the control of floral initiation. Investigations in two species of *Banksia* has shown that different triggers are responsible for the production of inflorescences, indicating that in cultivation, management may be required. This indicates that research into floral initiation of new crop species is critical, and the location of commercial plantations is another important management aspect to consider. *Banksia hookeriana* grown at low temperature failed to flower whereas daylength was of greater importance for *Banksia coccinea*. Increased production of flowers, outside the normal season, has long been an important aspect of floriculture. By examining flower production control, there is the potential of extending flowering time.

Improving the management of existing plantations is important to ensure the maintenance of supply to established markets, but the development of new cultivars can increase the volume of sales and open new markets. The lack of registered cultivars in the Australian floriculture industry is a hindrance to overseas exports where quality and consistency are expected. Recently three cultivars of *Banksia* have been released and in the future, as a result of genetic variation studies of the type described here, more targeted selection will be possible. In addition the paternity of potentially hybrid seeds can be confirmed using the RAPD technique, further advancing the production of novel commercial varieties.

The production of new improved cultivars is necessary for a floriculture species, whether through selection, breeding or hybridisation. The *Banksia* genus is characterised by the production of low seed numbers, which is a hindrance to the production of cultivars. Understanding the mechanisms which limit seed production may ultimately lead to an increase. Structural and spatial limitations documented in *Banksia* inflorescences may contribute to low seed production. Spatial limitations exist due to the size of the follicle and the infructescence (Collins and Rebelo, 1987; Fuss and Sedgley, 1991a) and structurally the style of *Banksia menziesii* has been found to be unique. A limited amount of transmitting tissue is present, which may restrict pollen tubes growth and hence seed production (Clifford and Sedgley, 1993). During this study mate selection was found to be prevalent, in two species of *Banksia*. This indicates that genetic selection of offspring is occurring and may result in selective abortion of unfavourable genotypes. Other research has indicated that outcrossed pollen is favoured by species in this genus either through self-

incompatibility mechanisms or fruit abortion (Collins and Spice, 1986; Carthew *et al*, 1988; Fuss and Sedgley, 1991a; Vaughton and Carthew, 1993). Paternity determination indicated that most seeds produced by *Banksia prionotes* and *Banksia praemorsa* were outcrossed, and the genetic variation levels in *Banksia coccinea* and *Banksia menziesii* indicate outcrossing breeding systems. Incompatibility mechanisms, structural and spatial limitations, preference for outcrossed pollen and mate selection seem to be operating in tandem to reduce the overall number of seeds produced by *Banksia* species.

Understanding the ecology of a species is fundamental to conservation (Ellyard, 1987). For a number of years various aspects of the *Banksia* genus has been investigated in relation to ecology. Initially descriptive research included aspects such as germination requirements (Sonia and Heslehurst, 1978), a comprehensive survey of population locations (Taylor and Hopper, 1988) and potential pollinator descriptions (Carolin, 1961). Emphasis later shifted to breeding systems (Carthew *et al*, 1988; Fuss and Sedgley, 1991a) and pollinator behaviour studies (Whelan and Burbridge, 1980; Collins and Spice, 1986; Ramsey, 1989). More recently research has concentrated on the effects of perturbation (Lamont *et al*, 1994a; Lamont *et al*, 1994b; Witkowski *et al*, 1994) and reproductive biology (Vaughton and Carthew, 1993; Vaughton, 1993). Fecundity of roadside populations of *Banksia hookeriana* and *Banksia menziesii* was found to be higher due to better access to water and nutrients (Lamont *et al*, 1994a; Lamont *et al*, 1994b), indicating these disturbed populations are important to conservation of these species. The impact of commercial harvesting of *Banksia hookeriana* has also been investigated, indicating that canopy

area and volume were affected as well as bloom and seed production (Witkowski *et al*, 1994). All this research, when combined, develops a comprehensive picture of the *Banksia* genus in relation to ecology and conservation. Many populations of this genus are located in sensitive areas such as agricultural or private land often with low numbers of plants. Most species investigated were partially self-incompatible, most likely with a mixed mating system. Reproductive biology information is also beginning to indicate that outcrossing is favoured, by mate selection mechanisms. The vast majority of pollinators are birds, with some evidence of marsupial activity (Carthew, 1994). Significantly the effects of perturbations have been found to affect population numbers, levels of genetic variation and long term viability of populations. Any conservation strategies of species belonging to the *Banksia* genus must take all these factors into account.

The goal of horticultural improvement need not be at the expense of species ecology or conservation. Horticultural management of a species can improve understanding of many aspects of reproductive biology and population ecology. This study has demonstrated that improving a specie's horticultural potential can be linked to conservation management strategies.

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