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# Complex and dynamic hybridization between *Banksia robur* and *Banksia oblongifolia* is revealed by genetic and morphological surveys and experimental manipulations

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Complex and dynamic hybridization between *Banksia*  
*robur* and *Banksia oblongifolia* is revealed by genetic  
and morphological surveys and experimental  
manipulations

A thesis submitted in fulfillment of the  
requirements for the award of the degree

Doctor of Philosophy

from

University of Wollongong

by

Annette Vivian Usher B.Sc (Hons) UOW

Department of Biological Sciences

2011

## **Certification**

I, Annette V. Usher, declare that this thesis, submitted in partial fulfillment of the requirements for the award of Doctor of Philosophy, in the School of Biological Sciences, University of Wollongong, is wholly my own work unless otherwise referenced or acknowledged. The second chapter has been published in the journal *Molecular Ecology Notes* and the third chapter published in the journal *Annals of Botany*. The remaining work has not been submitted for qualifications to any other academic institution.

Annette V. Usher

4 March 2011

## Abstract

Several models have been proposed to describe the main processes involved in the evolution or maintenance of hybrid zones. These differ in the type (genetic or environmental) and amount of selection acting on hybrids. Hybridizing species are often associated with specific habitats and empirical studies have revealed that the processes involved in maintaining hybrid zones are often complex and involve genotypic and environmental interactions. Short-lived species are often used to study hybrid fitness, because fitness can be assessed over the whole life-cycle within typical study periods. Hence, long-lived plants have been under-represented in hybrid zone studies.

The life histories of two *Banksia* species, *Banksia robur* and *B. oblongifolia*, provide an excellent opportunity to study the effects of hybridization in long-lived, re-sprouting plants. The hybrid zones contain the products of past hybridization as modified by subsequent natural selection. However, the extent of hybridization and selection after a single recruitment event is unknown. I took advantage a fire in the Sydney region in January 2002, which burned a number of swamps and woodlands releasing seed from the *Banksia* canopy.

I developed genetic markers to determine the genetic structure of mature plants and seedlings within the hybrid zones and to detect hybrids. I developed eight primer pairs for *Banksia* microsatellite markers and genotyped individuals from *B. oblongifolia*, *B. robur* and *B. paludosa* populations, as the latter species potentially hybridized with the other two species. All eight loci were polymorphic, with the exception of two loci in *B. robur*. Numbers of alleles ranged from 1 to 13 and observed average heterozygosity ranged from 0.000 to 0.833. No linkage disequilibrium ( $P < 0.01$ ) was observed between any pair of loci. At least four loci completely distinguished *B. robur* from *B. oblongifolia* and three separated *B. paludosa* from *B. oblongifolia*. Seven of these primers amplified DNA from at least two of three other local *Banksia* species.

To determine whether genetic data could both separate the taxa and predict morphology, I assessed *B. robur* and *B. oblongifolia* plants and seedlings using seven microsatellite markers and seven morphological characters. Pure stands of *B. oblongifolia* and *B. robur* mature plants formed genetically and morphologically identifiable groups. However, hybrid plants were not always intermediate. Morphology separated the two taxa grown in pots and hybrids had the same morphology as parentals. In contrast, seedling morphology in the field was highly variable. Greater phenotypic variation among field seedlings,

due to drought conditions, particularly affected *B. oblongifolia* seedlings as they were growing in the dryer soils than *B. robur* seedlings. Therefore, *B. oblongifolia* seedlings had relatively fewer leaves making the two species morphologically more similar in the field than the two species in pots.

A range of hybrid genotypes was detected among seedlings. Continuing hybridization and introgression was revealed by the composition of the genotypes among seedlings and their maternal plants. The proportion of hybrids appeared to be consistent between generations (6.6% vs 6.2%).

Performance of the two species and their hybrids was assessed in the different environments of the hybrid zones, using indicators of fitness. Germination, survival, growth and herbivore damage were all monitored and assessed. Three methods were used to assess relative seedling performance. A transplant experiment was set up within the hybrid zones; transects of emerging field seedlings were monitored across the hybrid zones; and seedlings from the hybrid zones were raised in pots. Mature plants were also measured to determine whether hybrid plants produced as much seed and recovered as well as the parental species after fire.

Hybrid seedlings and plants were found in all three habitat types within the hybrid zones and, on average, germinated, survived and grew as well as

parental species. Most hybrids were either similar to, or intermediate between, the two parental species in performance. Individual hybrids varied greatly however, and there was some evidence for hybrid superiority and also inferiority in a few seedlings. Whether these prove to be novel hybrid phenotypes depends on how they continue to develop.

There was no evidence for endogenous (intrinsic or genetic) selection against hybrid seedlings in hybrid zones or in pots. However, there was some direct and indirect evidence for selection against immigrant alleles in *B. oblongifolia* habitat (exogenous or environmental selection). There was less evidence for selection against *B. oblongifolia* and hybrid seed or seedlings in *B. robur* habitat. No selection was observed in intermediate habitat and each of the genotypic groups (*B. robur*, *B. oblongifolia* and hybrids) germinated, survived and grew equally well.

The *Banksia* hybrid zones appear to be maintained by gene flow assisted by common pollinators, adaptation of the two species to different environments and selection against immigrants within at least one parental habitat. Hybridization was restricted by the very narrow bands of intermediate habitat where the two species co-exist, partial temporal isolation through separation in peak flowering periods and infrequent recruitment events.



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Chapter Three **Window of opportunity: An episode of recruitment  
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## *Chapter 1*

### **General Introduction**

#### *1.1 Natural hybridization*

Recent progress in the study of natural hybridization has advanced the view that natural hybridization is not only common but is also of evolutionary significance. Indeed, some hybrid zones have become viewed as regions of speciation in progress (Ungerer *et al.* 1998, Arnold 2004, Gross and Rieseberg 2005, Mallet 2007, Bunje *et al.* 2007). Furthermore reticulate evolution, where the genomes of species have converged through, for example, hybridization (species hybridize resulting in either a new species or the transfer of genes from one species to another), has come under greater scrutiny resulting in a reassessment of evolutionary processes and lineages (Linder and Rieseberg 2004, Nakhleh *et al.* 2005).

Natural hybridization occurs when species or genetically divergent populations interbreed to produce individuals of mixed ancestry without human intervention. Hybrid zones are areas of contact between species or genetically

divergent populations where individuals of mixed ancestry are found (see Arnold 1997 for this and further definitions).

### 1.2 *Consequences of hybridization*

Hybridization can have several consequences that may affect the fate of the hybridizing species. It can result in the transfer of genes from one species to another through backcrosses (termed 'introgression', Anderson and Hubricht 1938, Anderson 1948). The transfer of genes can increase genetic variation in the recipient species and lead to changes in adaptation and geographic range (Martinsen *et al.* 2001, Arnold 2004). Novel characteristics can be formed through the re-assortment of genes in hybrids and result in the invasion of new habitats by hybrids (Arnold *et al.* 1991, Rieseberg 1997, Rieseberg and Carney 1998, Wang *et al.* 2001, Rieseberg *et al.* 2003, Howarth and Baum 2005, Gompert *et al.* 2006). Hybridization can also result in the loss of a species. If a less abundant species is swamped by pollen or sperm from a more abundant species (Rieseberg and Carney 1998, Petit *et al.* 2003), whole genomes can be lost. However, not all hybridization results in change. Some hybrid zones appear to be stable (Barton and Hewitt, 1985) and hybrids can come and go without any discernable effect on the hybridizing species.

There have been several approaches used to study the consequences of hybridization. Cline dimensions are used to determine the extent and direction

of gene flow and the amount and type of selection (Slatkin 1973, Endler 1977, Bridle *et al.* 2001, Brennan *et al.* 2009). Barton (2001) used computer models to determine the likely fitness of hybrids. Morphological and genetic markers and population genetic techniques (Brennan *et al.* 2009) are also used to determine the structure of existing hybrid zones, continuing hybridization, the extent and direction of hybridization and introgression, and selection. Empirical studies make use of experimental manipulations such as glasshouse and transplant experiments to detect selection acting on parental and hybrid genotypes within the different environments associated with the hybrid zones. Gene mapping is used to observe gene rearrangements and determine where hybrids have originated, how they have changed genetically and how these changes relate to phenotypic expression (Rieseberg *et al.* 2003). Not all of these approaches are suitable for every hybrid zone. For example, cline dimensions are not suitable for the study of hybrid zones in which the species have a very restricted or patchy distribution (Slatkin 1973).

### 1.3 Hybrid zone models

In an attempt to predict the consequences of hybridization and describe how species can change over time, several models have been proposed which describe the main processes in the evolution or maintenance of hybrid zones. Each of the models differs in the relative type and amount of selection acting

on hybrids. The comparative fitness of hybrids and their parental species over their complete life-cycle and in the various habitats associated with the hybrid zones is likely to affect the fate of hybridizing species. Therefore, consequences of hybridization cannot be studied fully without consideration of the relative fitness of hybrids (Arnold and Hodges 1995, Rieseberg and Carney 1998).

Barton and Hewitt (1985) proposed the ‘tension zone’ model in which a stable hybrid zone was formed through a balance between hybrid inferiority and gene flow into hybrid zones from parental populations. Rand and Harrison (1989) proposed the ‘mosaic’ model in which a patchy distribution of habitat gives rise to a patchy distribution of parental forms and hybrids which inhabit unoccupied regions. In both models hybrids are less fit within all of the habitats associated with the hybrid zone. Hybrid superiority within intermediate habitat of hybrid zones, but not in parental habitats, is termed the ‘bounded superiority’ model (Moore 1977). In the ‘evolutionary novelty’ model (Arnold 1997) hybrid fitness varies in all habitats and hybrids can be as fit, or fitter, than the parental species even in parental habitats.

An ecological gradient, along which the parental genotypes vary in relative fitness (Slatkin 1973, Endler 1977), is an assumption of most hybrid zone models. Mosaic hybrid zones are of particular interest in studies of genotype–

by-environment interactions because parental species are associated with specific habitats in close proximity to one another. The different character states between species reflect the underlying selection pressures imposed by the environment (Harrison 1986). In this model it is assumed that selection against hybrids is greater than the differential success of each species in their preferred habitats. Hybrid success within parental habitats would depend on the type of alleles (hence phenotypes) carried by the hybrids. It is expected that the 'mosaic' hybrid zone will exist while there is unoccupied or intermediate habitats. Nevertheless the two species are expected to maintain their integrity and remain separate species within their preferred habitats.

Hybrid zones can be narrow and restricted by limited gene flow. Most studies on seed and pollen dispersal have been modeled on plants that are wind and insect pollinated which mainly have leptokurtic dispersal distances. *Banksia* species are predominantly pollinated by birds. Krauss *et al.* (2009) found that, although seed dispersal distances in a population of *Banksia hookeriana* were leptokurtic at a very fine scale, pollen dispersal was at a scale that corresponded to the distribution of the plants in the population. Highly mobile nectar feeding birds spread pollen randomly throughout the population (Krauss *et al.* 2009). Schibeci (1994) found considerable opportunity for inter-specific pollination between *B. robur* and *B. oblongifolia*. The two species had common pollinators including birds (mainly honeyeaters) (Taylor and Hopper,

1988; George, 1984; and small mammals (*antechinus*). Where dispersal is restricted in many species of plants, it is unlikely that *Banksia* hybrid zones will remain very narrow through restricted gene flow alone.

#### 1.4 *Types of selection and hybrid fitness in empirical studies*

Two types of selection can operate on hybrids, endogenous (intrinsic or genetic) and exogenous (extrinsic or environmental) selection. Endogenous selection that acts against hybrids (a decrease in hybrid fitness) is thought to be due to genic incompatibilities or a disruption of co-adapted gene complexes, resulting in developmental and physiological disturbances (Dobzhansky 1937, Templeton 1981, Burton *et al.* 1999). Endogenous selection that causes hybrids to be fitter than parental species, termed heterosis, is common in first generation hybrids (Anderson 1948, Rieseberg and Carney 1998). Heterosis appears to be due to either the masking of deleterious recessive genes (dominance) or other advantageous genetic combinations (overdominance and epistasis) (Mitchell-Olds 1995, Rieseberg and Carney 1998). Exogenous selection occurs when fitness varies within different habitats (Anderson 1948, Johnston *et al.* 2001, Campbell and Waser 2001, Campbell *et al.* 2008) and over time (Miglia *et al.* 2005). Recently, exogenous selection and habitat adaptation are emerging as important factors in the maintenance of hybrid zones (Nosil *et al.* 2005, Carson *et al.* 2008). Most commonly, transplant

experiments, using specific hybrid classes, are performed to separate environmental and genetic effects. Cytoplasmic DNA markers are used to separate maternal effects on fitness as mitochondrial and chloroplast genes are mainly transferred by only one parent, usually the maternal parent (except for chloroplast DNA in gymnosperms).

Empirical studies have revealed that selection can be a combination of nuclear and cytoplasmic genetic effects, and environmental effects. The processes involved in forming and maintaining hybrid zones appear to be complex and specific to the hybridizing species (Rand and Harrison 1989). Empirical studies have revealed a number of examples that appear to fit one or other of the models. For example, the ‘tension zone’ model was supported by Scopece (2008), who found that 11 species of naturally hybridizing, Mediterranean, food-deceptive orchids had reduced hybrid fitness. Support was also found in an avian hybrid zone (Mettler and Spellman 2009). The ‘bounded superiority’ model was supported by Wang *et al.* (1997) (but also see Miglia *et al.* 2005) who found that hybrids were fitter than the two parental species within the hybrid zone. An excess of fertile F<sub>1</sub> hybrids, found in some hybrid zones, could have been the result of habitat-mediated selection within hybrid zones (Milne and Abbott 2008, Kumeyama *et al.* 2008). The ‘mosaic model’ was supported in plant (Howard *et al.* 1997), fish (Costedoat *et al.* 2005) and insect (Rand and Harrison 1989) hybrid zones. However, the majority of studies

appeared to have supported the ‘evolutionary novelty’ model. Computerized simulations (Barton 2001) and empirical studies (see review by Arnold and Hodges 1995, Grant and Grant 1996, Emms and Arnold 1997, Campbell 2003, Rocas *et al.* 2004, Himrane *et al.* 2004, Carballo *et al.* 2005, Matondo *et al.* 2007, van de Sluijs *et al.* 2008, Campbell *et al.* 2008), indicated that certain hybrid genotypes can be as fit or fitter (Campbell *et al.* 2005, Facon *et al.* 2005) than their parental progenitors even within the parental habitats. In a review on plant hybridization Rieseberg and Carney (1998) found that hybrid fitness was heterogeneous within and between generations and emphasized the importance of determining fitness of individual hybrids and not analyzing them as a single group.

Hybrid fitness can be influenced by a combination of factors including ecological and genomic (nuclear and cytoplasmic) interactions. For example, first and second generation hybrid cichlid fish (van der Sluijs *et al.* 2008) and first and second generation hybrid *Ipomopsis* plants (Campbell and Waser 2001, Campbell *et al.* 2008) were as fit as their parental species; however hybrid fitness was dependent on the maternal genotype. In the *Ipomopsis* example, reduced survival occurred only in hybrids of one cytoplasmic background, indicating cyto-nuclear gene interactions, and only within the parental environment, indicating ‘complex genotype-by-environment interactions’ (Campbell *et al.* 2008). These examples illustrate that factors



affecting hybrid fitness can be complex (also see Rieseberg and Ellstrand 1993).

### *1.5 Determination of hybrid fitness in long-lived species*

Hybridization and hybrid fitness is most commonly studied in species exhibiting annual recruitment events (Grant & Grant 1994, Jiggins *et al.* 1996, Burke *et al.* 1998, Carney *et al.* 2000, Bridle *et al.* 2001, Reed *et al.* 2003, Yanchukov *et al.* 2006, Campbell *et al.* 2008), because short life-cycles allow the measurement of fitness over the whole life of the organism and over several generations. Long-lived species are underrepresented in hybridization studies as life-time fitness and temporal variation in fitness are both difficult to obtain (Rieseberg and Carney 1998). However, despite the difficulties in drawing conclusions based on performance at different stages of the life cycle and often during single events, some inferences can be made (Rieseberg and Carney 1998). Furthermore, genetic techniques can be used to determine factors such as the extent and direction of hybridization and introgression (Perron and Bousquet 1997, Howard *et al.* 1997, Petit *et al.* 2003, Muir and Schlotterer 2005, Zha *et al.* 2008) as well as selection.

The problems associated with studying long-lived species are exacerbated when recruitment is rare, episodic or unpredictable (Meyerscough *et al.* 2000, Knox and Morrison 2005). Consequently there may be few opportunities to

assess the effects of hybridization during recruitment when selection is often most intense. Indeed, very long-lived plants may be slow to respond to selection (Ackerly *et al.* 2000) apart from recruitment events.

### 1.6 *Banksia* species

*Banksia* species are woody, evergreen plants that range from prostrate shrubs to 25 meter tall trees and all but one species are endemic to Australia (George 1984, Mast and Thiele 2007). Some species have a canopy-stored seed bank that is released, *enmasse*, after fire. This mass seed release usually produces cohorts of seedlings of the same age. As mass recruitment depends on fire, recruitment events are unpredictable and often infrequent. Other *Banksia* species shed seed as it matures. Some *Banksia* species re-sprout from lignotubers after fire (George 1984). They are slow growing and may persist over long periods of time creating populations comprising many cohorts. Re-sprouting aids in the persistence and the potential spread of genotypes throughout populations. In other species, adults die even in low intensity fires, and persistence of the population depends on a single cohort of recruitment from the seed bank (George 1984).

Some *Banksia* species hybridize readily and natural hybridization has been observed between species that coexist, especially in Eastern Australia. (George 1984, Taylor and Hopper 1988, Schibeci 1994). Hybridizing species include

*B. oblongifolia* x *B. robur*, *B. paludosa* x *B. marginata*, *B. paludosa* x *B. integrifolia* var. *integrifolia* and *B. oblongifolia* x *B. integrifolia* var. *integrifolia* (Taylor and Hopper 1988). *B. paludosa* is closely related to *B. oblongifolia* and *B. robur* (Mast and Givnish 2002) and is found in close proximity to both species in the study area. *Banksia oblongifolia*, *B. robur* and *B. paludosa* all re-sprout from lignotubers and broadcast seed after fire from a canopy-stored seed bank.

#### 1.7 *Banksia oblongifolia* and *Banksia robur*

*Banksia oblongifolia* and *B. robur* are two closely related species (Mast and Givnish 2002) that hybridize readily where they co-exist (Turner 1976, Elphinstone 1980, George 1984, Taylor and Hopper 1988, Schibeci 1994). Hybrid zones between *B. oblongifolia* and *B. robur* occur intermittently along 1500km of the east coast of Australia. A putative hybrid of intermediate morphology between *B. oblongifolia* and *B. robur* was collected in 1793 (see George 1984), and the hybrid zones existed well before European settlement over 200 years ago. *Banksia robur* plants have large shiny leaves, and *B. oblongifolia* plants have a greater number of smaller, thinner leaves on more branches than *B. robur*. The difference in leaf morphology was found to be genetically inherited (Schibeci 1994).

The origin of the hybrid zones is unknown. Each species could have adapted to different environments in allopatry, and converged again after population expansion due to climatic changes (Mayr 1963, Hewitt, 2001). Alternatively, the two species could have experienced divergent selection for extreme characters in sympatry (Endler, 1977; Bolnick and Fitzpatrick, 2007). Perhaps there has been a mixture of the two extremes with divergence starting in allopatry and continuing in sympatry or divergence starting in allopatry and convergence continuing in sympatry. This dilemma is unlikely to be resolved (Endler, 1977), but it is possible to make inferences about the present state and fate of hybrid zones by studying the genetic structure and fitness of plants within them.

### *1.8 Study area and hybrid zone habitats*

The study area was located within the O'Hares Creek Catchment, on the Woronora Plateau near Darkes Forest (34°14'S 150°54'E), 45km southwest of Sydney. Davis (1941) described the vegetation as a mosaic of moorlands and eucalypt woodlands and forests (see Keith and Myerscough (1993) for a detailed description of the study area). One site (Darkes Forest) is protected by National Parks and the other site (Appin Road) is protected by Sydney Water Catchment Authority. The two sites chosen for this study are both on inclines. The Darkes Forest site is gently sloping from eucalypt woodlands down to an

upland swamp. The Appin Road site is steeper from eucalypt woodlands to a small deep creek.

Soil groupings (Young 1986) and floristic composition, including the *Banksia* species, are closely coincident with the gradient of soil moisture (Keith and Myerscough 1993). *Banksia oblongifolia* is found in sands on drier upper slopes and *B. robur* is found in black organic fine soils in wet seepage zones. Between the two habitats, intermediate slopes of grey-black organic soils and intermediate seepage zones are found (Keith and Myerscough 1993). As the hybrid zones are composed of a mosaic of parental and intermediate habitats it is difficult to assess whether either species or their hybrids occupy intermediate habitat.

### 1.9 Previous studies

Information about *B. robur* and *B. oblongifolia* populations has been compiled over the range of the two species (Taylor and Hopper, 1988; George, 1984) and hybrid zones within the same study area have been studied in detail (Schibeci 1994, Elphinstone 1980, Turner 1976). *Banksia oblongifolia* and *B. robur* plants from hybrid zones and pure populations, as determined by morphology, have been studied to determine whether they are separate species and whether they form genetic hybrids (Turner 1976, Elphinstone 1980, Schibeci 1994). Leaf phytochemistry analyses (Turner 1976) revealed

phenolics exclusive to each of the two species. A combination of parental phenolics and unique phenolics were found in plants exhibiting intermediate morphology (Turner 1976).

Elphinstone (1980) tested two allozyme markers and found them to be identical for the two species. Viability of seeds and pollen was the same for both species and intermediate forms. In the Elphinstone (1980) study, apart from morphological differences, there was no evidence to separate the two species. It has since been established that these closely related species have low allozyme variability (Schibeci 1994); however it is interesting that Elphinstone (1980) did not find any difference between the two species or intermediate forms in viability, suggesting that these hybrids were as fit as the parental species.

Schibeci (1994) used morphometric and allozyme markers to characterize the two species and their hybrids. Her findings (Table 1.1) suggested that hybridization was extensive and that fertile hybrids had produced a range of hybrid genotypes including putative  $F_1$  hybrids and backcrossed individuals. Morphological characters separated the two species into two identifiable groups. Morphology of mature plants was a good indicator of genotype and hybrids were predominantly, but not exclusively, of intermediate morphology. *Banksia robur* and *B. oblongifolia* were similar to other Proteaceous plants

(Carthew *et al.* 1988; Coates and Sokolowski 1992; Ayre *et al.* 1994) in that they had low allozyme variability (Schibeci 1994) which restricted the identification of some hybrids and hybrid classes.

(Schibeci 1994) found no direct evidence for hybrid inferiority or infertility. *B. oblongifolia*, *B. robur* and hybrid plants were categorized using both genetic and morphological indices scores and four key characteristics were used to assess survivorship, vigour and fecundity (Schibeci 1994). In the Schibeci (1994) study there was no one group showing significantly greater vigour or fecundity as measured by the proportion of *B. robur*, *B. oblongifolia* and hybrid plants, plant height, the total inflorescences produced and mean number of follicles per infructescence. However there was indirect evidence for hybrid inferiority in the Schibeci (1994) study. There were heterozygote deficits in the hybrid zones but not in parental populations indicating fewer hybrids than expected under Hardy-Weinberg equilibrium. Linkage disequilibrium was detected in the hybrid zones but not in the parental populations and high levels of effective selection pressure ( $s^*$ ) was found in the hybrid zones, but not in parental populations (Schibeci 1994). However, Schibeci (1994) also found that the hybrid zones were not simple clines, but were a complex mixture of genotypes, which could have confounded the use of cline parameters to estimate selection.

Table 1.1 Summary of past findings from Schibeci (1994) concerning hybrid zones between *B. oblongifolia* and *B. robur*

Questions	Findings
Is there hybridization between the two species, or are intermediate forms due to phenotypic plasticity?	There is hybridization and hybrids are genetic hybrids.
Is there evidence for reinforcement of reproductive barriers within contact zones?	No, there is no difference in flowering times between mixed stands or pure stands, except that in mixed stands, there is greater overlap due to hybrid plants of intermediate flowering times. No pollinator preferences were obvious.
Are hybrids fertile?	Hybrids are fertile. Backcrossing and introgression were confirmed.
Is there evidence for selection against hybrids?	<p>Heterozygote deficits, linkage disequilibrium and high coefficient of effective selection (<math>s^*</math>) within hybrid zones provide indirect evidence of selection against hybrids.</p> <p>No selection against hybrids was observed after establishment within the hybrid zone.</p> <p>Parental types were found within specific regions.</p>
What is the origin of the hybrid zones?	Still speculative. The zone appears to be environmentally dictated. Lack of genetic variation within and between species suggests recent divergence.



Schibeci (1994) found that peak flowering times for each of the two species were closer to one another within contact zones than in allopatric stands. Floral phenology could have been affected by the more variable environment of the contact zone and differences in the availability of resources, by a change in the number of flowers and size of plants (Lamont *et al.* 2003). Alternatively undetected introgression could have contributed to the closer flowering times within the contact zones, as the flowering times of hybrid plants were intermediate between the two species (Schibeci, 1994). Even though the flowering times of the two species overlapped, their peak flowering times differed (Schibeci, 1994; Taylor and Hopper, 1988; George, 1984) resulting in some restriction of gene flow between species. Even though peak flowering times were closer within the hybrid zones than pure species populations, the difference in floral phenology probably still acted to restrict hybridization and increase reproductive isolation between the two species, especially if the phenology was highly heritable and coupled with ecological preferences (Templeton 1981, Hendry and Day 2005, Gerard *et al.* 2006).

In conclusion, there was evidence to suggest that the two species were associated with specific environments and there was indirect evidence to support hybrid disadvantage. *B. robur* and *B. oblongifolia* hybrid zones appear

to fit the 'mosaic' hybrid zone model. Hybrids occur where the two species and their preferred habitats converge often in a patchwork of parental and intermediate habitats. Models using cline widths to ascertain selection may not be suitable for this system as populations of *B. robur* are often narrow and restricted to small areas along creeks or within swamps. The hybrid zones themselves are very narrow and a mosaic of habitat types (Schibeci 1994). Transplant experiments which take into account microhabitats could clarify many of the questions regarding selection within the hybrid zones.

The use of intermediate morphology to classify hybrids in past studies could have resulted in some misclassification of hybrids with parental morphology. Even when allozyme markers were used to detect hybrids, low variation prevented accurate classification and probably identification of hybrids. Variable genetic markers are needed to accurately classify hybrids.

#### 1.10 *This Study*

Two recent developments have allowed a unique opportunity to study the dynamics of *Banksia* hybrid zones in more detail, and to study selection amongst seedlings during a recruitment event. First, an extensive wildfire, which burned through sites containing *B. robur* and *B. oblongifolia* and their hybrids, provided the opportunity to seek evidence for recruitment of hybrid individuals from the seed bank and to study selection amongst seedlings of the

same generation and age group (avoiding the complications arising from age structure, Arnold and Wade 1984).

Second, I developed primers for microsatellite markers (Usher *et al* 2005) providing greater variation than allozyme markers and allowing me to more completely describe the genetic composition of these hybrid zones. Traditionally, morphological markers have been used to distinguish between species and detect hybrids. However, intermediate morphology is not always a suitable predictor of hybrid origin. A review of literature on plant hybridization (Rieseberg and Ellstrand 1993) revealed that hybrids generally, and even first generation hybrids, tended to exhibit a mosaic of parental and intermediate characteristics. Plant phenotypes, in particular, can vary even within species, when influenced by environmental variation (Bradshaw 1965, Sultan and Bazzaz 1993a,b,c, Ackerly *et al.* 2000, Donohue *et al.* 2001). Moreover, plants often show greater phenotypic plasticity to environmental stress (Ackerly *et al.* 2000, Close *et al.* 2003, Dudley 2004). Therefore, the use of morphology to predict hybridization can lead to an under-estimation of hybrid individuals.

Molecular markers allow hybrids to be detected at a genetic level and are now commonly used in conjunction with morphology. Some advantages of molecular markers over morphological markers include low levels of non-

heritable variation or phenotypic variation (Hillis 1987, Rieseberg and Wendle 1993) and often selective neutrality (Kimura 1982, Rieseberg and Wendle 1993).

Several elements of hybrid fitness should be considered. Hybrid fitness can vary between generations and between hybrid classes. Here I compare a cohort of seedlings from the same generation. Rieseberg and Carney (1998) pointed out that pooling of hybrid data even if individuals are from the same hybrid class could lead to erroneous conclusions. Therefore, in this study, I examine both the distribution of individual hybrid performance measurements and the mean hybrid performance and compare them with the mean for the parental species.

It is important to determine fitness within a natural setting as fitness is often habitat-dependent (Rieseberg and Carney 1998, Campbell and Waser 2001). Hybrid zones are often found where different environments come together and plants are particularly useful in elucidating the strength and type of selection operating within hybrid zones because they can easily be transplanted into different habitats (Linhart and Grant 1996, Wang *et al.* 1997, Campbell and Waser 2001). In this study I transplant seed taken from across the *Banksia* hybrid zones into parental species habitats and intermediate habitats.

To determine the effect of field conditions generally on seedlings of all genotypes and to determine whether there is evidence for endogenous selection when environmental variation is kept to a minimum, I raised seedlings in pots under more uniform conditions, away from the natural setting, and compared their germination, survival and growth between genotypic groups and with that of seedlings in the field.

Although measurement of life-time fitness is important as fitness can vary during different stages of the life-cycle and it is overall fitness that affects reproductive success, it is not possible to study life-time fitness in these long-lived species. Nevertheless selection is likely to be most intense during the recruitment stage of development because of the genetic diversity created amongst seedlings and often high mortality in the first few years of existence (Lytle and Merritt 2004). In this study, I assess the performance of cohorts of seedlings from seed dispersed after fire. Germination and survival are measured along with indirect performance measurements such as growth.

Herbivore damage can potentially affect plant fitness. Herbivore attack has been found to vary between hybridizing species and their hybrids and even between hybrid genotypes (Rieseberg and Carney 1998, Yarnes *et al.* 2008) due to differences in the biosynthesis of chemical defenses (Yarnes *et al.*

2008). In this study herbivore damage was estimated and compared between the various genotypes occupying the *Banksia* hybrid zones.

To make further inferences about production and survival of seedlings from the various genotypes found in the hybrid zones, I assessed the genetic structure of established plants and cohorts of seedlings using genetic markers and population genetic techniques. Here I examined the genotypes of seedlings from maternal plants of known genotypes, which allowed me to make inferences about selection from the very early stages of recruitment.

#### 1.11 *Aims of thesis*

The aim of this study is to determine whether or not the *Banksia* hybrid zones fit the ‘mosaic’ model among seedlings after recruitment to two years of age. That is, are the *Banksia* hybrid zones maintained by a balance between gene flow, intrinsically unfit hybrids and the availability of unoccupied or intermediate habitats?

The null hypothesis of this thesis is that there is no difference in seedling establishment and range of performance measures between *B. oblongifolia* x *B. robur* hybrids and their parental species within parental and intermediate habitats associated with the *Banksia* hybrid zones during recruitment after fire.

To test this, I aimed to:

1. develop microsatellite markers for *B. oblongifolia* and *B. robur*.
2. determine the effectiveness of the microsatellite markers and morphological characters in distinguishing pure populations of the two species and in detecting hybrids amongst mature plants and amongst seedlings.
3. establish the genetic and geographic distribution of mature plants and seedlings across the different habitats associated with hybrid zones.
4. compare the capacity of the two species and their hybrids to re-sprout after fire
5. look for evidence of endogenous or exogenous selection during recruitment of seedlings across the hybrid zones.
6. determine whether there evidence for endogenous selection when environmental variation is kept to a minimum.
7. test whether the environment of maternal plants affect seedling performance.
8. estimate the extent and direction of hybridization during the recent recruitment event.

9. determine whether selection is acting either directly or indirectly on leaf morphology across the hybrid zones.



## *Chapter 2*

### **Microsatellites for Eastern Australian *Banksia* species**

Primer Note printed in Molecular Ecology Notes:

Usher AV, Ayre DJ, Whelan RJ (2005) Microsatellites for eastern Australian *Banksia* species. *Molecular Ecology Notes*, **5**, 821-823.

Shrubs and small trees of *Banksia* species are prominent in southern Australian forests and woodlands (George 1984). They have been the focus of many studies, including processes facilitating hybridization (Lamont *et al.* 2003). *Banksia robur* and *B. oblongifolia* are two species known to hybridize, in sites where they co-occur in eastern Australia (George 1984).

Natural hybrid zones provide a powerful opportunity to investigate speciation processes, and studies of hybridization are being facilitated by the power of variable genetic markers to detect different combinations of hybrid genotypes (Arnold and Hodges 1995). Australian shrubs in the family Proteaceae, such as *Banksia*, have shown little allozyme variation (Carthew *et al.* 1988, Coates and Sokolowski 1992), so highly variable, codominant, microsatellite markers are needed to provide sufficient power to investigate the genetic structure of

complex hybrid zones. I developed microsatellite markers for *B. robur* and *B. oblongifolia* as part of a larger study to investigate the dynamics of the hybrid zone between these species and to test the relative fitness of different hybrid genotypes.

DNA was extracted from 5 plants displaying *B. robur* morphology and 5 plants displaying *B. oblongifolia* morphology, using a Qiagen DNeasy® Plant Mini Kit. The DNA from each species was pooled and used in the isolation of microsatellite markers. The protocol MsatMan2000 Travis Glenn ([www.uga.edu/srel/DNA\\_Lab/Msat\\_Easy\\_Isolation\\_2000.rtf](http://www.uga.edu/srel/DNA_Lab/Msat_Easy_Isolation_2000.rtf)) was used. The DNA was cut with RsaI, ligated to dsSNX linkers, hybridized to biotinylated microsatellite oligonucleotides (30bp CA, GA, ATT and CAT probes), captured on Streptavidin MagneSphere® Paramagnetic Particles (Promega) beads and unwanted DNA washed away. Captured DNA was recovered by polymerase chain reaction (PCR) with SNX-f and cloned using the pGEM®-T Easy vector system (Promega). Positive colonies were amplified using M13 primers, and PCR products were sent to Westmead Millennium Institute, DNA sequencing facility, Sydney, to be sequenced. Microsatellite primer pairs were designed using Primer3 (Rozen and Skaletsky 2000).

Primer optimization was performed in a 20µL volume using an Eppendorf Mastercycler gradient thermocycler. PCR final concentrations were, 2µL of

10X buffer supplied with taq (Promega), MgCl<sub>2</sub> (1.5mM - 4.5mM), 200μM for each dNTP, 1 unit of Taq (Promega), 10pmol of primers, and ~ 60ng DNA template. The thermocycler parameters were, 5min at 94° C then 35 cycles of 30s at 94° C, 30s at annealing temperatures (45° C to 65°C), and 1min at 72 °C followed by 5min at 72 °C. PCR products were scored on a 2% agarose gel.

To screen for variable loci, I genotyped six *B. oblongifolia* and six *B. robur* plants using optimal annealing temperatures and 2mM MgCl<sub>2</sub> plus 1μM dUTP fluorescent dye (Rhodamine Green™-5 Molecular probes, Bioscientific) and sized on an ABI 377 sequencer. Results were analyzed using GENESCAN and GENOTYPER software (Applied Biosystems). Eight loci were selected to genotype individuals from 4 populations of *B. oblongifolia* (total of 40 plants) and 4 populations of *B. robur* (38 plants). I suspected that *B. paludosa* might also hybridize with *B. oblongifolia*, so we included 3 populations of *B. paludosa* (27 plants). I found that all eight loci could be amplified at an annealing temperature of 50 °C for 35 cycles, and all readily PCR multiplexed with the exception of Br3.

All eight loci were polymorphic in each species, with the exception of two loci in *B. robur* (Table 2.1). Numbers of alleles ranged from 1 to 13 across the three species. Heterozygosities were calculated for each population using GenAlEx (Peakall and Smouse 2006) and then averaged for each species. The

average heterozygosities ranged from 0.000 to 0.833. Heterozygosity varied between populations within species, probably as a result of small sample sizes. However, heterozygote deficits were consistent across *B. robur* populations at locus Br3 (Fixation Indices = 0.130 to 0.467) and across *B. paludosa* populations at locus Br 23 (FI = 0.294 to 0.529). The large heterozygote deficits at locus Bo16, in *B. robur* (FI = 0.111 to 0.586) and *B. paludosa* (FI = 0.775 to 1.00) populations, along with a lack of amplification in some instances, could indicate null alleles. Linkage disequilibrium tests using GENEPOP (Raymond and Rousset 1995) indicated no linkage disequilibrium ( $P < 0.01$ ) between any pair of loci within populations. At least four loci separated *B. robur* from *B. oblongifolia*, three loci separated *B. oblongifolia* and *B. paludosa*, and one locus separated *B. robur* and *B. paludosa*. Across the eight loci, 12 alleles were unique to *B. oblongifolia*, 21 were unique to *B. paludosa* and 5 were unique to *B. robur* (Table 2.1).

We also tested amplification of the eight primer pairs in three other *Banksia* species that occur in the same region: *B. spinulosa* (50 plants), *B. serrata* (one plant) and *B. ericifolia* (3 plants). Seven of the eight loci amplified in at least two species (Table 2.1).

Table 2.1 Primer details for eight *Banksia* microsatellite loci developed with *Banksia oblongifolia* (Bo) and *Banksia robur* (Br) DNA; allelic diversity for the hybridizing species, and positive amplification in three other local species.

Locus	Repeat	Primer Sequence (5'-3')	Hybridizing species	Range of allele sizes (bp)	No. alleles	No. indiv (pops)	MeanHo/Pop	Mean He/Pop	Amplification in other local <i>Banksia</i> species (range of allele sizes bp)
Bo3	(AG) <sub>11</sub>	<b>F:</b> AGATGGAGGTGGATGGTCTG <b>R:</b> GTTAACTTGCGCCGCTTTAG	<i>B.oblongifolia</i> <i>B.robur</i> <i>B.paludosa</i>	141-145 145-149 138-157	3 3 5	29(4) 35(4) 21(3)	0.554 0.485 0.648	0.504 0.464 0.642	<i>B. serrata</i> (134) <i>B. spinulosa</i> (151-177) <i>B. ericifolia</i> (150-168)
Bo7	(AG) <sub>11</sub>	<b>F:</b> GGCAAGCACTTTACCCATGT <b>R:</b> GATAACTTAGAAAGAGACACATCC	<i>B.oblongifolia</i> <i>B.robur</i> <i>B.paludosa</i>	142-151 151-161 142-165	4 5 7	30(4) 36(4) 21(3)	0.135 0.572 0.456	0.202 0.648 0.662	none
Bo16	(AG) <sub>9</sub>	<b>F:</b> GAACGGGTGCAACTGAGAAT <b>R:</b> TCCTAATGCACTGTCCCACA	<i>B.oblongifolia</i> <i>B.robur</i> <i>B.paludosa</i>	127-132 125-134 120-159	5 6 4	30(4) 27(4) 15(3)	0.632 0.281 0.042	0.573 0.411 0.458	<i>B. serrata</i> (143) <i>B. spinulosa</i> (138-164) <i>B. ericifolia</i> (126)
Bo17	(AG) <sub>4</sub> C (AG) <sub>7</sub>	<b>F:</b> CAACAAATTTCCGCCCTAAA <b>R:</b> CGTCCACCTGAAATATGGAGA	<i>B.oblongifolia</i> <i>B.robur</i> <i>B.paludosa</i>	89-91 87 87-93	2 1 multiple bands	30(4) 36(4) 22(3)	0.351 0.000 0.000	0.290 0.000 0.000	<i>B. serrata</i> (83) <i>B. spinulosa</i> (83-91)
Bo22	(AG) <sub>10</sub>	<b>F:</b> GCTCGAGTATTCGACCCAAA <b>R:</b> TTTGAATGCCTCCATTCCTC	<i>B.oblongifolia</i> <i>B.robur</i> <i>B.paludosa</i>	220-224 220 220-224	3 1 3	30(4) 34(4) 22(3)	0.648 0.000 0.111	0.539 0.000 0.208	<i>B. serrata</i> (223) <i>B. spinulosa</i> (212-218) <i>B. ericifolia</i> (217)
Br3	(AG) <sub>8</sub> G (AG) <sub>8</sub>	<b>F:</b> GGTCTCCACATGGGGTTTTA <b>R:</b> CATAGAGGTGGTGGGGACAC	<i>B.oblongifolia</i> <i>B.robur</i> <i>B.paludosa</i>	150-156 138-152 132-154	4 7 10	30(4) 33(4) 22(3)	0.592 0.396 0.611	0.469 0.567 0.740	<i>B. serrata</i> (133) <i>B. spinulosa</i> (136-158)

Locus	Repeat	Primer Sequence (5'-3')	Hybridizing species	Range of allele sizes (bp)	No. alleles	No. indiv (pops)	MeanHo/Pop	Mean He/Pop	Amplification in other local <i>Banksia</i> species (range of allele sizes bp)
Br13	(AG) <sub>13</sub>	<b>F:</b> TAGCCCAATCAAAACCGAAC <b>R:</b> CTTTGTGCTGAATCCCCTCAG	<i>B.oblongifolia</i>	173-181	5	30(4)	0.238	0.370	<i>B. serrata</i> (238-248)
			<i>B.robur</i>	167-169	2	35(4)	0.292	0.262	<i>B. spinulosa</i> (220-240)
			<i>B.paludosa</i>	167-181	7	22(3)	0.833	0.712	<i>B. ericifolia</i> (224-228)
Br23	(AG) <sub>9</sub> AA (AG) <sub>3</sub>	<b>F:</b> GCGAGGAAAATCAAAATGGA <b>R:</b> CTACGGTGGCTACTCCCAA	<i>B.oblongifolia</i>	219-232	6	30(4)	0.417	0.520	<i>B. serrata</i> (366)
			<i>B.robur</i>	215-229	2	35(4)	0.087	0.082	<i>B. ericifolia</i> (231)
			<i>B.paludosa</i>	219-244	7	22(3)	0.411	0.684	

H<sub>o</sub>, observed and He, expected heterozygosity. GenBank Accession nos AY953295-AY953318 (includes unused sequences)

## *Chapter 3*

### **Window of opportunity: An episode of recruitment in a Banksia hybrid zone demonstrates continuing hybridization and phenotypic plasticity**

Printed in *Annals of Botany*:

Usher AV, Whelan RJ, Ayre DJ (2010) Window of opportunity: an episode of recruitment in a *Banksia* hybrid zone demonstrates continuing hybridization and phenotypic plasticity.

*Annals of Botany* **105**, 419-429.

#### *3.1 Introduction*

Hybrid zones are often viewed as dynamic systems in which speciation and introgression (the incorporation of genes from one species into another) can proceed rapidly (Ungerer *et al.* 1998, Arnold 2004, Gross and Rieseberg 2005, Mallet 2007, Bunje *et al.* 2007). An understanding of the processes leading to evolutionary change during hybridization requires knowledge of the frequency of interbreeding between the hybridizing species, and the intensity of selection

operating on the hybrids and their parental species. In many published studies on both plants and animals, hybrids are relatively easy to recognize morphologically, and hybrid fitness can therefore be evaluated within annual recruitment events (Grant and Grant 1994, Jiggins *et al.* 1996, Burke *et al.* 1998, Carney *et al.* 2000, Bridle *et al.* 2001, Campbell 2003, Reed *et al.* 2003, Yanchukov *et al.* 2006). However, there are other systems in which hybrid and parental phenotypes are not easily distinguishable (Hopper 1977, McKinnon *et al.* 2001) and in which recruitment of hybrids can be rare or episodic (Myerscough *et al.* 2000, Knox and Morrison 2005). Moreover, the phenotypes of plants can vary in response to environmental variation (Bradshaw 1965, Sultan and Bazzaz 1993a,b,c, Ackerly *et al.* 2000, Donohue *et al.* 2001), and in response to environmental stress, such as drought (Ackerly *et al.* 2000, Close *et al.* 2003, Dudley 2004). An understanding of the dynamics of hybridization within mixed stands of *B. oblongifolia* and *B. robur* therefore requires evaluation of the capacity of genetic and morphological data to identify the two species and their hybrids in the field, and upon having the opportunity to study an episode of recruitment.

Morphological intermediates between the monoecious shrubs, *Banksia robur* and *B. oblongifolia* (Figure 3.1), occur across a series of widely distributed swamps along the east coast of Australia (Taylor and Hopper, 1988). Hybridization may have been occurring for a long time, because a specimen



collected in 1793 has intermediate morphology (George, 1984). However, although Schibeci (1994) used allozyme data to provide evidence that hybrid plants are present, the composition and dynamics of these zones remained obscure. Only adult plants were present within her study sites; and the relatively low variation revealed by allozyme markers (Schibeci, 1994) has meant that we have had little capacity to quantify the composition of hybrids within the hybrid zones. Moreover, individual plants may be very long-lived having the capacity to re-sprout from lignotubers after fire destroys the canopy. Seed stored in the canopy is usually released only after fire, which can be infrequent and has occurred only once in the last 20 years in the study area. Very long lived plants may be slow to respond to selection (Ackerly *et al.* 2000) particularly if selection occurs mainly during recruitment events.



Figure 3.1 *Banksia* seedlings showing different phenotypes with *B. robur* on the left, *B. oblongifolia* on the right and intermediate forms are in the middle.

Two developments have allowed a unique opportunity to study the dynamics of the *Banksia* hybrid zones in more detail. First, an extensive wildfire, which burned through two sites containing *B. robur* and *B. oblongifolia* and their putative hybrids, has provided the opportunity to seek evidence of recruitment of hybrid individuals from the seed bank. Second, the development of primers for microsatellite markers (providing greater variation than allozyme markers),

allowed me to more completely describe the genetic composition of these hybrid zones. Importantly, this recruitment event also allowed a test of the match between seedling morphology and genotype within variable and invariable environments, which will facilitate future studies of this hybrid zone.

I had several interrelated aims;

(1) To determine the effectiveness of microsatellite markers and morphological characters in distinguishing pure populations of two species of *Banksia*, *B. robur* and *B. oblongifolia*.

(2) To determine the genotypic composition of sets of mature plants and recruits within two *Banksia* hybrid zones.

(3) To determine whether or not mature hybrid plants and seedlings exhibit intermediate morphology and hence whether morphology can be used to identify hybrids.

(4) To determine the effect of environment on seedling morphology by comparing the morphology of seedlings grown in pots with those found growing naturally within the hybrid zone

In this study, I first categorised the microsatellite genotypes and morphology of mature plants in apparently pure and mixed stands. Second, I categorised the genotypes and morphology of seedlings growing in mixed stands, and of seedlings grown in pots. The seeds for these latter seedlings were taken from plants of known maternal genotype growing across the mixed stands.

### 3.2 *Materials and Methods*

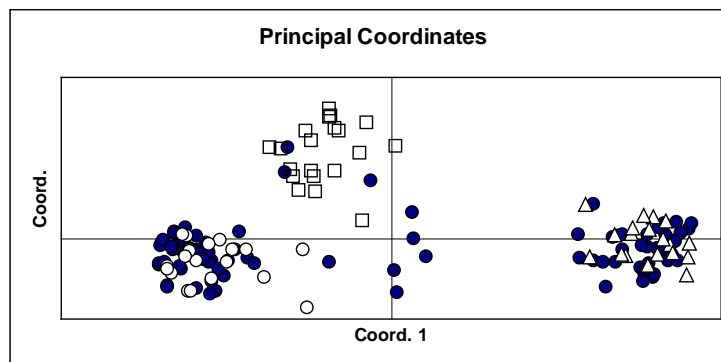
#### 3.2.1 *Study area*

The study area is located within the O'Hares Creek Catchment, on the Woronora Plateau near Darkes Forest (34°14'S 150°54'E), 45km southwest of Sydney. The site has been described as a mosaic of moorlands and eucalypt woodlands and forests (Davis 1941, Keith and Myerscough 1993). *Banksia robur*, bearing large shiny leaves, grows in the upland swamps and *B. oblongifolia* bearing smaller, more numerous leaves, grows along swamp margins and in surrounding eucalypt woodland. The study area contained pure and mixed stands of *B. oblongifolia* and *B. robur*.

*Banksia paludosa* plants, often found in close proximity to *B. oblongifolia* and *B. robur*, were found on the periphery of one of the hybrid zone sites in *B. oblongifolia* habitat. The genetic composition and morphological appearance

(Figure 3.2) of four mature plants revealed that *B. paludosa* may also hybridize with the other two species. Therefore, these plants and their seedlings were excluded from this study.

A



B

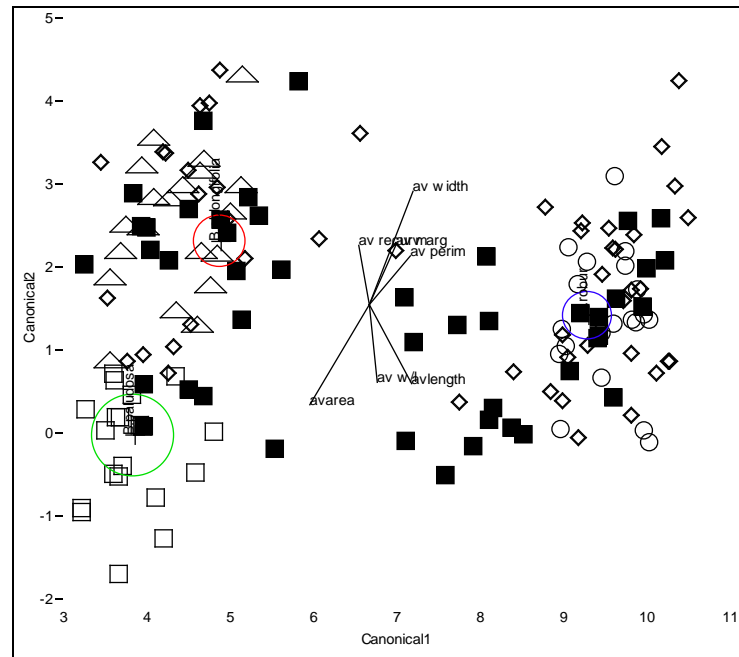


Figure 3.2 A. Genetic and B morphological distinction between mature plants in *B. robur* (o), *B. oblongifolia* ( $\Delta$ ) and *B. paludosa* ( $\square$ ) pure populations and mature plants within the two hybrid zones ( $\bullet$ ). Circles show 95% confidence limit for the mean.

### 3.2.2. Mature plant samples

To determine whether the mature plants of *B. oblongifolia* and *B. robur* were genetically distinct, and to measure the morphological variation within the pure

stands, I used morphology to select two large, apparently pure stands of each species. In 2005, after three years of re-growth from lignotubers, I collected leaves from ten plants from each stand (for a total of 20 plants from each species); all towards the centre of each stand (at least 300m from plants of the other species).

To determine the genetic and morphological composition of mature plants within *Banksia* hybrid zones, and to determine whether hybrid plants exhibit intermediate morphology, I sampled two mixed stands in 2005, including 45 plants at one site and 48 plants at the other site. I collected plant tissue and leaves for genetic and morphometric analyses.

### 3.2.3 *Seedling samples*

I was able to visit the two hybrid zones so soon after fire in 2002 that seeds had still not been broadcast from the burned fruits, though they were readily removed from follicles. I collected seeds from a random selection of plants (which we subsequently genotyped) across the hybrid zones and raised them in pots. After the fire, the above-ground biomass of mature plants consisted only of burnt stems and canopy-stored fruits. Consequently, during seed collection, I was unable to use leaf morphology to indicate hybrid plants. Some 840 of the seed that I collected were placed in seedling trays to germinate. After 2 months, the seedlings that germinated (70%) were potted into 6 cm pots and

kept in a glasshouse for 4 months. They were then re-potted into 12.5 cm pots and placed in the open for the next 8 months. At this time they became too big for the pots so they were removed and measured. As the maternal genotypes were already determined, I selected 31 seedlings, 15 at random from *B. oblongifolia* and 15 from *B. robur* maternal plants along with one seedling selected from a hybrid plant.

I determined the genotype and the morphology of each seedling that developed in the glasshouse trail and of a subset of 53 seedlings growing within the hybrid zones, three years after the fire. These seedlings, growing within the hybrid zone, were selected randomly (maternal genotypes were unknown) from across the width of the hybrid zones along one metre transects. Although the seedlings raised in pots were measured almost a year and a half before the wild seedlings, they were at a more advanced stage of development in terms of overall size and branching.

#### 3.2.4 Analysis of microsatellite data

For genetic analysis, we collected tissue from new leaves and stored it at -80°C. Seven microsatellite loci, Bo3, Bo7, Bo17, Bo22, Br3, Br13, Br23, were used to genotype the plants collected for this study. The molecular markers and genotyping methods are described in Usher *et al.* (2005). The average number of alleles per locus was 3.6 for both *B. oblongifolia* and *B. robur*. Each



species had a unique set of alleles at five of the seven loci, and I detected both shared and unique alleles at the remaining two loci.

As a measure of genetic distance between the two species, Nei's genetic distance and  $F_{ST}$  values were calculated using the population genetics software GenAlEx 6 (Peakall and Smouse, 2006). To visualize the genetic grouping of plants in pure stands, in hybrid zones and in potted and field seedlings, PCA plots were generated using the same software. The pure stand plants were included in the analysis of the hybrid zones mature plants to compare the genetic composition of pure and mixed stands.

I used the Bayesian methods implemented in the software STRUCTURE version 2.0 (Pritchard *et al.*, 2000) to perform population assignment of individual plants and to identify putative hybrid individuals. This model-based clustering method uses multilocus data to assign individuals to clusters. Probabilities were used to assign individuals to one of two populations ( $K = 2$ ). The program was run with 10 000 iterations as the burn-in period, and 100 000 iterations for Markov chain convergence. Plants were assigned as hybrids if they had an assignment value ( $q_i$ ) of less than 0.90. Once hybrids were identified, the individual genotypes of hybrids were scrutinized to determine the origin of alleles at each locus. Pure populations of *B. oblongifolia* and *B. robur* proved to have fixed differences at five of the seven microsatellite loci

and it was possible to infer whether hybrids were most probably  $F_1$  hybrids (displaying an allele from each species at each locus), backcrossed (more similar to one of the parental species), or later generation hybrids (with some loci only having alleles unique to one species and some loci only having alleles unique to the other species).

### 3.3.5 *Morphology*

The same plants selected for genetic analysis were used for morphological analysis. For each mature plant, four mature leaves were collected from different branches and from each direction of the compass (following the method of Schibeci, 1994). For each seedling we measured the four largest leaves. The leaves were scanned, while still fresh using a flatbed scanner, and the length, width (at the widest section), perimeter, and area were measured using NIH Image software (<http://rsb.info.nih.gov/nih-image/Default.html>) for Macintosh computers. The number of veins that curved away from the margin of the leaf and the number of veins that ended on the margin of the leaf were also counted along one side of each leaf. The width (at the widest point) and length ratio was used to represent leaf shape. Measurements of four leaves from each plant were averaged for analysis.

A one-way ANOVA analysis was performed on each leaf measurement for both pairs of pure stands from each species to test the significance of inter-

stand variation in individual leaf measurements (SAS - JMP 5.1). A canonical discriminant analysis (CDA) (SAS - JMP 5.1) was performed to determine the power of all leaf measurements to distinguish between the two species and hence to determine whether it is possible to distinguish hybrids of intermediate morphology from pure species. Plants with a probability of assignment to the parental species of less than 0.90 were assigned as hybrids. Before analysis, plants were classified into species (the classification variable) according to their genotype. As with the genetic analysis, the pure stands were included in the analysis of the hybrid zones mature plants, to improve the accuracy of the assignment inference and to determine any phenotypic differences between pure and mixed stands.

### *3.3.6 Genetic and Morphometric Correlations*

To determine the relationship between morphology and genotype of mature plants in pure stands and in mixed stands and to determine the relationship between morphology and genotype of seedlings in the field and in pots, Mantel tests were performed (permutations,  $n = 999$ ) using the software 'GenAlEx 6' (Peakall and Smouse 2006). The first two canonical points from the morphological discriminant analysis (SAS - JMP 5.1) were used in the place of geographic distances in the GenAlEx Mantel test. Tests were also performed without genetic hybrids, to determine whether any change in the

correlation between morphology and genotype between treatments was due to hybrids or to phenotypic plasticity.

### *3.3 Results*

A summary of the number of individuals classified as pure and hybrid based on either genetic or morphological analyses for pure stands, mixed stands and seedlings is shown in Table 1.

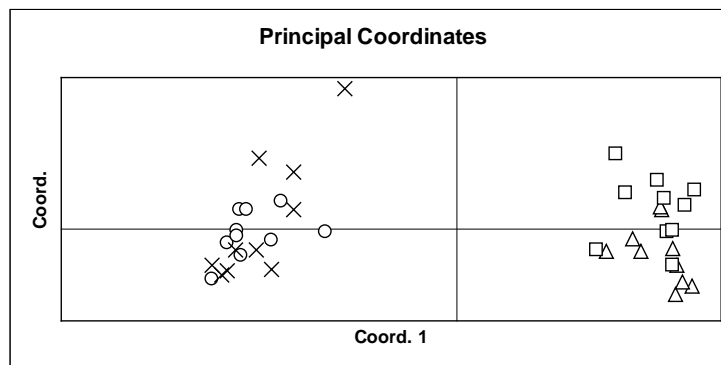
Table 3.1 Number of pure species and hybrid plants found within each population as determined by genotypes and leaf morphology.

	Genetic analyses	Morphological analyses
<b>Pure populations</b>	$q_i < 0.90$	$P < 0.90$
<i>B. oblongifolia</i>	20	20
<i>B. robur</i>	19	20
hybrids	1	0
<b>Mixed populations</b>		
<i>B. oblongifolia</i>	36	36
<i>B. robur</i>	42	49
hybrids	11	4
<b>Potted seedlings</b>		
<i>B. oblongifolia</i>	16	16
<i>B. robur</i>	14	19
hybrids	5	0
<b>Natural seedlings</b>		
<i>B. oblongifolia</i>	24	31
<i>B. robur</i>	17	19
hybrids	12	3

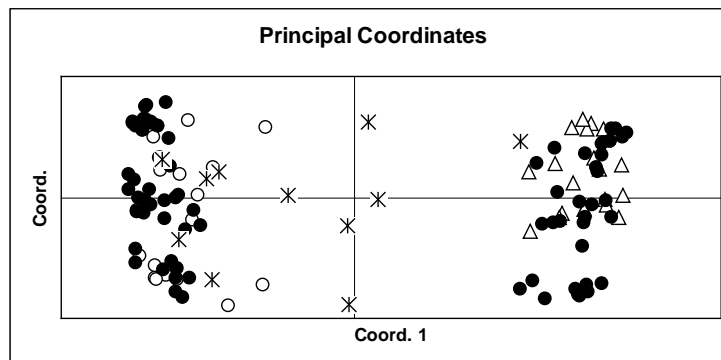
### 3.3.1 *Pure stands*

My genetic (Figure 3.3A and 3.3C) and morphometric (Figure 3.4A) analyses revealed that, with one exception, the 40 plants within the putatively pure stands formed two distinct groups. CDA clusters thus allowed me to characterize mature plants from the two parental species.

A



B



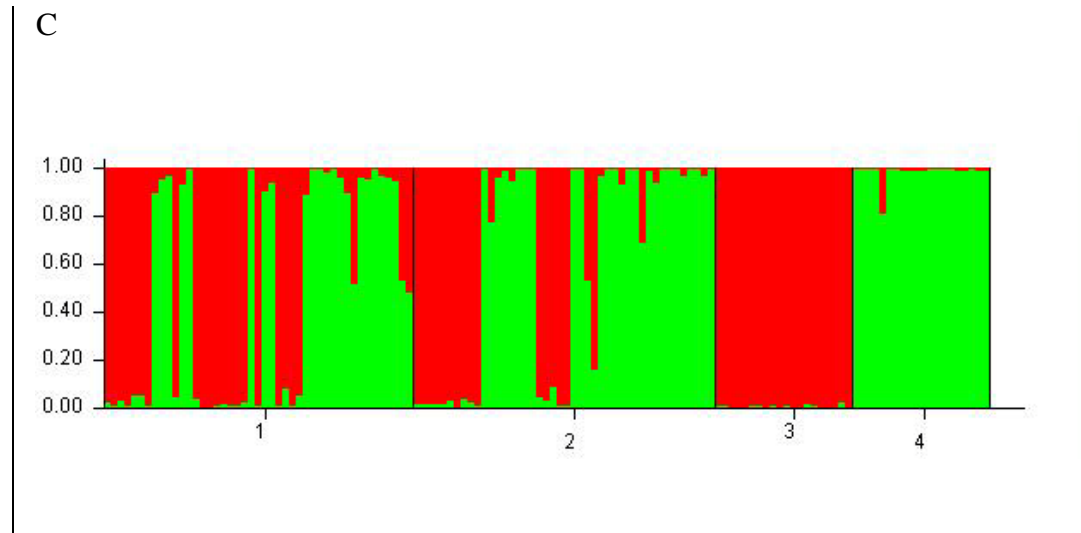


Figure 3.3 A. Genetic distinctions between the four pure stands, two *B. oblongifolia* ( $\Delta$ ,  $\square$ ) and two *B. robur* stands (o, x). B. Genetic distinction between plants from the pure stands (*B. oblongifolia*  $\Delta$  and *B. robur* o) and each of the hybrid zones (parentals  $\bullet$  and genetic hybrids  $*$  as determined by STRUCTURE). C. Bar plot of the probabilities of assignment for the 93 plants in the two hybrid zones (1 & 2), and in the 20 plants each in pure stands of *B. oblongifolia* (3) and *B. robur* (4).

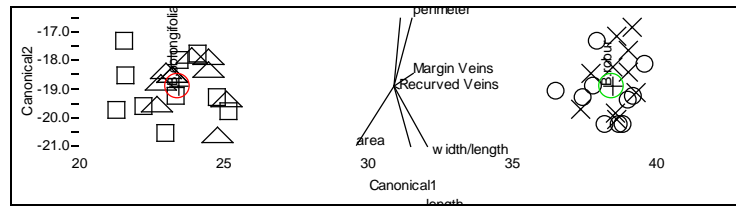
STRUCTURE confirmed the genetic pattern that the two putative *B. oblongifolia* stands were genetically distinct from the two putative *B. robur* stands (Figure 3.3C) and this was reflected in Nei's genetic distance values ( $D = 2.28 - 2.42$ ) and the mean  $F_{ST}$  of 0.414. Within species, the two *B. robur* stands and the two *B. oblongifolia* stands showed little differentiation ( $D = 0.09$  and  $0.02$ , and  $F_{IS} = 0.048$ ). Five loci, Bo17, Br23, Bo22, Br13 and Bo7,

each showed fixed differences between the two species. The other two loci revealed shared and unique alleles. There was some evidence for introgression within a pure stand of *B. robur* plants with the presence of a *B. oblongifolia* allele in one plant (Figure 3.3A and 3.3C).

Moreover, my discriminant analysis of the morphological data from the pairs of pure stands confirmed that the two species formed distinct clusters (Wilks' Lambda = 0.016,  $F_{(7, 32)} = 272.69$ ,  $P < 0.0001$ ) (Figure 3.4A). This analysis also revealed some morphological differentiation of the two *B. robur* stands ( $P = 0.039$ ) and this was supported by the ANOVA analysis for four of the seven leaf characters, length ( $P = 0.005$ ), area ( $P = 0.048$ ), perimeter ( $P = 0.003$ ) and width/length ratio ( $P = 0.026$ ). In contrast, both CDA analysis ( $P = 0.75$ ) and ANOVAs (each measurement  $P < 0.0001$ ) for individual characters revealed no significant morphological differences between the two *B. oblongifolia* stands.



### A Discriminant Analysis - Canonical Plot



### B Discriminant Analysis - Canonical Plot

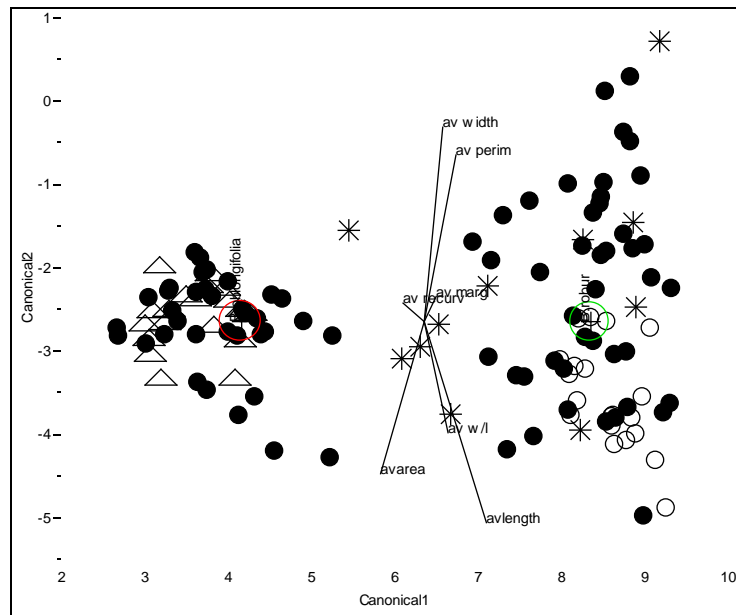


Figure 3.4 A. Morphological discrimination between the four allopatric stands, two *B. oblongifolia* ( $\Delta$ ,  $\square$ ) and two *B. robur* stands ( $\circ$ ,  $\times$ ). B. Morphological

discrimination within the two hybrid zones (●). Genetic hybrids (as determined by STRUCTURE) from both zones (\*). The pure stands are included (*B. oblongifolia* Δ and *B. robur* o). Circles show 95% confidence limit for the mean

### 3.3.2 Hybrid Zones

In contrast to the clear separation of parental species in the pure stands, genetic (Figure 3.3B and 3.3C) and morphometric (Figure 3.4B) analyses of mature plants occupying the two mixed sites revealed both parental types and a range of intermediates.

My analysis of the genetic data revealed that the 89 mature plants within the mixed stands displayed both pure species plants (42 *B. robur* and 36 *B. oblongifolia* plants) and a range of hybrid genotypes (11 plants) (Figure 3.3B and 3.3C). Four plants possessed one *B. robur* and one *B. oblongifolia* allele at each locus (as expected for first generation hybrids) and two plants displayed the genotype of one species with the exception of a single allele from the other species (as expected for introgressed plants). One plant displayed alleles indicative of *B. oblongifolia* at all loci, but one, which displayed two alleles expected only in *B. robur* plants (as expected for a later generation hybrid). One plant displayed four loci with alleles indicative of *B. robur* and three loci with one allele from each species (a pattern predicted for a backcrossed

hybrid). Some apparently backcrossed or later generation hybrid genotypes could have been generated through self-pollination. The remaining three plants were classified as hybrids by STRUCTURE ( $q_i < 0.90$ ). However, when individual loci were scrutinized, it was unclear why they were classified as hybrids and appeared to be like *B. robur* (Figure 3.3B).

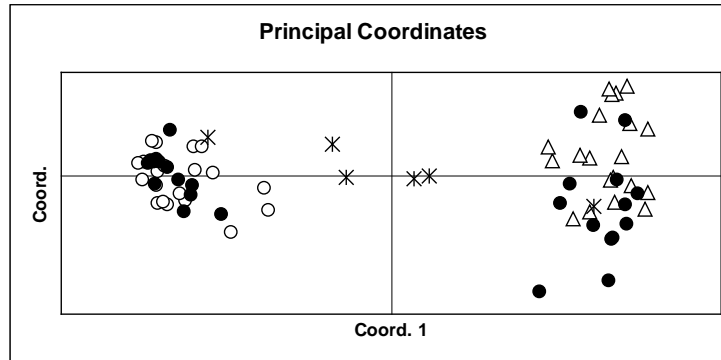
Similarly, the contact zone stands contained some plants with either typical *B. robur* or *B. oblongifolia* morphologies and plants with a range of intermediate morphologies (Wilks' Lambda = 0.18,  $F_{(7, 121)} = 77.16$ ,  $P < 0.0001$ ) (Figure 3.4B). The discriminant analysis revealed that only four of the 11 plants with hybrid genotypes were morphologically intermediate along with one plant that was genetically classified as *B. oblongifolia* ( $q_i = 0.91$ ).

### 3.3.3 Potted seedlings

As expected, the genetic analysis of 31 seedlings, from known mature plants growing in the hybrid zones, revealed a majority (29) of parental genotypes that were indistinguishable from the clusters formed by pure species adults (Figure 3.5A and 3.5B). Within this sample, two hybrid seedlings were originally detected, one from the single hybrid adult and one from a *B. robur* maternal plant. As there were so few hybrids, four more seedlings from the same maternal plants were also genotyped, two more from each plant. As the genotype of the maternal parent was known, I was able to infer the genotype

of the paternal parents by assessment of the maternal and their seedling genotypes. All hybrid seedlings were sired either by *B. oblongifolia* or hybrid plants. The range of hybrid genotypes reflected the diversity of known maternal genotypes along with further inter-specific crosses. For example, of three hybrids produced by a *B. robur* maternal plant; two were first generation hybrids sired by a *B. oblongifolia* plant (and genetically intermediate to the two parental species) and one was apparently a backcrossed hybrid (with five complete loci from *B. robur* and two loci bearing one *B. oblongifolia* allele) sired by a hybrid plant. One of the seedlings from the hybrid parent plant was classified as *B. oblongifolia* by STRUCTURE ( $P < 0.90$ ) and displayed one *B. robur* allele.

A



B

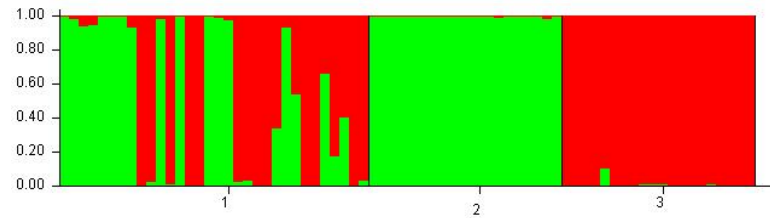
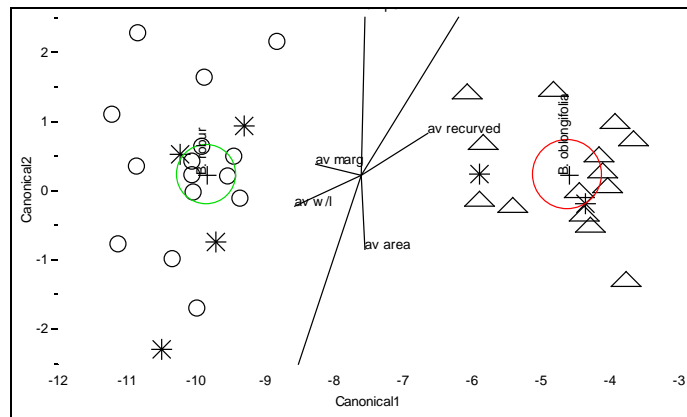


Figure 3.5 A. Genetic discrimination of seedlings raised in pots (parentals ● and Genetic hybrids \* as determined by STRUCTURE) and mature plants from pure stands (*B. oblongifolia* Δ and *B. robur* ○). B. A plot of the probabilities of assignment for the 35 potted seedlings (1) and for the 40 mature plants from pure stands of *B. oblongifolia* (2) and *B. robur* (3).

There was clear morphological distinction between *B. oblongifolia* and *B. robur* potted seedlings (Wilks' Lambda = 0.12,  $F_{(7, 27)} = 27.56$ ,  $P < 0.0001$ ) (Figure 3.6A). However, leaf morphology proved ineffective in detecting hybrid seedlings. None of the hybrids detected by genotyping had intermediate morphology ( $P > 0.90$ ) (Figure 3.6A). Even apparent first generation hybrids were phenotypically similar to parental species. Interestingly, two seedlings, from a hybrid maternal plant exhibiting extreme *B. robur* morphology, both retained *B. robur* morphology although one was sired a *B. robur* plant and the other by a *B. oblongifolia* plant.

#### A Discriminant Analysis - Canonical Plot



## B Discriminant Analysis - Canonical Plot

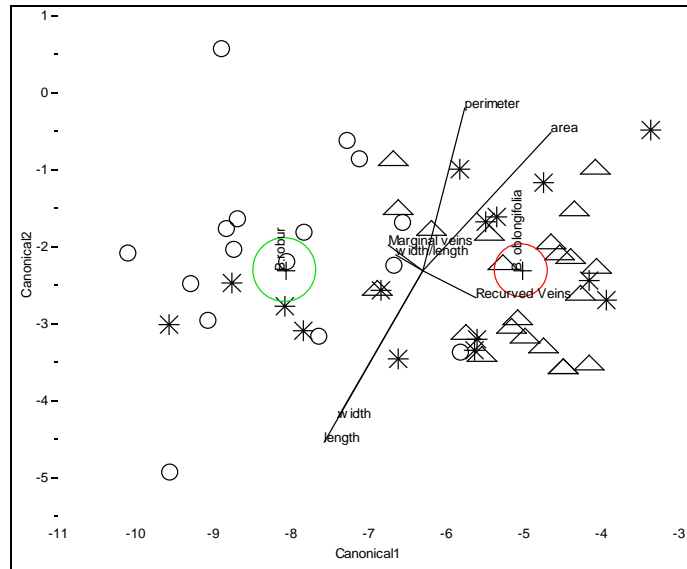


Figure 3.6 Morphological discrimination between seedlings; A. grown in pots and B. growing naturally in the field. Plants were classified genotypically by STRUCTURE *B. robur* (o), *B. oblongifolia* ( $\Delta$ ) and hybrids (\*). Circles show 95% confidence limit for the mean.

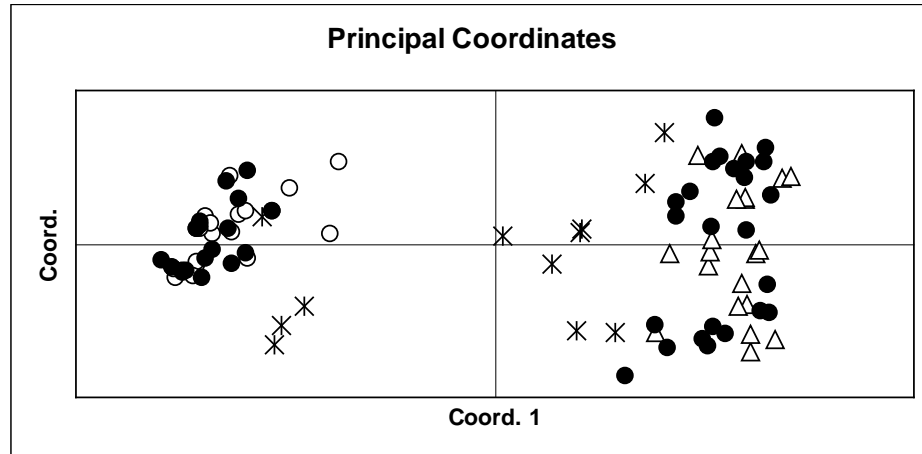
### 3.3.4 Seedlings in the field

I detected 12 hybrid seedlings ( $q_i < 0.90$ ) and 41 parental seedlings among the seedlings that germinated within the hybrid zones (Figures 3.7A and 3.7B). However, none of the hybrids in this sample appeared to be first generation

hybrids. Hybrid seedlings were comprised of what appeared to be seven *B. oblongifolia* backcrosses, one *B. robur* backcross and four complex later generation hybrids.



A



B

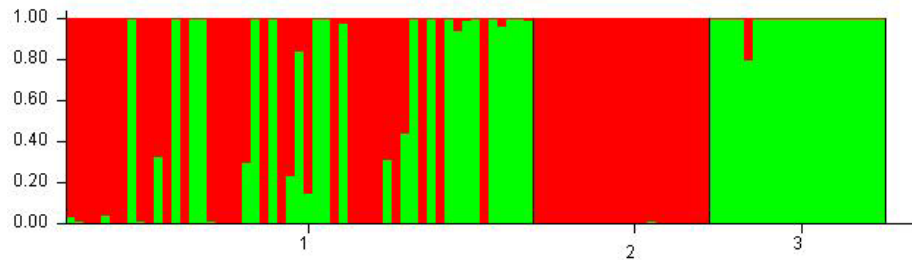


Figure 3.7 A. Genetic discrimination of the seedlings growing naturally in the two hybrid zones (parents ● and Genetic hybrids \* as determined by STRUCTURE). Pure stand plants (*B. oblongifolia* Δ and *B. robur* ○) were included. B. A plot of the probabilities of assignment for the 53 seedlings growing naturally in the hybrid zones (1), and for the 40 mature plants from pure stands of *B. oblongifolia* (2) and *B. robur* (3).

Leaf morphology proved very ineffective in detecting hybrid plants. Although most individuals with parental *B. robur* or *B. oblongifolia* genotypes formed pure species clusters (Wilks' Lambda = 0.30,  $F_{(7, 45)} = 15.09$ ,  $P < 0.0001$ ) (Figure 3.6B) these were relatively indistinct. The discriminant analysis misclassified seven of the parental seedlings as hybrids ( $P < 0.90$ ) and only three of the 12 genetic hybrids were classified as morphological hybrids.

### 3.3.5 Genetic and Morphometric Correlations

The environment of the hybrid zone does not appear to affect the range of morphologies displayed by mature pure species plants since this was similar to that seen within pure populations. There was a strong correlation between genetic distance and morphological distance when *B. oblongifolia* and *B. robur* pure stand plants were analyzed together ( $r = 0.92$ ;  $P < 0.001$ ). The morphological and genetic markers also correlated well for hybrid zone plants ( $r = 0.79$ ;  $P < 0.001$ ) and this correlation improved when hybrid plants were removed from the analysis of the hybrid zone ( $r = 0.90$ ;  $P < 0.001$ ). There was also strong correlation between genetic distance and morphological distance for potted seedlings ( $r = 0.69$ ;  $P < 0.001$ ) and this again improved when hybrids were removed from the analysis ( $r = 0.87$ ;  $P < 0.001$ ). The correlation between genetic distance and morphological distance for seedlings growing in the field was less than potted seedlings ( $r = 0.57$ ;  $P < 0.001$ ) and there was little change

when hybrid genotypes were removed ( $r = 0.59$ ;  $P < 0.001$ ) indicating that hybrid plants were not contributing to the observed morphological variation.

### 3.4 Discussion

My study confirmed that areas of mixed stands between *B. robur* and *B. oblongifolia* are indeed zones of active hybridization. Of the seedlings that appeared in response to fire, at least 21% were hybrids, and first generation hybrid genotypes were found amongst the seeds collected from plants in the hybrid zones. Although pulses of recruitment may be rare in populations of these re-sprouting plants, the presence of hybrid plants (approximately 9% amongst mature plants within contact zones) do not simply reflect historical hybridization events. Moreover, the genotypes of mature plants proved a powerful indicator of morphology, and both data sets can be used to distinguish pure species. The findings here support those of a previous study (Schibeci 1994) which found a good correlation between genotype and morphology using morphological and allozyme markers to characterize the hybrid zones. However, I found that hybrid morphology was less reliable because not all genetic hybrids were morphologically intermediate between the two species. Even one apparently first generation hybrid was morphologically indistinguishable from other *B. robur* adults. For seedlings

growing in pots, there was good morphological distinction between the two species. However, seedlings of the two species growing in the field, within mixed populations, were morphologically less distinct than the potted seedlings. In both potted and field seedlings, most genetically intermediate hybrid seedlings were morphologically indistinguishable from pure species seedlings.

#### 3.4.1 *Patterns of hybridization and introgression: as revealed by genetic data*

Although, the genetic categorization of 'pure' and 'hybrid' individuals is predictably difficult in species that hybridize freely and form sympatric populations (Rieseberg *et al.* 1998), our genetic data revealed that *Banksia* populations in their native heath-land can form a patchy mosaic of almost pure species stands and complex hybrid swarms. This implies that, although hybridization may be common under favourable conditions (that appear to be repeated within many drainage basins) isolating mechanisms are sufficient to prevent widespread introgression and imply that hybrid genotypes must be less fit outside environmentally intermediate hybrid zones. Indeed, only one individual of the 40 *B. robur* and *B. oblongifolia* sampled in the 'pure' stands (initially based on leaf morphology) appeared to be of mixed ancestry.

Within areas of hybridization, mixed stands of *B. robur* and *B. oblongifolia* appear to consist of pure species plants, as well as, first and later generation hybrid plants. Boecklen and Howard (1997) used mathematical models to

determine the number of diagnostic markers needed to correctly classify a series of backcrossed (BC) individuals as hybrids and not pure species and found that, for an error rate less than 10%, 8 markers would be needed to detect a second generation BC individual and up to 70 markers for a fifth generation BC individual. Self fertilization further complicates the distinction between later generation hybrids and backcrossed individuals. Nevertheless, simple inspection of my data revealed plants that were genetically intermediate between pairs of pure species, displaying one allele characteristic of each species at each locus. These plants perfectly match my expectations for F1 hybrids whereas other plants displayed genotypes predicted for backcrosses to either of the species and also more complex genotypes.

Schibeci (1994) found that overlapping flowering times of *B. oblongifolia* and *B. robur* plants along with common pollinators provide opportunity for inter-specific pollination. The flowering times of hybrid plants overlap much of the flowering times of the two parental species, and several examples in this study demonstrate a role for backcrossing in the facilitation of gene flow from one species to another. Although direct observation of a small number of hybrid seedlings from two known maternal plants revealed only backcrossing toward *B. oblongifolia*, the genotypes of field seedlings suggested that introgression occurs in both directions at the contact zones.

### 3.4.2 Morphology as an indicator of hybridization

Although hybrid plants are often recognized on the basis of morphologically intermediate mature plants, Rieseberg & Ellstrand (1993) noted that the unpredictability of hybrid character expression makes the use of morphology for hybrid identification unreliable. My data show that classification based on morphological intermediacy alone would result in an underestimation of the frequency of hybridization between *B. robur* and *B. oblongifolia* within groups of mature plants, seedlings raised in pots and seedlings growing naturally within the hybrid zone. This agrees with findings of Craft *et al.* (2002) who discovered that for *Quercus lobata* and *Quercus douglassi* not all intermediate plants were hybrids and not all hybrids were intermediate. This suggests that hybridization in general could be more common than is apparent by morphological observation and emphasises the need for genetic analyses in the detection hybridization.

Rieseberg and Ellstrand (1993) found that a large proportion of first and later generation hybrids exhibited extreme or novel characters and that hybrids of all generations were generally no more likely to display intermediate character states than parental character states (see Rieseberg & Ellstrand (1993) for a summary of some causes for transgressive segregation and /or novel characters). Three of the four mature first generation genetic hybrids between

*B. robur* and *B. oblongifolia* plants had leaf morphology intermediate between the two species; the fourth had *B. robur* morphology. Later generation hybrids showed a range of intermediate to parental characteristics. These results are consistent with multigenic control (Grant 1975) of the leaf characteristics measured and mostly quantitative traits (Rieseberg & Ellstrand 1993). However, the morphology of the potted seedlings suggests that leaf morphology is determined by more than simple additive genetic variation. All hybrid seedlings, including putatively first generation hybrids, had parental morphology and all but one had *B. robur* morphology like the maternal parent. This could reflect either nuclear or cytoplasmic effects or interactions between maternally inherited cytoplasmic genes and nuclear genes (Campbell *et al.* 2008).

#### 3.4.3 *Phenotypic plasticity amongst seedlings*

Identification of hybrids can be obscured by phenotypic plasticity within a variable environment (Bradshaw 1965, Dudley 1996) and phenotypic plasticity is hypothesized to influence ecological niche breadth (Bradshaw 1965, Sultan and Bazzaz 1993*a,b,c*, Oyama 1994, Sultan *et al.* 1998). For *Banksia* seedlings, morphology proved ineffective in predicting hybrid seedling genotypes, particularly for seedlings growing naturally in the hybrid zones. Phenotypic plasticity appears to play a role in seedling survival in the

variable environment of the hybrid zones, as seedlings of the two species were closer in size and more variable in field conditions than those grown in pots. The ability of *Banksia* seedlings to adapt to various stresses may also extend the niche range of seedlings of each species.

During this study, there was a severe drought. Prolonged drought conditions can have severe deleterious affects on plant metabolism (Bradford *et al.* 1982, Sultan and Bazzaz 1993b). Furthermore, growth can be restricted by biochemical disruption and reduced cell enlargement, leading to smaller leaves and plants can be at an earlier ontogenetic stage (Gedroc *et al.* 1996). Many traits can change with size and age (Gedroc *et al.* 1996, Dudley 2004). Compared to the development of the potted seedlings, the development of the field seedlings was significantly retarded. It could be argued that different developmental stages and water stress experienced by the field seedlings, contributed to the difference in comparative sizes of *B. oblongifolia* and *B. robur* seedlings growing in the field and in pots.

Despite the abundance of hybridization and gene exchange within the *Banksia* hybrid zones, the genetic and morphological integrity of the different species appear to be maintained. Although there appears to be some evidence for selection against early generation hybrids amongst the field seedlings, physical and partial temporal separation along with pollinator preferences, and selection



within different environments, could all contribute to the maintenance of pure species genotypes. The role of environmental variation in determining seedling survival, growth and susceptibility to herbivory has not been tested in the *Banksia* hybrid zones and will be investigated in a future study using transplant experiments, including independent measurements of the environment, and by comparing field and potted seedlings. A comparison between seedlings in pure stands with the seedlings in mixed stands may bring further clarity to the role of the environment in the morphological variation and selection amongst seedlings. Further studies should include the use of cytoplasmic markers and transplant experiments to ascertain any influence of cytoplasmic genes on inheritance and morphology of hybrids. The possibility of *B. paludosa* as a third species in a *Banksia* hybrid complex with *B. robur* and *B. oblongifolia* should be further investigated.

## *Chapter 4*

### **Post-fire distribution and performance of re-sprouting adult plants and performance of transplanted seed within *Banksia* hybrid zones**

#### *4.1 Introduction*

Although closely related (Mast and Givnish 2002), *Banksia oblongifolia* and *B. robur* are identifiable both on genetic and on morphological characters. There appears to be little introgression of genes from one species to the other away from contact zones (Schibeci 1994, Usher *et al.* 2010). Nevertheless, after a recruitment event, scrutiny of seedling genotypes has revealed that hybridization continues to create a range of hybrid genotypic classes within the contact zones (Usher *et al.* 2010). Continuing hybridization without widespread introgression suggests strong selection against hybrid genotypes, particularly within parental habitats. Here, I assess evidence for environment-dependent selection or genotype-by-environment interactions within the *Banksia* hybrid zones amongst mature plants and seedlings during, and two years after, a recruitment event.

#### 4.1.1 Selection in long-lived species

*B. oblongifolia*, *B. robur* and their hybrid plants have characteristics that are problematic when assessing their comparative fitness and performance. First, they are long-lived species. *B. robur* plants do not reach sexual maturity for three to five years and *B. oblongifolia* plants for five to seven years (George 1984). Mature plants can persist for decades as they re-sprout from lignotubers after fire. Therefore, I am unable to determine fitness for the whole life-cycle of individual plants. As fitness of individual plants can vary between stages of the life-cycle, inferences about performance are restricted to the stages under study. Second, recruitment events are infrequent (intervals between fires can be up to ten years or more) and unpredictable making it impossible to consider repeat episodes of recruitment within a single study. Despite these difficulties, it is important to study hybridization in long-lived species to gain a more comprehensive view on the effects of hybridization.

There are several critical life-cycle stages where selection is likely to be intense. *B. robur* and *B. oblongifolia* plants re-sprout from lignotubers after fire. Recovery of mature plants is important because re-sprouting results in the persistence of genotypes through successive fire events. As yet, it is unknown how well *Banksia* hybrid plants re-sprout and recover in comparison with their parental species after fire.

Another important life-cycle stage is the reproductive stage. The quantity and quality of seed produced by an individual plant is indicative of how well a plant has performed throughout its life-cycle. However reproductive output in long-lived plants can vary with different episodes of recruitment, so one episode does not completely quantify the success of each plant. Nevertheless, a comparison between hybrids and their parental species, even in a single recruitment event, can be informative. Furthermore, intense selection is expected after fire when seeds disperse, germinate, grow and develop. Little is known about selection amongst seed and seedlings of *B. oblongifolia*, *B. robur* and their hybrids.

#### 4.1.2 *Environment-dependent selection*

Hybridizing species are often associated with habitats that are different from one another (Adamik and Bures 2007, Carson *et al.* 2008). In chapter 3, it was found that the environment of the hybrid zone had a profound affect on the size and development of seedlings compared to those raised in pots. Field seedlings were much smaller and less well developed than potted seedlings (Usher *et al.* 2010), probably due to drought stress (Bradford *et al.* 1982, Sultan and Bazzaz 1993b). The *Banksia* seedlings remained small and underdeveloped for at least two years during drought conditions (Usher *et al.* 2010). Furthermore, the morphology of field seedlings was more variable than

potted seedlings. Seedlings of the two species were closer in size to one another than potted seedlings. Many traits can change with size and age (Gedroc *et al.* 1996, Dudley 2004); however greater similarity in size between the two species could have been due to phenotypic plasticity within the variable environment of the hybrid zones (Bradshaw 1965, Dudley 1996). Furthermore, phenotypic plasticity may have resulted in an extension of niche range of the two species (Bradshaw 1965, Sultan and Bazzaz 1993<sup>a,b,c</sup>, Oyama 1994, Sultan *et al.* 1998). *B. oblongifolia* and *B. robur* are associated with different habitats (Schibeci 1994) along a gradient of the soil moisture from dry sandstone ridges to (normally) wet seepage swamps (Keith and Myerscough 1993). It is unknown what effect the different habitats have on seedling size and development; or whether the different habitats effect parental species and hybrids in the same way.

I chose three habitat types for this study, *B. robur*, *B. oblongifolia* and intermediate habitats. As vegetation within the two hybrid zones was completely burnt and virtually unrecognizable at the beginning of my study, habitats were initially defined by their position on the slope. I designated *B. oblongifolia* habitat to areas high on the slope amongst eucalypt forests. I defined areas between the eucalypt forests and low-lying swamps and streams as intermediate habitat. *Banksia robur* habitat, I designated to areas of low

lying swamps and along stream edges. However, as the vegetation began to recover and plants re-sprout, fine scale habitat differences were observed over very small distances within the habitat types that I had defined. Johnson *et al.* (2001) compared genotypic classes with environmental variables and found environment-dependent selection, at a very fine scale, contributed to the structure of *Iris* hybrid zones. The *Banksia* hybrid zones are not simple clines but a mosaic of areas with small moist gullies and depressions and flatter dryer areas. I therefore sampled soil moisture content at regular intervals or wherever there were obvious changes in vegetation to identify, as far as possible, habitat types across the hybrid zones and redefine some habitats.

Indirect evidence for environment-dependent selection acting on mature plants across the hybrid zones is found in the natural relationship between genotype and environment. In chapter 3, I determined the genetic and morphological structure of the two hybrid zones. In this chapter, I compare the genetic composition of mature plants within *B. oblongifolia*, *B. robur* and intermediate habitats across the hybrid zones.

More direct evidence for environment-dependent selection is ascertained through experimental reciprocal transplantation of seed from each species and their hybrids into the various habitats associated with hybrid zones (Sambatti *et al.* 2008, Campbell *et al.* 2008, Emms and Arnold 1997). Transplant

experiments can reveal endogenous and exogenous selection acting on hybrids. Schibeci (1994) transplanted 900 seeds collected from plants in hybrid zones between *B. oblongifolia* and *B. robur* into three habitat types, *B. oblongifolia*, intermediate and *B. robur*. In her study, only 4% of seeds germinated, mainly *B. robur* seeds in *B. robur* habitat. However, hybrid seeds germinated in low numbers within all three habitats suggesting hybrid fitness across the hybrid zone.

#### 4.1.3 *Components of hybrid performance*

Key components of fitness include germination, an early life stage of development (Kahmen and Poschlod, 2000), and survival. Herbivore resistance can be fitness related. Herbivore damage was visible on the *Banksia* plants with all morphologies (*B. oblongifolia*, *B. robur* and hybrid) and may represent a significant selective force. Greater herbivore grazing on hybrid seedlings may result from disruption of co-adapted genes (Burton *et al.* 1999, Endler 1977) causing a breakdown in normal plant defenses. Also, seedling growth can be fitness related if larger seedlings have survival advantage or if larger seedlings grow into larger plants with greater reproductive output (Farris and Lechowicz 1990). Vegetative growth, such as height of seedlings and the number of leaves can be used as surrogate measures of fitness or comparative performance.

Hybrid performance can be extremely variable (Rieseberg and Carney 1998). Often the analysis of hybrid fitness of different genotypic classes is performed together. However, low average hybrid fitness does not preclude some hybrid genotypes from being as fit, or fitter, than parental genotypes (Rieseberg and Carney 1998). Therefore, in this study, I compared the performance of individual hybrids to determine how they varied in comparison with the mean for the parental species. I also divided hybrids into broad genotypic classes (see Usher *et al.* 2010) that allowed me to determine whether performance could be predicted by the hybrid genotype. For example, I wanted to know if hybrid performance was the same for all intermediate genotypes; and whether hybrid performance depended on how closely related they were to one or other of the parental species

#### 4.1.4 Aims

In this chapter, I compare the performance of *B. oblongifolia*, *B. robur* and their hybrid plants and seedlings, to determine whether hybrid performance during post-fire recovery and recruitment matches the predictions of one or more of the following models. (a) Hybrids are intrinsically less fit than parental species (Barton and Hewitt, 1985). (b) Hybrids are intrinsically less fit than parental species but inhabit unoccupied habitats (Rand and Harrison 1989). (c) Hybrids are fitter than parental species in intermediate habitats only



(Moore 1977). d) Some hybrids can be as fit, or fitter than, parental species in all habitats (Arnold 1997). To assess which of the models of hybrid zones might best describes the *Banksia* hybrid zone during recruitment, first I define three habitat types. Then, I compare and contrast the two species and their hybrids in the three habitats in the following ways. I determine the structure of genotypes amongst mature plants within the three habitats of the hybrid zones. I compare the recovery of each of the genotypes amongst mature plants as they re-sprout after fire. I determine the reproductive output of mature plants. I monitor germination, survival and performance of seed and subsequent seedlings transplanted into the hybrid zone.

I address the following questions.

1. What moisture content is characteristic of the habitats occupied by each species and what moisture content is intermediate?
2. What is the distribution of hybrid and parental mature plants within *B. oblongifolia*, *B. robur* and intermediate habitats across the hybrid zones?
3. Is there a difference in the capacity of parental species and their hybrids to re-sprout after fire?

4. Is there a difference in seed number and quality between parental species and their hybrids?
5. Is there evidence for environment-dependent selection acting on parental and hybrid seedlings during recruitment?
6. Is there evidence for endogenous selection against hybrid genotypes? That is, are hybrids intrinsically less fit across all habitats?

#### *4.2 Methods and Materials*

##### *4.2.1 Habitat types*

The two contact zones were criss-crossed with a myriad of natural depressions dotted amongst flatter, drier areas. Soil moisture and soil type varied from waterlogged peaty soil to drier sandy soils sometimes over distances of only a few metres. Because soil moisture within the hybrid zones coincided with soil nutrients and floristic composition (Keith and Myerscough, 1993), soil moisture was used as a guide for the determination of habitat types. To determine the amount of variation in soil water content across the hybrid zones and, as far as possible, to divide the hybrid zones into three habitat types, water content of the soil was measured across the hybrid zones. Soil moisture

was measured four times from 2002 to 2004 (Aug and Oct 2002, Oct 2003, Mar 2004) by taking a sample core with a diameter of 2cm and 12cm depth and weighing it, drying it at 60-70 degrees Celsius for 3 days, and weighing it again. For the transplant experiments soil samples were collected near every second or third cage (2 to 3 meters). For the natural seedlings (Chapter 6) and mature plants, soil samples were collected from within one metre wide transects that extended from *B. oblongifolia* habitat to *B. robur* habitat. Samples were taken every 6 metres or wherever there were obvious changes in habitat type. From the Appin Road site, 51 samples were collected, 14 from transect one, 16 from transect two, 10 from transect three and 11 from transect four. From the Darkes Forest site 38 samples were collected, 15 each from transects one and two, and 8 from transect three.

#### 4.2.2 *Mature plants and seed collection*

Mature plants were reduced to burnt stems supporting open follicles on fruits after fire in 2002. Fruits were collected across the hybrid zones from 142 plants, 70 at Appin Road and 72 at Darkes Forest, and tags were placed on the burnt stems. Only plants with fruits were randomly selected. All available fruits, which varied from plant to plant, were collected from each plant. Seeds were removed from the follicles and damaged seeds or seeds affected by fungus were counted and discarded. The number of fruit and healthy seed from

each plant was also counted and recorded. At the time that seeds were collected from burnt plants, hybrid plants were impossible to detect, even if they were morphologically intermediate because there was no identifiable foliage. The healthy seed was divided into two groups, one for the transplant and the other for the potted (chapter 5) experiment.

The charred plants within the two hybrid zones described above were revisited 10 weeks after the fire. New leaf tissue was taken for genotyping from 129 of the tagged plants that were relocated and re-sprouting (62 from Darkes Forest and 67 from Appin Road). The habitat of each plant was determined as described in 4.2.1 (*Habitat Types*). The numbers of parental species and hybrids from different genotypic groups (Usher *et al.* 2010, see chapter 3) found in each habitat, *B. oblongifolia*, *B. robur* and intermediate habitat, were counted.

To make a comparison between hybrid and parental plants in their recovery after fire, the 129 plants were all measured as follows. The length and width of the lignotuber of each plant was measured and the area calculated. For each plant, the numbers of shoots were counted and the tallest shoot measured. *B. oblongifolia* and *B. robur* plants re-sprout after fire and plants within the hybrid zones were of different ages hence have lignotubers at different stages of development. The size of the lignotuber is likely to determine the amount of

re-growth. Lignotuber volume may have been a better measure of lignotuber size; however volume could not be measured without disturbing the plants. Therefore, to establish if lignotuber area could determine the amount of re-growth within each species, the relationship lignotuber area and shoot number, and the relationship between lignotuber area and the tallest shoot, was calculated. Herbivore damage was also assessed as an estimate of percentage of damage on leaves in the field. Plant measurements were compared between genotypes, sites and habitats within sites.

#### *4.2.3 Transplant Experiment*

To determine whether selection was acting on hybrid and parental seedlings within the different habitats associated with the hybrid zones, 840 seeds, collected from 110 of the tagged plants that had produced healthy seed, were pooled (to maximize the number of seeds planted within each habitat at each site) and transplanted back into the two hybrid zones. The number of seeds used in the transplant experiment varied from one to 30 seeds per plant as the number of seeds collected from each plant varied widely, so there were some sibling seeds. As the maternal plants were genotyped, the number of seed expected to grow from each maternal genotype was known (Table 4.1). The number of plants from each genotypic group varied because genetic markers were not yet developed at the time of collection so genotypes were unknown.

Table 4.1 The number of plants from which seed was collected, and the number of seeds used for the transplant experiment from each genotypic group, *B. oblongifolia*, *B. robur*, *B. paludosa* and hybrids, as determined by mature plant genotypes. Fine scale habitat was determined by soil moisture content.

Site	Appin Road			Darkes Forest			Tot
Fine Scale habitat	<i>B. oblongifolia</i>	<i>B. robur</i>	Inter-mediate	<i>B. oblongifolia</i>	<i>B. robur</i>	Inter-mediate	
Genotype							
Number of plants							
<i>B. oblongifolia</i>	11	0	10	6	5	9	41
<i>B. robur</i>	0	18	10	0	18	8	54
<i>B. paludosa</i>	0	0	0	6	0	1	7
Hybrids	2	0	2	0	2	0	6
Tot No. of plants	13	18	22	12	25	18	108

Number of seeds (Maternal Genotypes)							
<i>B. oblongifolia</i>	74	0	72	30	71	66	313
<i>B. robur</i>	0	161	70	0	137	71	439
<i>B. paludosa</i>	0	0	0	33	0	18	51
Hybrids	14	0	11	0	12	0	37
Tot	88	161	153	63	220	155	840

The 840 seeds were grouped into three groups according to their position on the slopes from where they were collected (*B. oblongifolia* habitat, *B. robur* habitat, and intermediate habitat). As recruitment events after fire were temporally and spatially unpredictable, time constraints made it impossible to make a detailed appraisal of the study area before transplantation of seed. Therefore predictions were made concerning the habitat types before soil water content was measured. *Banksia robur* habitat was predicted to be at the bottom of the slope in swamp consisting of peaty soils in sedge-heathland and was dotted with freshwater crayfish holes at both sites. *Banksia oblongifolia* was predicted to occur on flat, or gently sloping, sandstone ridges in open forest around swamp margins in sandy soil (George 1984, Taylor and Hopper 1988, Davis 1941, Keith and Myerscough 1993). Intermediate habitat was positioned between the two parental habitat types. Seeds collected from *B. oblongifolia* and intermediate habitat types (120 seeds) were transplanted into *B. oblongifolia* habitat. Seeds collected from all three habitat types (180 seeds) were transplanted into intermediate habitat. Seeds collected from intermediate and *B. robur* habitat types (120 seeds) were transplanted into *B. robur* habitat. I used an unbalanced design (Figure 4.1). Seed taken from *B. oblongifolia* and *B. robur* habitats were expected to fare poorly in the habitat of the other species, so seeds collected from *B. oblongifolia* and *B. robur* habitats were omitted from the habitat of the opposite species to boost numbers planted in



the parental and hybrid habitats. However, seed from both parental species was collected from plants in intermediate habitats and these were transplanted into the parental habitats of each species, so comparisons between parental genotypes within opposite parental habitats were possible. Although it was not possible to keep track of seed from each maternal plant, the proportion of seed from each genotypic group, *B. oblongifolia*, *B. robur* and hybrid was known and used to calculate the proportion of each genotypic group that germinated and survived.

To allow the seeds to be relocated and protect new seedlings from damage by wallabies and wombats, twelve seeds were placed under each of 70 (35 at each site) 22 cm square cages, 11cm high and made from 1 cm ‘snake’ wire mesh, and the seeds were dabbed with a small spot of whiteout. Ten cages were placed in *B. oblongifolia* habitat, five containing seeds taken from *B. oblongifolia* habitat and five containing seeds from intermediate habitat. Ten cages were placed in the *B. robur* habitat, five containing seeds taken from *B. robur* habitat and five containing seeds taken from intermediate habitat. Fifteen cages were placed in the intermediate habitat, five containing seeds taken from *B. robur* habitat, five containing seeds taken from intermediate habitat, and five containing seeds taken from *B. oblongifolia* habitat. The cages were randomly positioned in sets of two in *B. oblongifolia* and *B. robur*

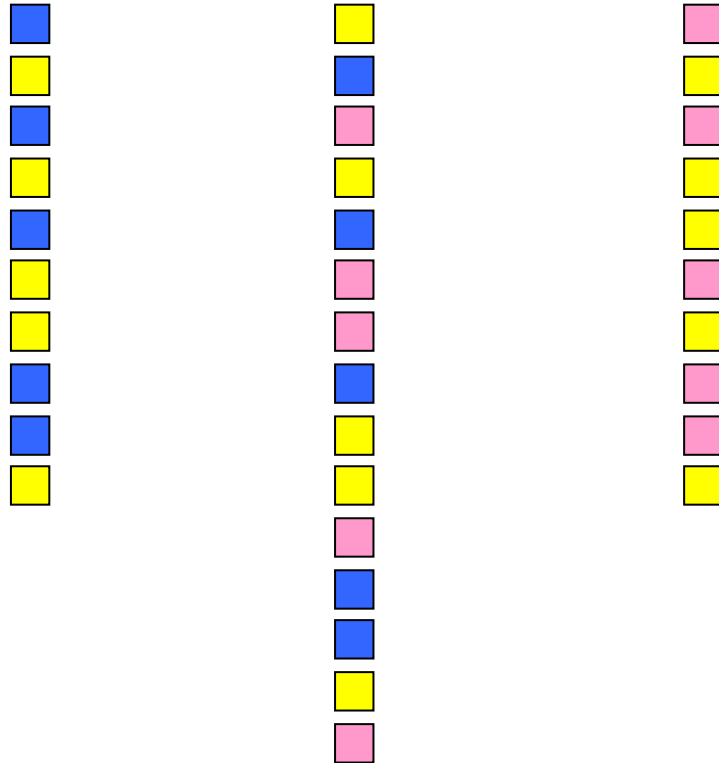
habitat and in sets of three in intermediate habitat (Figure 4.1), one metre apart in the three different habitat types. Cages were removed after 7 months when seedlings began to reach the height of the cages. Seedling measurements were made periodically over two years in total.

## Habitat

*B. oblongifolia*


hybrid

*B. robur*



## Cage Contents

 Seeds taken from plants in *B. oblongifolia* habitat

 Seeds taken from plants in hybrid habitat


 Seeds taken from plants in *robur* habitat

Figure 4.1 Layout of cages containing 12 transplanted seed at each site, Appin Road and Darkes Forest.

#### 4.2.4 Genetic Techniques

A one centimetre tip from new leaves of all plants and seedlings was collected as soon as possible after re-sprouting of plants and sufficient development of seedlings, and stored at -80°C. The techniques for extraction and genotyping are described in Usher *et al.* (2005) or chapter 2. Seven microsatellite loci, Bo3, Bo7, Bo17, Bo22, Br3, Br13, Br23, were used to genotype the plants (Usher *et al.* 2005). To allow me to determine the various hybrid classes, in broad terms, (as described in chapter three) the following assignments were made: first generation ( $F_1$ ) had one allele from each species at all distinguishing loci; later generation ( $F_2$ ) had complete loci from each species; back crossed to *B. oblongifolia* (BC-o) had some *B. robur* alleles and some complete *B. oblongifolia* loci; back crossed to *B. robur* (BC-r) had some *B. oblongifolia* alleles and some complete *B. robur* loci; *B. oblongifolia* genotypes with one *B. robur* allele (I-o); *B. robur* genotypes with one allele *B. oblongifolia* (I-r).

#### 4.2.5 Seedling Fitness and Performance Measurements

In the transplant experiment, I measured seedling performance from germination to two years of growth and development. Key components of performance such as germination, the ability to survive and resistance to

herbivore damage were determined. Seeds transplanted into the two hybrid zones were observed every three days for four weeks and the number of seeds germinating recorded. To determine whether there was a difference in survival rate between hybrids and parental species during the first two years of growth, the number of surviving seedlings after two years was recorded for all seedlings and compared with those originally germinating.

To determine whether hybrid vigour (heterosis) (Baack and Rieseberg 2007) or hybrid inferiority was evident amongst hybrid seedlings within habitats, the height from ground to leaf tip was measured and the number of leaves counted.

To determine whether hybrid seedlings were more susceptible to herbivore damage than parental species seedlings, the overall damage to each seedling was assessed as a percentage. The numbers of damaged leaves were counted and the amount of damage on each leaf estimated. The total amount of damage was then compared with the total area of all leaves. The transplantation of seed into different habitats could also have subjected seedlings to different types of herbivores than normally experienced in their natural habitats. Therefore, the type of herbivore damage was also recorded in following three categories. Damage by leaf miners (miner) was observed when the inside of leaves were

eaten resulting in discoloured squiggles. Some insect herbivores grazed the edge (edge) of leaves and others made holes (hole) in the leaves.

All seedlings were measured and assessed once a month for 3 months, and again after 3 months, and then after every 6 months, until two years from germination.

#### 4.2.6 Statistical analyses

##### **Maternal plants**

To determine differences between the two species and hybrids during regrowth of plants from lignotubers, one way ANOVAs were performed on the averages for *B. oblongifolia*, *B. robur* and hybrid genotypes in shoot number/lignotuber area, height of tallest shoot and herbivore damage. As lignotuber surface area may not reflect the volume of the lignotuber and hence the amount of re-growth, correlation between lignotuber area and shoot number and between lignotuber area and the height of the tallest shoot, within species, was tested using a pairwise multivariate analysis (SAS - JMP 5.1). Simple nested ANOVAs (SAS - JMP 5.1) were used to determine if the amount of regrowth differed between sites and habitats within sites for each species. A compound nested ANOVA was performed to test the amount of

herbivore damage on the different genotypes between sites and habitats within sites.

To determine whether there was evidence for hybrid inferiority in individual hybrids during re-sprouting, one way ANOVA analyses (SAS - JMP 5.1) were performed on each of the re-growth measurements for each species. Individual hybrid plant measurements were compared with the 95% confidence levels of the means of the two parental species measurements. Hybrids were grouped into the broad genotypic classes described in '*Genetic Techniques*' above.

To determine whether seed production differed between the two parental species, habitats and sites a compound nested ANOVA analysis (SAS - JMP 5.1) was performed on the numbers of fruits, healthy seeds, fungus infected seeds and damaged seeds.

To determine whether individual hybrids produced less seed or poorer quality seed, the number of fruit, number of healthy seed, number of fungus infected seed and otherwise damaged seed of individual hybrid plants was compared with parental species performances. One way ANOVA analyses (SAS - JMP 5.1) were performed on measurements of the parental species and individual hybrid measures were compared with the 95% confidence levels of the means

from the parental species. Hybrids were grouped within the broad hybrid classes described in ‘*Genetic Techniques*’ above.

### **Transplant experiment**

A contingency analysis (SAS - JMP 5.1) was conducted on transplanted seed to determine whether genotypic frequencies between maternal plants and their seedlings remain the same between generations. The number of *B. oblongifolia*, *B. robur* and hybrid seedlings germinating and then surviving to two years were compared with the number of seeds within *B. oblongifolia* and *B. robur* that were originally transplanted as derived from the maternal genotypes. Comparisons were made over all habitats and sites, within sites and within habitats.

Germination differences between sites and habitats within sites were tested using a simple nested ANOVA. Survival differences between genotypes within sites and within habitats within sites were tested using a compound nested ANOVA. To determine whether the mean performance of plants differed between genotypes, sites and habitats within sites, compound nested ANOVA analyses (SAS - JMP 5.1) were performed on growth, leaf number, herbivore damage, and types of herbivory.



To determine how individual hybrids perform in comparison with parental species, one way ANOVA analyses (SAS - JMP 5.1) were performed on measurements of the parental species and individual hybrid measures were compared with the 95% confidence levels of the means from the parental species. Hybrids were group within the broad hybrid classes described in ‘*Genetic Techniques*’ above.

### 4.3 Results

#### 4.3.1 Characterization of habitats

To determine the amount of variation in soil moisture content across the hybrid zones and, as far as possible, to divide the hybrid zones into three reasonably consistent habitat types, the moisture content of soil was measured on four separate occasions (14 Aug 2002, 25 Oct 2002, 24 Oct 2003 and 25 Mar 2004) along each location of the transplanted seed. Soil moisture was variable between habitat types designated by position on the slope. In August 2002 soil moisture content in the designated *B. robur* habitat was above 50%, designated intermediate habitat was between 30% and 50% and designated *B. oblongifolia* habitat was below 30% (Figure 4.2). Percentage moisture in the soil varied over the two year period, but followed similar trends in differences

between habitat types, except for *B. robur* habitat at Appin Road which did not dry out as much as the other regions (Figure 4.2), probably due to proximity to a running creek. Therefore, the relative percentage moisture content was used as a guide to assign habitat types at a fine scale level (Figure 4.2). However, at the Appin road site, the transplant section that was thought to be *B. oblongifolia* habitat, as ascertained by position on the slope, had intermediate soil moisture content. The vegetation, after re-growth, also confirmed this distinction as it was more characteristic of areas not normally occupied by *B. oblongifolia* plants. Reclassification of *B. oblongifolia* habitat to intermediate habitat at the Appin road meant that analysis of *B. oblongifolia* habitat was restricted to a single site (Darkes Forest). However, because seeds were taken from the same mixture of maternal plant genotypes (an advantage of not separating the seed from the two sites) and placed in the two different habitat types (although at different sites), it presented the opportunity to compare seed germination and seedling growth within the different habitat types.

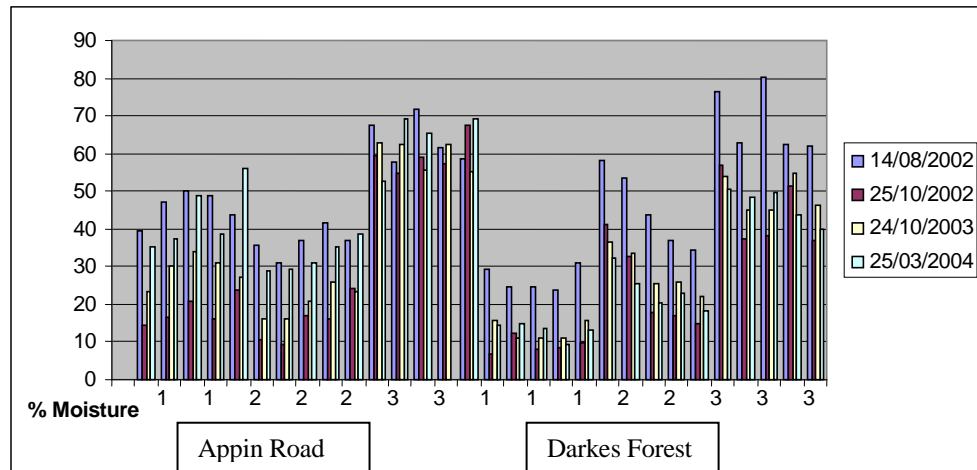


Figure 4.2 Percentage moisture content of soil samples taken four times over two years from within the transplanted seed locations, *B. oblongifolia* (1), intermediate (2), and *B. robur* (3) habitats, as designated by position on the slope, at the Appin road site and at the Darkes Forest site.

#### 4.3.2 Maternal Plants

Of 142 plants selected because they bore fruits, 12 (9.2%) were hybrid, 43 (33%) were *B. oblongifolia*, 69 (53%) were *B. robur* and 6 (4.6%) were *B. paludosa* (or *B. paludosa* hybrid) plants (Table 4.2). Twelve plants were not genotyped as they were either not relocated or had not re-sprouted.

Plants with hybrid genotypes were found in all three habitats at Appin Road in small numbers, 2 in *B. oblongifolia* habitat, 3 in *B. robur* habitat and 2 in

intermediate habitat. At Darkes Forest hybrids were only found in *B. robur* habitat (five plants) (Table 4.2) All hybrid plants in *B. oblongifolia* and intermediate habitats at Appin Road only had one *B. robur* allele (introgressed) and there were no hybrids in these habitats at Darkes Forest. First generation and backcrossed hybrids were found only in *B. robur* habitat at both sites.

As expected, *B. oblongifolia* and *B. robur* genotypes were predominant in *B. oblongifolia* and *B. robur* habitats respectively (Table 4.2). In *B. robur* habitat at Appin Road none of the 27 plants were *B. oblongifolia* plants. In *B. robur* habitat at Darkes Forest only three of the 29 plants had *B. oblongifolia* genotypes. In *B. oblongifolia* habitat at Appin Road none of the 15 plants were *B. robur* plants. In *B. oblongifolia* habitat at Darkes Forest only one of the 11 plants was a *B. robur* plant. However, almost equal numbers of the two species were found in intermediate habitat. At Appin Road 12 of plants were *B. oblongifolia* and 11 were *B. robur*. At Darkes Forest 9 were *B. oblongifolia* and 12 were *B. robur*. *B. paludosa* plants were found at the Darkes Forest site only (five plants in *B. oblongifolia* habitat and one in intermediate habitat).

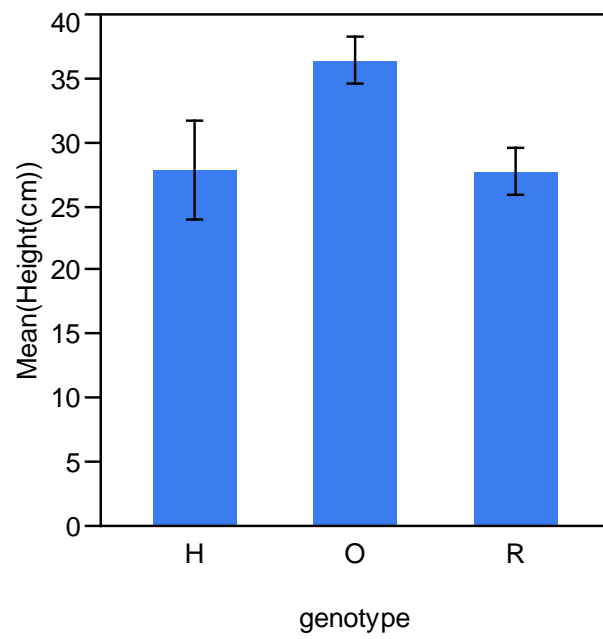
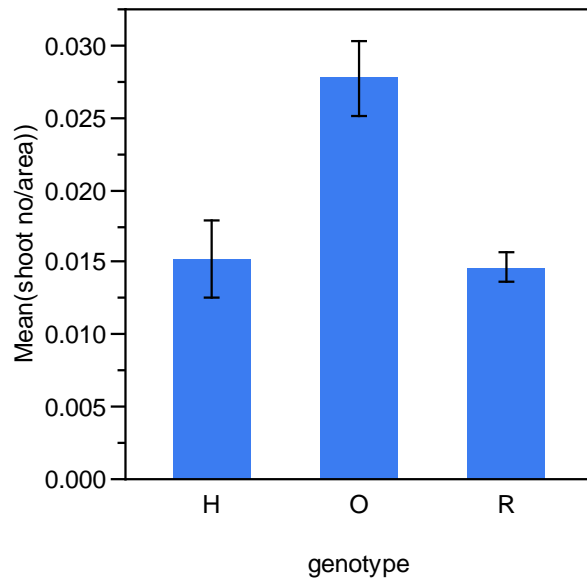
Table 4.2 Distribution of genotypes of plants that were sampled across the two hybrid zones 10 weeks after fire to genotype and measure re-growth. Hybrids classes, first generation (F1), backcross to *B. oblongifolia* (BC-o) or *B. robur* (BC-r), introgressed by a *B. robur* allele (I-o) or *B. oblongifolia* allele (I-r).

site	Appin Road			Darkes Forest			Total
Habitats	<i>B. oblongifolia</i>	Inter-mediate	<i>B. robur</i>	<i>B. oblongifolia</i>	Inter-mediate	<i>B. robur</i>	
Genotypes							
<i>B. oblongifolia</i>	13	12	0	6	9	3	43
<i>B. robur</i>	0	11	23	1	12	21	69
<i>B. paludosa</i>	0	0	0	5	1	0	6
Hybrids	2 I-o	2 I-o	2 BC-o 1 F1			2 BC-r 2 F1 1 I-r	12

#### 4.3.3 Re-sprouting of mature plants

There were characteristic differences in morphology between the two species (Figure 4.4) in the measurements selected to monitor re-growth. The shoot

number and lignotuber area ratio was used to determine the amount of re-growth within species, as there was good correlation between lignotuber area and shoot number (*B. oblongifolia*,  $r = 0.87$ ; *B. robur*,  $r = 0.67$ ). The numbers of shoots per lignotuber area of *B. oblongifolia* ( $0.028 \pm 0.02$  s.e) was about twice that of *B. robur* ( $0.015 \pm 0.002$  s.e) ( $F_{2,117} = 15.21$ ,  $P < 0.0001$ ), and the mean of hybrid measures ( $0.15 \pm 0.004$  s.e) was the same as *B. robur* plants (Figure 4.3). The height of the tallest shoot was used as a measure of performance within species on its own, as the relationship between the tallest shoot and lignotuber dimensions was poor (*B. oblongifolia*,  $r = 0.32$ ; *B. robur*,  $r = 0.28$ ). *B. oblongifolia* plants were taller ( $36.36 \pm 2.14$ cm s.e) than *B. robur* plants ( $27.70 \pm 1.71$ cm s.e;  $F_{2,117} = 5.30$ ,  $P = 0.006$ ), and again, the hybrid average ( $27.83 \pm 4.00$ cm s.e) was similar to *B. robur* (Figure 4.3). Herbivore damage was extremely variable across all genotypes, but on average, similar for both species, *B. oblongifolia* ( $15.24 \pm 3.30\%$  s.e), *B. robur* ( $14.82 \pm 2.63\%$  s.e) and their hybrids ( $16.67 \pm 6.17\%$  s.e;  $F_{2,117} = 0.04$ ,  $P = 0.96$ ), (Figure 4.3).



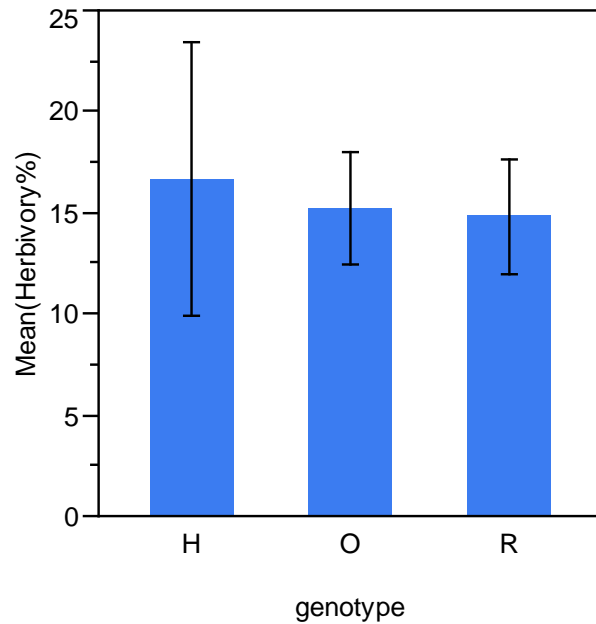


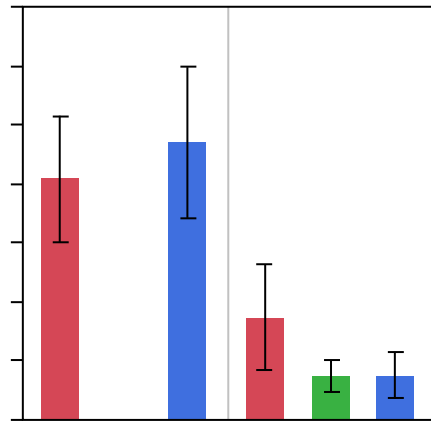
Figure 4.3 Re-growth and herbivory on plants re-sprouting across two hybrid zones 10 weeks after fire destroyed the canopy. The different genotypic groups are hybrid (H), *B. oblongifolia* (O) and *B. robur* (R). Error bars = standard error.

Regrowth of both species (separately from one another) was examined between sites and habitats (Figure 4.4). Habitat comparisons were restricted to the species own habitat and intermediate habitat as there were only three *B. oblongifolia* plants in *B. robur* habitat and only one *B. robur* plant in *B. oblongifolia* habitat (Table 4.2).

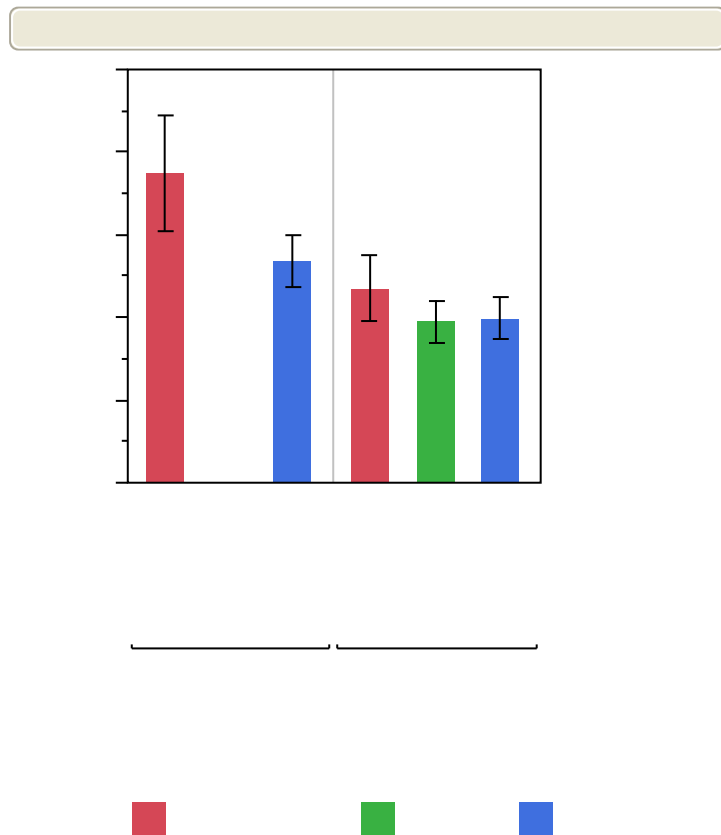


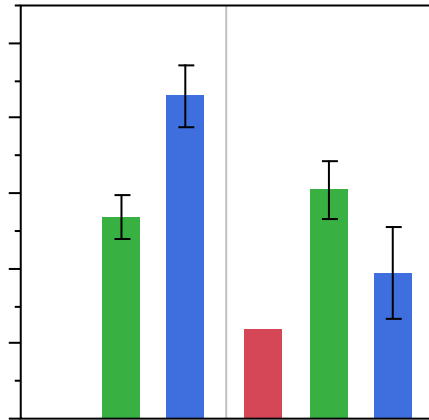
*B. oblongifolia* plants had the same number of shoots per lignotuber area between sites and among habitats ( $F_{4,36} = 1.89$ ,  $P = 0.13$ ). *B. robur* plants had more shoots per lignotuber area at Appin Road ( $0.017 \pm 0.0015$ ), particularly in intermediate habitat ( $0.021 \pm 0.0025$ ) than Darkes Forest ( $0.011 \pm 0.003$  s.e) ( $F_{4,58} = 3.07$ ,  $P = 0.023$ ) (Figure 4.4 A).

*B. oblongifolia* plants were taller at the Darkes Forest site ( $44.05 \pm 2.81$  cm) than at the Appin Road site ( $31.30 \pm 2.10$  cm s.e) ( $F_{4,36} = 3.90$ ,  $P = 0.0099$ ), but the same height between habitats. *B. robur* plants were the same height between sites and habitats ( $F_{4,58} = 0.61$ ,  $P = 0.66$ ) (Figure 4.4 B). There was no difference in herbivore attack between genotypes, sites or habitats ( $F_{14,100} = 1.75$ ,  $P = 0.056$ ) (Figure 4.5).

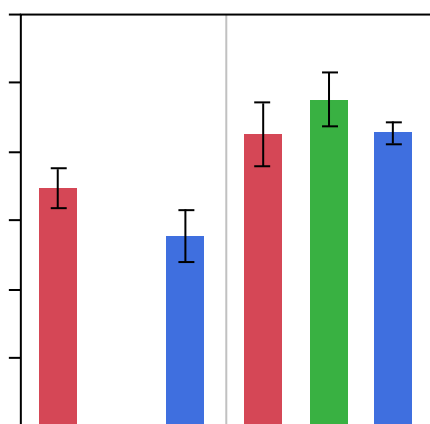
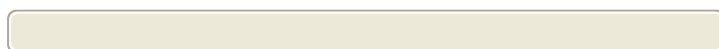


A.





B.



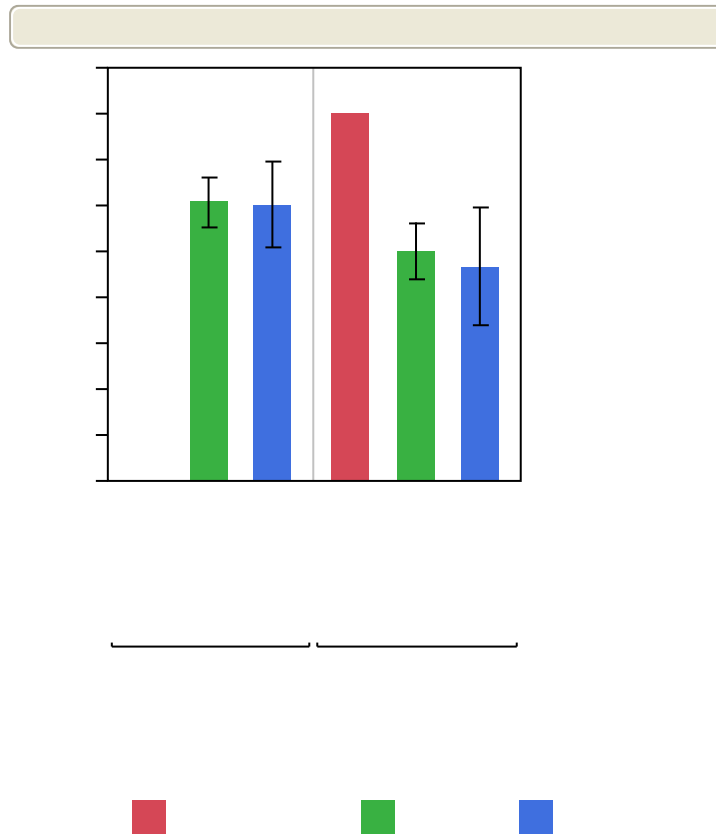


Figure 4.4 Regrowth of *B. oblongifolia* (A) and *B. robur* plants (B), between sites and within habitats, 10 weeks after fire.

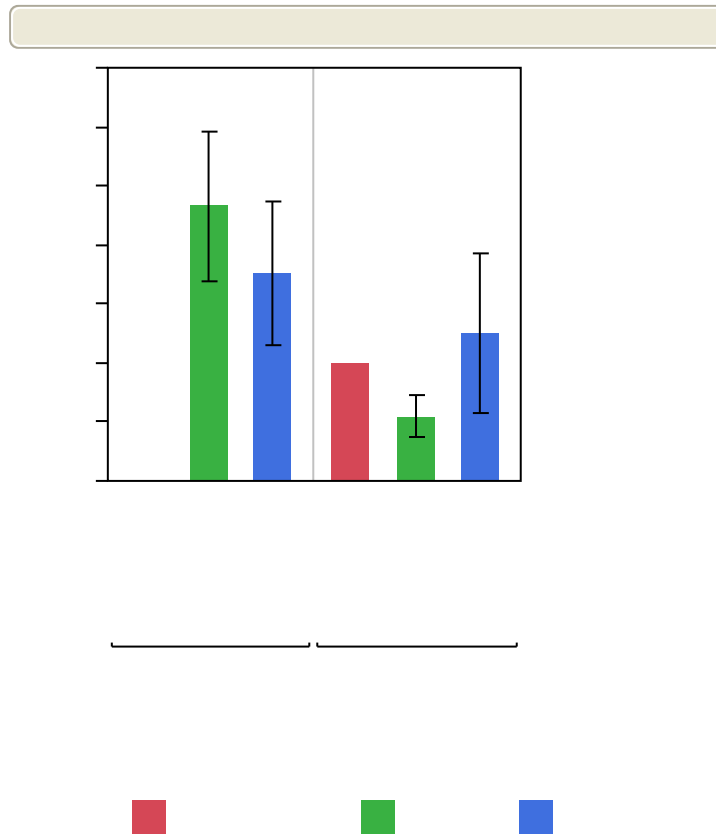


Figure 4.5 Herbivore damage on *B. oblongifolia* and *B. robur* re-shooting plants among sites and habitats 10 weeks after fire.

As there were some differences in re-growth within each species between sites, hybrids were compared with the 95% CLs of parental plants within sites and habitats. Also, where there were differences between species the lowest and highest limits were used regardless of species. There were no hybrids in *B. oblongifolia* or intermediate habitats at Darkes Forest in this sample of plants.

Individual hybrid measurements varied widely (Figure 4.6). Of the twelve hybrid plants (Table 4.2) performance measures varied from above to below 95% confidence limits (CLs) for the two species. As there were few hybrids patterns were not discernable, nevertheless intermediate genotypes ( $F_1$ ) and introgressed genotypes were found to have above average, average and below average performance measurements which differed amongst types of measurements. Backcrossed plants were found to have average or below average measurements.

When number of shoots per lignotuber area was compared between hybrids and parental species, five hybrids had average numbers (between the 95% CLs for the two species). These included an introgressed plant (0.061) in intermediate habitat (95% CL = 0.02 - 0.05), (Figure 4.6B), two backcrossed plants (0.01 each) in *B. robur* habitat (95% CL = 0.01 - 0.02), (Figure 4.6C) at Appin Road; and a backcrossed (0.007) and first generation (0.006) in *B. robur* habitat (95% CL = 0.009 - 0.03), (Figure 4.6F) at Darkes Forest. Five of the hybrids had less shoots per lignotuber area than parental plants (below the lower 95% CL for both species), (Figure 4.6). These included, two introgressed plants (0.016 and 0.021) at Appin Road in *B. oblongifolia* habitat (lower 95% CL = 0.022), (Figure 4.6A); a first generation plant (0.005) at Appin Road in *B. robur* habitat (lower 95% CL = 0.01), (Figure 4.6C); and a

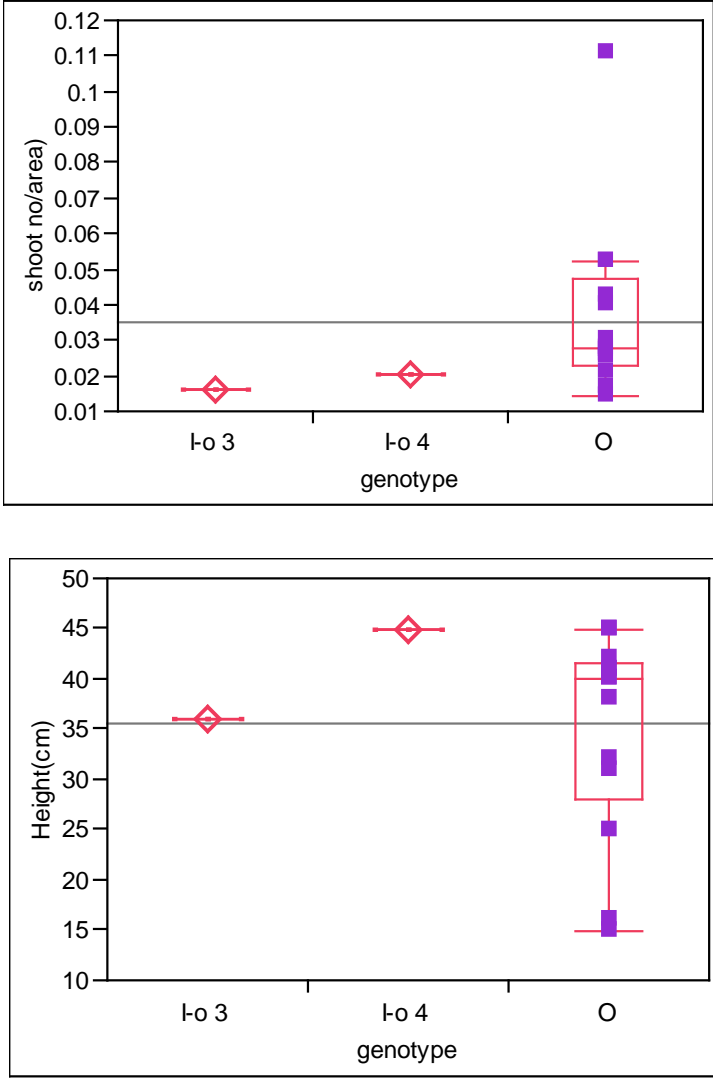


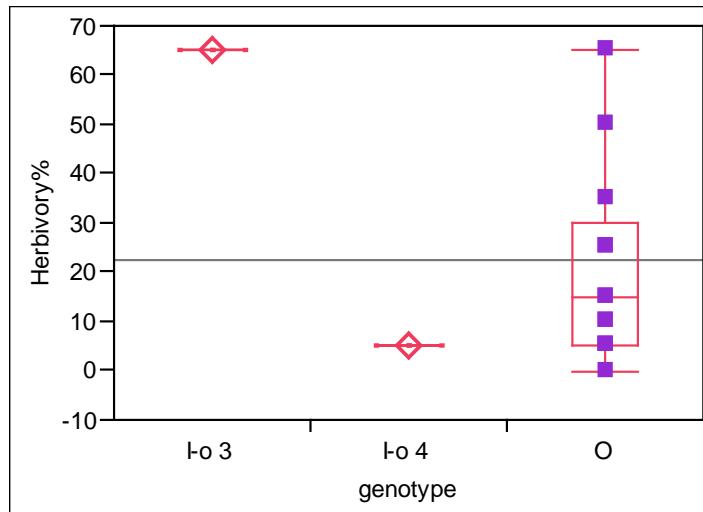
backcrossed plant (0.007) and a first generation plant (0.006) at Darkes Forest in *B. robur* habitat (lower 95% CL = 0.009) (Figure 4.6F). Two introgressed individuals, one in intermediate habitat (0.034), (Figure 4.6B) at Appin Road and one in *B. robur* habitat (0.066) (Figure 4.6F) at Darkes Forest, had above average shoots per lignotuber area (upper 95% CLs = 0.030 and 0.032).

Heights of the tallest shoot also varied amongst hybrids, but not in the same way as measurements for shoots per lignotuber area. Six hybrids had heights of tallest shoot that were below average for the two species (lower 95% limit = 21.73), all but one (an introgressed plant - 15cm) were backcrossed (18cm and 5cm) and first generation hybrids (18cm and 5cm); and all but one (a backcrossed plant in *B. robur* habitat at Appin Road (22cm); lower 95% CL = 24.68cm) were from *B. robur* habitat at Darkes Forest (Figure 4.6D). Four hybrids had average measurements for the two species, an introgressed plant (36cm) in *B. oblongifolia* habitat (95% CI = 28.49 – 40.90), and two (30 and 35cm) in intermediate habitat (95% CI = 19.29 – 39.1), and a backcrossed plant in *B. robur* habitat (25cm) at Appin Road. Two plants had above average heights, one introgressed plant in *B. oblongifolia* (45cm) habitat and one first generation plant in *B. robur* habitat (38cm) had above average measurements for those habitats (upper 95% CL = 40.9 and 36.10cm respectively) (Figure 4.6).

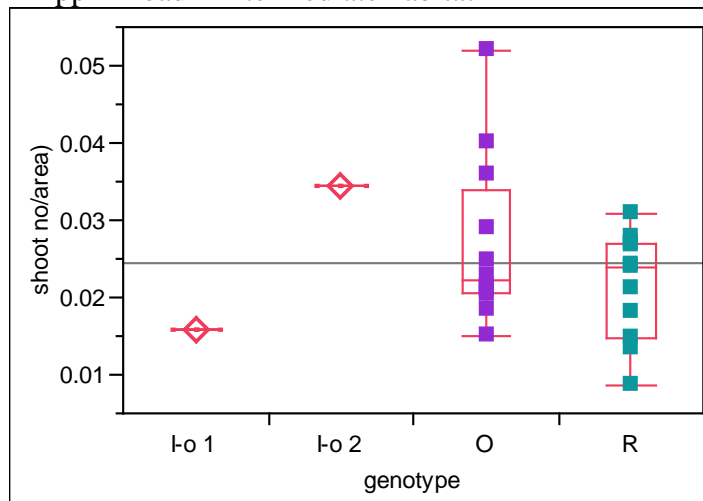
There was no evidence that most hybrid plants were attacked by herbivores any more than parental species plants. All hybrids except two introgressed plants (I-o) had low herbivore damage (< 20%, Figure 4.6).

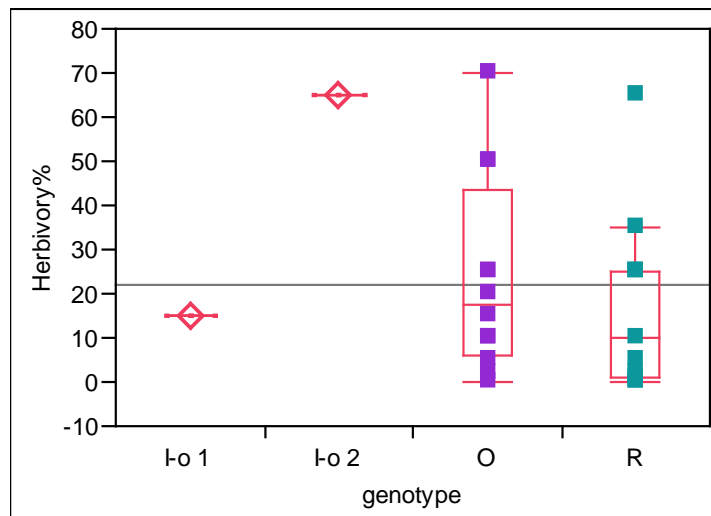
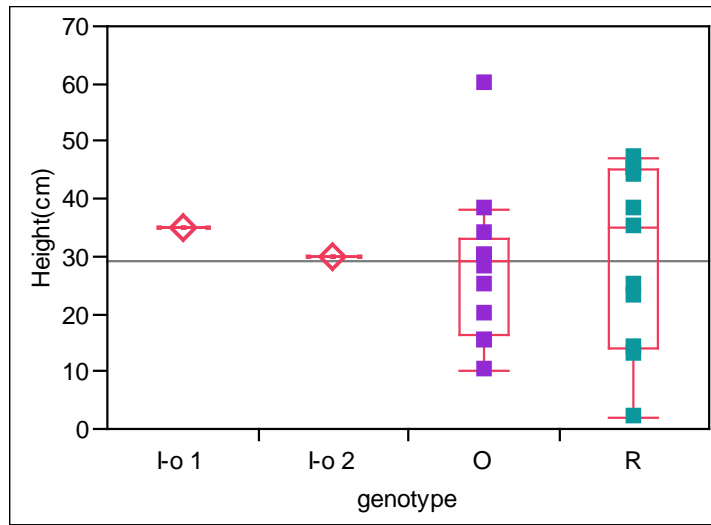
A Appin Road – *B. oblongifolia* habitat



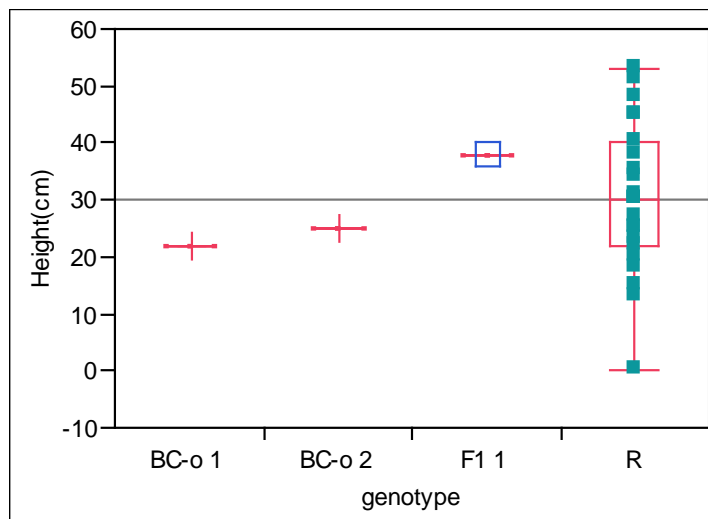
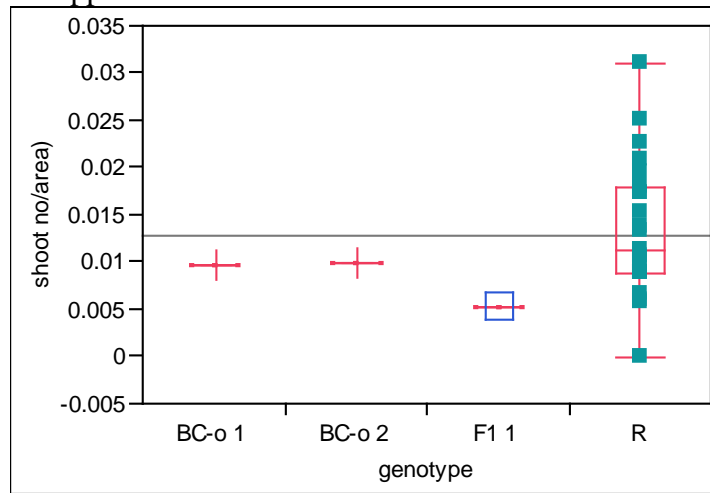


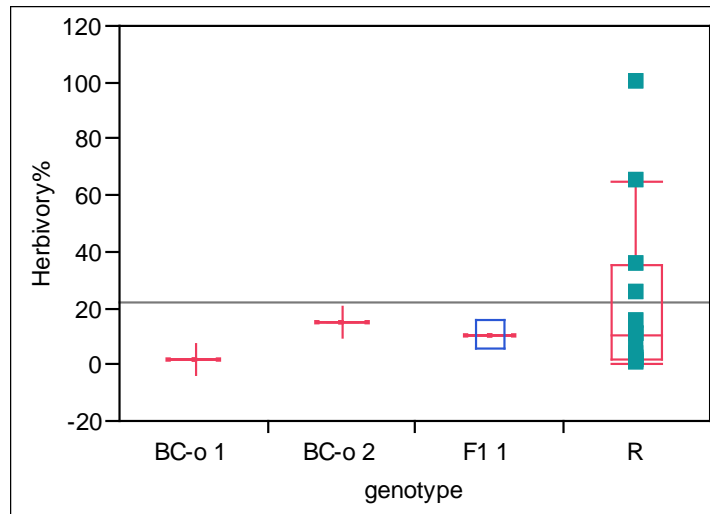
B Appin Road – intermediate habitat



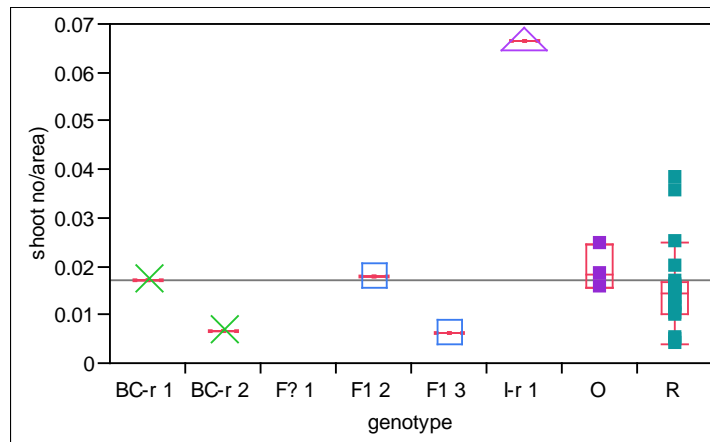


C. Appin Road – *B. robur* habitat





#### D. Darkes Forest – *B. robur* habitat



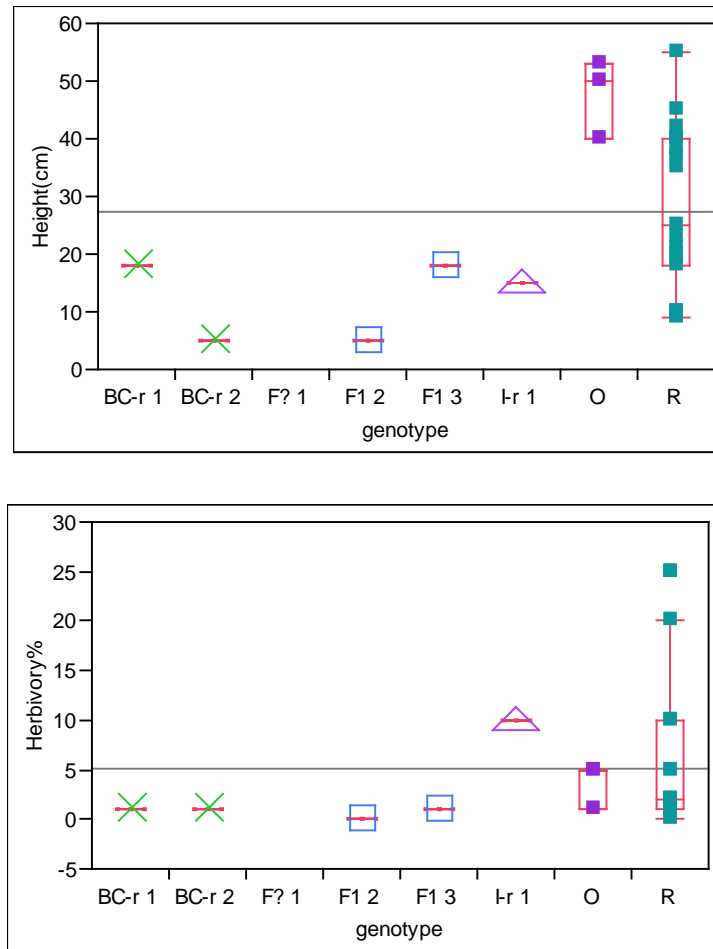
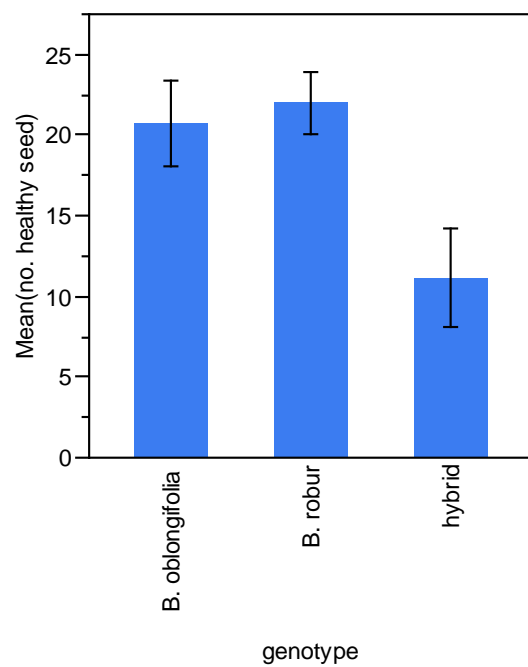
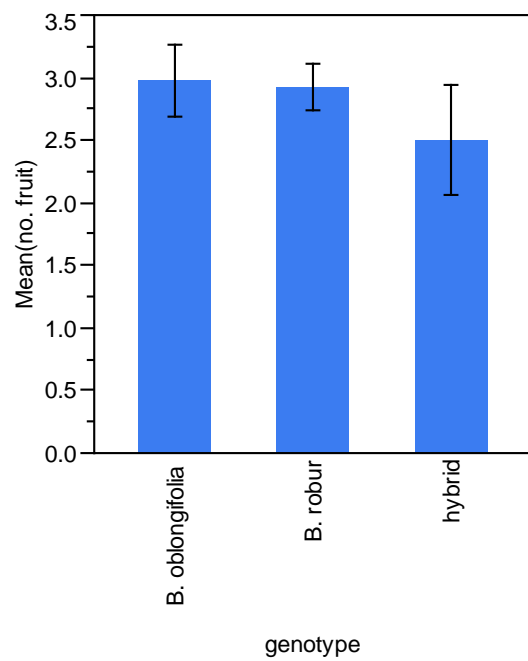


Figure 4.6 Individual measurements of re-growth and herbivore damage of plants that re-sprouted from lignotubers across the two hybrid zones 10 weeks after fire destroyed the canopy. Genotypes include *B. oblongifolia* (O) and *B. robur* (R) and hybrids: first generation (F1), backcross to *B. oblongifolia* (BC-o) or *B. robur* (BC-r), introgressed by a *B. robur* allele (I-o) or *B. oblongifolia* allele (I-r).

#### 4.3.4 *Fruit and seed collection from mature plants*

The distribution of plants, from which seed was collected, was similar to the plants used to measure re-growth (Table 4.2). The quantity and quality of seed collected two weeks after fire across the two hybrid zones was determined for each of the genotypic groups and individual hybrids. Hybrid seeds were too few (125 seeds from 12 plants) and variable between plants (0 to 29 seeds) to provide adequate statistical comparisons with parental species. Nevertheless, it is worth noting that the mean number of healthy seed retrieved from hybrid plants, although not statistically different ( $F_{2,125} = 2.18$ ,  $P = 0.21$ ) ( $\alpha = 0.05$ ,  $\sigma = 16.64$ ,  $\delta = 3.07$   $n = 128$ , Power = 0.44) from the parental species, was half ( $11.17 \pm 4.80$  *s.e*) that of the two parental species ( $20.69 \pm 2.55$ ,  $22.01 \pm 2.03$  *s.e*) (Figure 4.7).





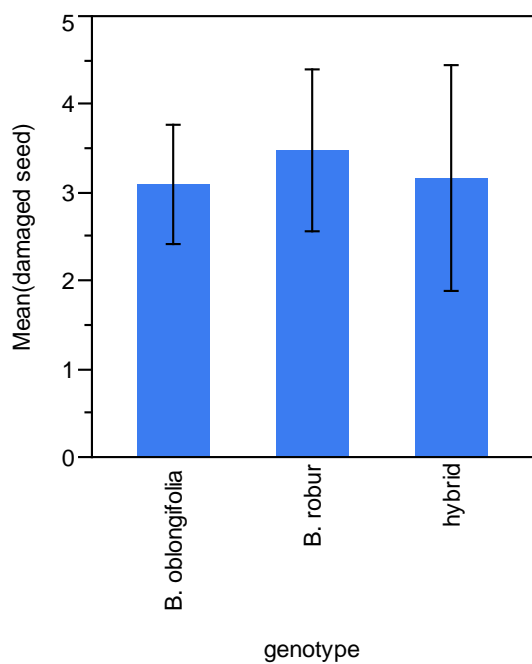
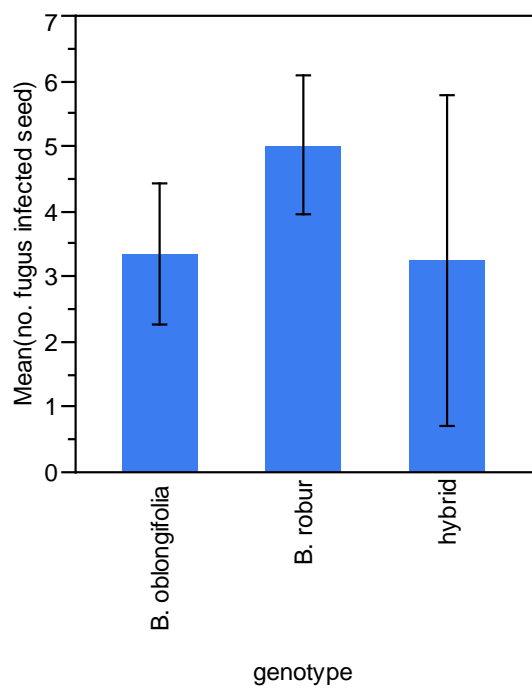


Figure 4.7 Mean quantity and quality of seed collected within the three genotypic groups across the two hybrid zones after fire. Error bars = standard error.

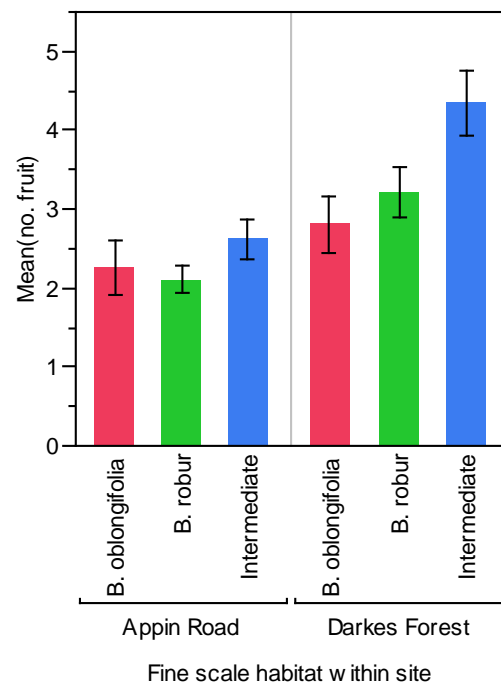
There was no difference between the two species in the average number of fruit collected from plants. There was more fruit collected per plant from plants at Darkes Forest (*B. oblongifolia*  $4.76 \pm 0.40$  and *B. robur*  $3.70 \pm 0.26$  s.e) than Appin Road (*B. oblongifolia*  $2.15 \pm 0.29$  and *B. robur*  $2.47 \pm 0.26$  s.e), particularly in *B. robur* habitat (*B. oblongifolia*  $7.00 \pm 1.01$  and *B. robur*  $3.12 \pm 0.29$  s.e) and in *B. oblongifolia* habitat for *B. oblongifolia* plants ( $3.00 \pm 0.29$  s.e) (  $F_{8,108} = 6.54$ ,  $P < 0.0001$ ) (Figure 48 A). There was no *B. robur* fruit found amongst fruit collected from *B. oblongifolia* habitat.

Although the number of fruit differed between sites, there were the same number of healthy seed collected per plant from the each site and habitat and species ( $F_{8,107} = 1.17$ ,  $P = 0.32$ ) indicating that more seed per fruit were produced at the Appin Road site than at the Darkes Forest site (Figure 48 B).

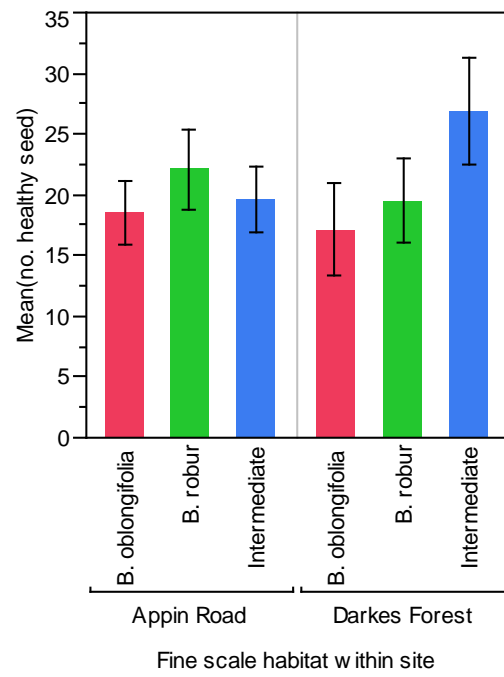
There were more fungus infected seed per plant at Darkes Forest (*B. oblongifolia* least sq mean  $7.03 \pm 2.19$  and *B. robur*  $9.34 \pm 1.40$  s.e) than Appin Road (*B. oblongifolia*  $0.93 \pm 1.58$  and *B. robur*  $1.39 \pm 1.41$  s.e) ( $F_{8,108} = 3.36$ ,  $P = 0.0018$ ), but no difference between habitats (Figure 48 C). The

numbers of damaged seed were the same for genotypes, sites and habitats  
 $(F_{8,108} = 1.41, P = 0.20)$  (Figure 4.8 D).

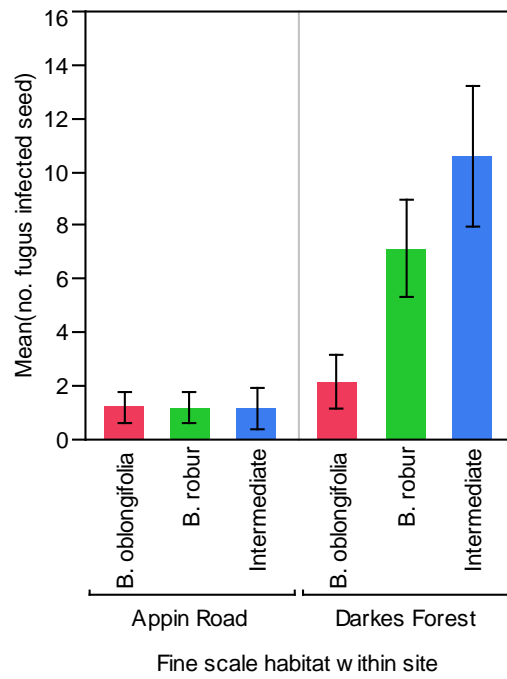
A



B.



C



D

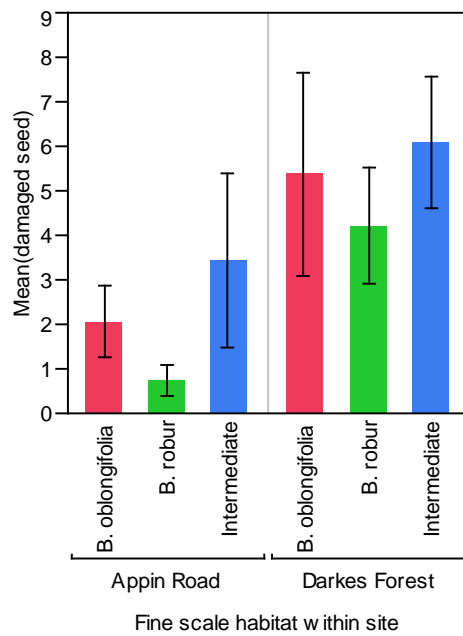


Figure 4.8 Mean quantity and quality of seed collected from plants within the three habitats of the two sites 2 weeks after fire. Error bars = standard error.

As there were some differences in numbers of seeds and fruits collected from parental species between sites and habitats, individual hybrids were compared with the mean and 95% confidence limits (CL) of the parental species within habitats and sites.

Individual hybrid measurements varied widely (Figure 4.10), and, as there were few hybrids (12), patterns may not have been discernable. Nevertheless, two introgressed plants had above average numbers of fruit and healthy seed. Although eight of the hybrid plants had average numbers of fruit, nine had below average numbers of healthy seed. But this was not due to infection or damage of seeds, as only one hybrid had above average fungal infection, and only three had above average damaged seeds.

Numbers of fruit on individual hybrid plants were compared with their parental species. At the Appin Road site (Figure 4.9A), of seven hybrids, two introgressed hybrids, one in *B. oblongifolia* habitat (4 fruits) and the other in intermediate habitat (6 fruits) had above average numbers (upper 95% CL = 2.33 and 3.43 fruits respectively). Two hybrids, an introgressed (2 fruits) in *B. oblongifolia* habitat (95% CL = 1.10 – 2.33) and a first generation hybrid (2

fruits) in *B. robur* habitat (95% CL = 1.86 – 2.56) both had average numbers of fruit. Three hybrids, two backcrossed in *B. robur* habitat (both 1 fruit) and one introgressed in intermediate habitat (1 fruit) had below average (lower 95% CL = 1.86 and 1.84 fruits respectively). At the Darkes Forest site all five hybrids were from *B. robur* habitat. Four hybrids (2 backcrossed and 2 first generation hybrids – 3 fruits each) had average numbers of fruit (95% CLs = 2.83 – 4.89). An introgressed hybrid had fewer than average (1 fruit) (Figure 4.9B).

Numbers of healthy seed from individual hybrid plants was compared with their parental species. At Appin Road, five hybrids, two backcrossed (4 and 12 seeds), one first generation (9 seeds) in *B. robur* habitat and two introgressed, one in *B. oblongifolia* habitat (9 seeds) and one in intermediate habitat (3 seeds), had below average numbers of healthy seed (95% CLs = 12.84 – 27.29 seeds). Two introgressed plants one in *B. oblongifolia* habitat (29 seeds) and one in intermediate habitat (28 seeds), had above average numbers of healthy seed (28 and 29 seeds), (Figure 4.9A). At Darkes Forest, four hybrids, a backcrossed (13 seed), 2 first generation (1 seed each) and an introgressed hybrid (0 seed) had below average numbers of healthy seed (95% CLs = 14.46 – 32.24 seeds). A backcrossed plant had average healthy seeds (25 seeds), (Figure 4.9B).

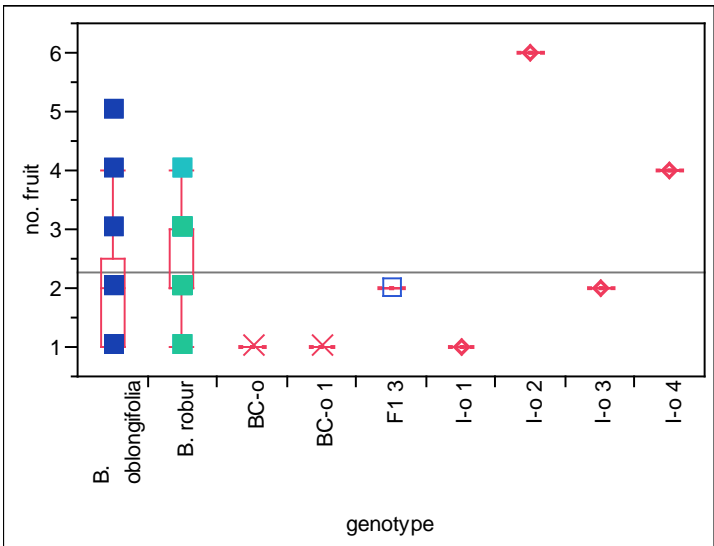


.Numbers of fungus affected seed from individual hybrid plants were compared with their parental species. At Appin Road, four hybrids (2 backcrossed, one first generation in *B. robur* habitat and introgressed plant in intermediate habitat) had no fungal infection of seeds. Three introgressed plants (in intermediate and *B. oblongifolia* habitats- 1 to 3 seeds) had an average number (95% CLs = 0 - 3 seeds) of fungal infected seed (Figure 4.9A). At Darkes Forest four hybrid plants had no seeds with fungal infection, one backcrossed (31 seeds) had above average (95% CLs = 1.55 – 12.10 seeds). (Figure 4.9B).

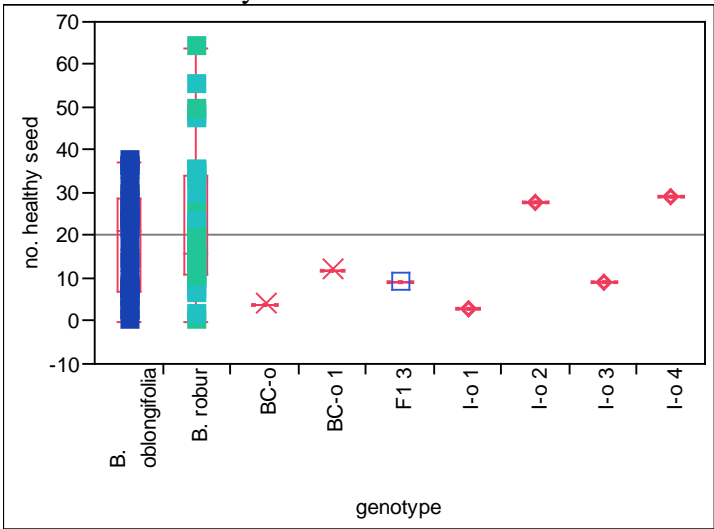
Numbers of damaged seed was also compared. At Appin Road, three hybrids, a first generation (in *B. robur* habitat) and two introgressed (in *B. oblongifolia* and intermediate habitats), had no damaged seed. Two hybrids, a backcrossed in *B. robur* habitat (6 seed) and an introgressed plant in *B. oblongifolia* habitat (11 seed) had above average damage (95% CLs = 0 – 4.06 seeds). An introgressed plant in intermediate habitat (4 seeds) and a backcrossed plant in *B. robur* habitat (1 Seed) had average damaged seed (Figure 4.9A). At Darkes Forest three hybrids, 2 backcrossed and an introgressed plant had no damaged seeds. One first generation had above average (12 seeds) and one first generation (4 seeds) had average seed damage (95% CLs = 1.43 – 7.91 seeds), (Figure 4.9B).

A. Appin Road

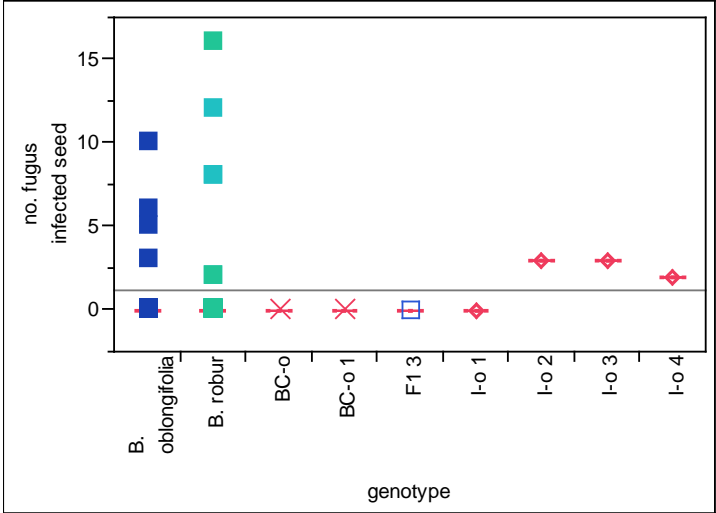
Numbers of fruits



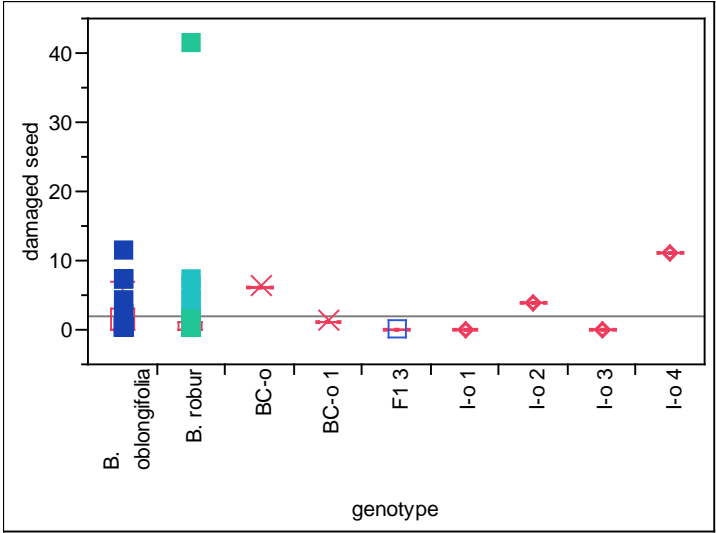
Numbers of healthy seeds



Numbers of fungus infected seeds

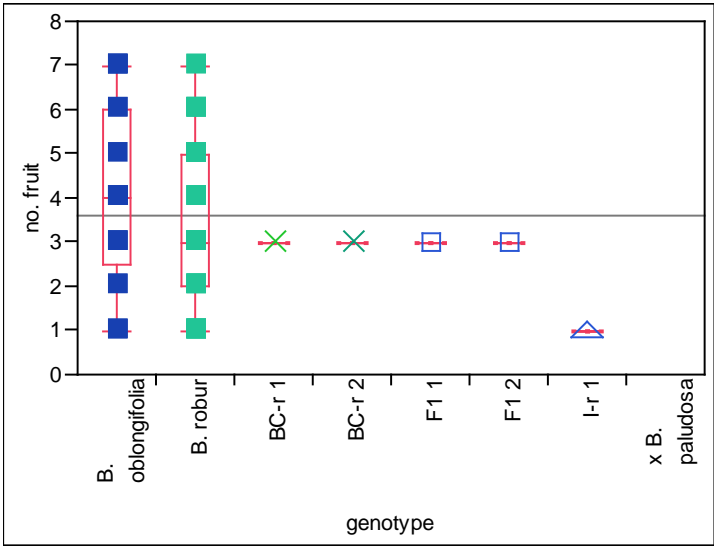


Numbers of damaged seeds

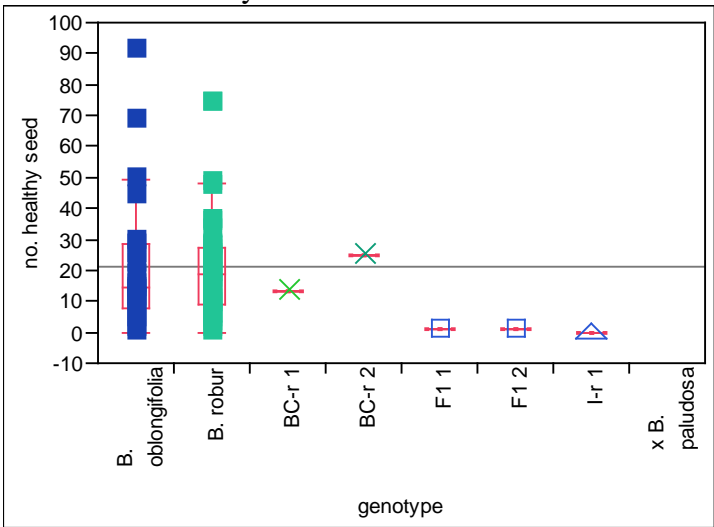


B. Darkes Forest

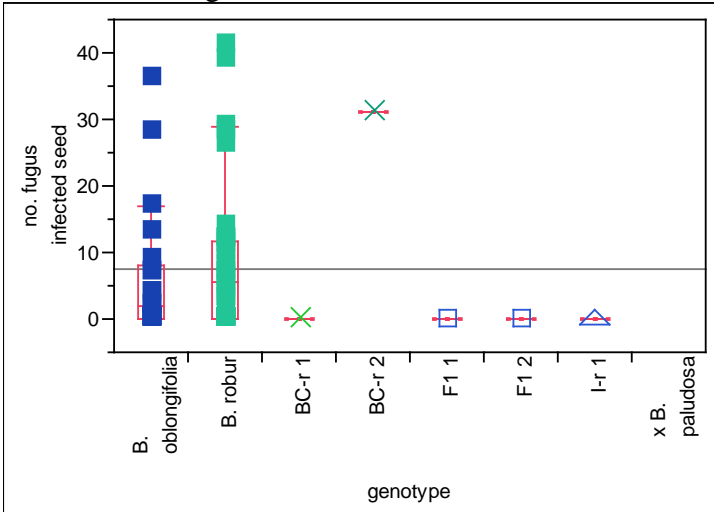
Numbers of fruits



Numbers of healthy seeds



Numbers of fungus infected seeds



Numbers of damaged seeds

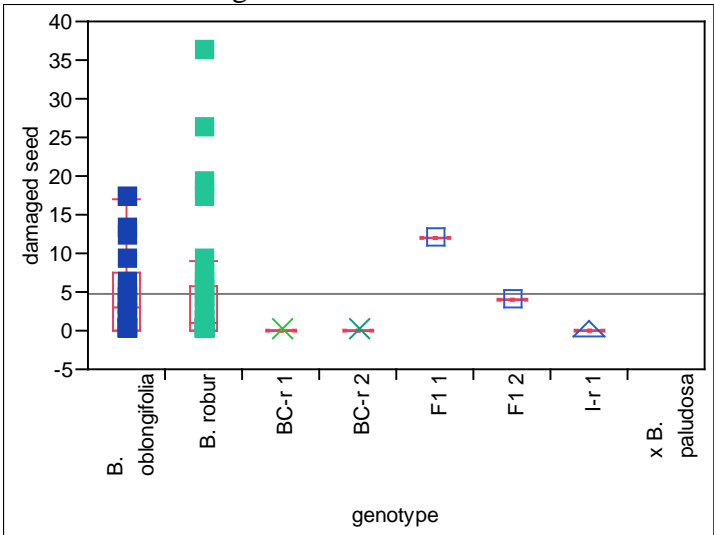


Figure 4.9 Number of fruits, healthy seeds, seeds affected by fungus and damaged seeds collected from individual mature plants across Darkes Forest and Appin Road hybrid zones. Hybrid classes: first generation, F1; backcross to *B. oblongifolia*, BC-o; backcross to *B. robur*, BC-r; *B. oblongifolia* with one *B. robur* allele, I-o; *B. robur* with one *B. oblongifolia* allele, I-r.

#### 4.3.5 Transplant experiment - Germination and survival

Germination of seeds transplanted into the field after 11 weeks (36%) was about half the number that germinated in seed trays (70%, see chapter 5). Some of the difference in germination rates was due to seeds being washed away by heavy rain after transplantation, mainly within the *B. robur* habitats. At the Appin Road site, nine of the ten cages were washed out in *B. robur* habitat and, at Darkes Forest, five of the ten cages in *B. robur* habitat and two of the fifteen in intermediate habitat were washed out. Therefore, 192 of the 840 (23%) seeds were washed away. If these seeds were removed from the original 840 and the germination rate recalculated, then the germination rate becomes almost 50% which is similar to the germination rate in intermediate habitat.

Only small numbers of seeds germinated in parental habitats. Of the seeds planted in *B. robur* habitat 18% germinated (only 10% of all seeds

germinating). Only one site contained *B. oblongifolia* habitat (see ‘*Characterization of habitats*’) in which 27% of seeds planted germinated, (representing only 14% of all seeds germinating). Therefore, germination results mainly reflected germination in intermediate habitats (76% of all seeds germinating) (Table 4.3 B).

Table 4.3 Seeds transplanted (tran), seed germinating (germ) and seedling survival (surv) after two years within the genotypic groups, *B. oblongifolia*, *B. robur*, and hybrid classes. Each habitat in A, B, and C contained the same proportion of seeds from known maternal plants, half of which was placed into each site (\* statistical differences between proportion transplanted and proportion germinating; <sup>+</sup> numbers small, ChiSquare suspect)

A

ATransplan t Habitats	<i>B. oblongifolia</i> (Darkes Forest)			Intermediate 1 (Appin Road)		
Genotypic groups	No. of seeds			No. of seeds		
	Tran	Germ	Surv	Tran	Germ	Surv
<i>B. oblongifolia</i>	61	21*	18	61	28*	23
<i>B. robur</i>	38	0*	1	38	30*	25
<i>B. paludosa</i>	13	2	1	13		
I-o	5	1		5	1	1
BC-r	1					
F?	2			3	1	
F1					1	1
BC-o		1	1		1	1
Unknown		7	6		8	6

B

Transplant Zones	Intermediate 2 (Darkes Forest)			Intermediate 2 (Appin Road)		
Genotypic groups	No. of seeds			No. of seeds		
	Tran	Germ	Surv	Tran	Germ	Surv
<i>B. oblongifolia</i>	61	14	14	60	33	26
<i>B. robur</i>	95	21	17	96	53	36
<i>B. paludosa</i>	13			13		
I-o	5	1	1	5	1	1
BC-r	3	1	1	3	1	1
F?	3			3	1	1
F1					2	2
BC-o					1	1
I-r		1	1			
Unknown		23	20		10	8

C

Transplant Zones	<i>B. robur</i> (Darkes Forest)			<i>B. robur</i> (Appin Road)		
Genotypic groups	No. of seeds			No. of seeds		
	Tran	Germ	Surv	Tran	Germ	Surv
<i>B. oblongifolia</i>	27	2*	1	27	5 <sup>+</sup>	4
<i>B. robur</i>	86	24*	17	86	6 <sup>+</sup>	5
I-o	2			2		
BC-r	3	2	1	3		
F?	2	1	1	2		
Unknown		3	1			



The proportion of seed transplanted from each genotypic group (a mixture of known maternal genotypes that were genotyped using microsatellite makers) was compared with the proportion seed germinating (genotyped using microsatellite makers) and the proportion seedlings surviving after two years. At the Appin Road site, in the first hybrid habitat (originally classified as *B. oblongifolia* habitat through position on slope, but redefined because of soil moisture content), there appeared to be selection against *B. oblongifolia* genotypes. Of the transplanted seed (8 hybrid, 61 *B. oblongifolia* and 38 *B. robur*), fewer *B. oblongifolia* seed germinated than *B. robur* seed (4 hybrid, 28 *B. oblongifolia* and 30 *B. robur*), ( $\chi^2 = 5.93$ ,  $DF = 2$ ,  $P = 0.05$ ), (Table 4.3A). Conversely, there was evidence for selection against the germination of *B. robur* seed in *B. oblongifolia* habitat ( $\chi^2 = 21.5$ ,  $DF = 2$ ,  $P < 0.0001$ ) at the Darkes Forest site (Table 4.3A). Of the transplanted seed (8 hybrid, 61 *B. oblongifolia* and 38 *B. robur*), no *B. robur* seed germinated (2 hybrid and 21 *B. oblongifolia*). Unfortunately there was no *B. oblongifolia* habitat at Appin Road to compare with this result

*B. robur* habitat at Appin Road was badly affected by heavy rain and only 11 of the 120 seeds germinated, 5 *B. oblongifolia* and 6 *B. robur* seedlings (Table 4.3C). Although *B. robur* habitat was not as badly washed out at Darkes Forest, the proportion of seed transplanted may have been biased. Although

hybrid numbers were extremely small (15) they germinated in all three habitat types (Table 4.3A, B and C). Twelve hybrids were found in intermediate habitat and, not surprisingly as seedlings were few, only two hybrids were found in *B. robur* habitat and one in *B. oblongifolia* habitat. Hybrids consisted of first generation hybrids (1% of all germinating seedlings) and a range of later generation, backcrossed and introgressed hybrids (5.7 % of all germinating seedlings).

The number of seeds germinating varied between sites but not between habitats within sites (excluding *B. robur* habitat) ( $\chi^2 = 24.34$ ,  $DF = 3$ ,  $P < 0.0001$ ). More seeds germinated at the Appin Road site (58.3%) than the Darkes Forest site (34.3%).

Overall 64 seedlings died, in the first two years, representing 20% of total seeds that originally germinated. There was no difference in the proportion of deaths between *B. robur* (37 seedlings or 25%) and *B. oblongifolia* (21 seedlings or 15%) when compared to seedlings that germinated ( $\chi^2 = 0.61$ ,  $DF = 2$ ,  $P = 0.74$ ). Hybrid survival rate after two years was very good with only one death (6%) amongst the 16 hybrids. There was no evidence for selection against either species or their hybrids between sites or between habitats within sites during the first two years of growth ( $\chi^2 = 21.28$ ,  $DF = 15$ ,  $P = 0.13$ ) (Figure 4.10).

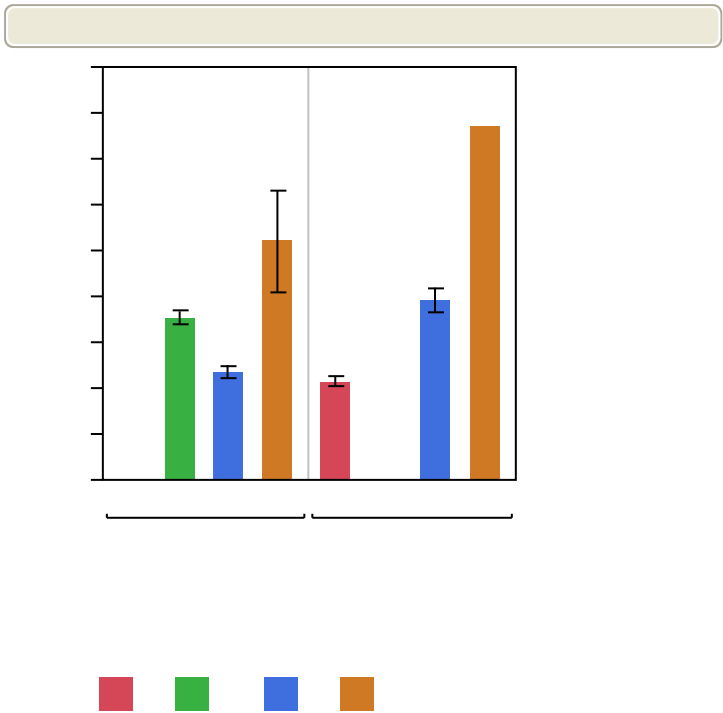
#### 4.3.6 Growth of transplanted seed after two years.

The mean growth of transplanted parental and hybrid seedlings after two years was compared. Because there were so few numbers of each species in their non-native habitats, height, leaf number and herbivore damage could not be compared between species in parental habitats. Therefore, comparisons between the two species are mainly made in intermediate habitat.

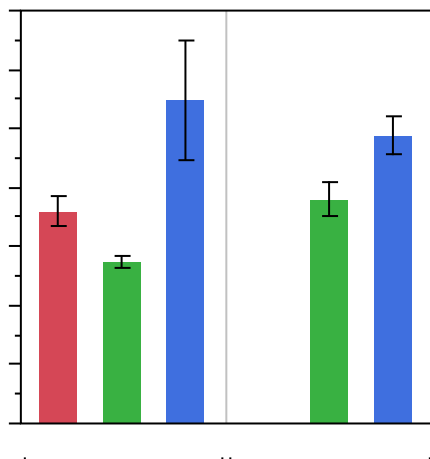
There were genotype, genotype within site, and genotype within zone within site differences in the heights of seedlings ( $F_{15,230} = 10.58$ ,  $P < 0.0001$ ). Hybrid seedlings were shorter, on average, ( $16.54 \pm 1.62\text{cm s.e}$ ) than *B. oblongifolia* ( $20.72 \pm 1.13\text{cm}$ ) and *B. robur* seedlings ( $20.70 \pm 0.66\text{cm}$ ). *B. oblongifolia* seedlings were shorter at Appin Road ( $18.49 \pm 1.08\text{cm}$ ) than at the Darkes Forest site ( $22.95 \pm 1.98\text{cm}$ ). *B. oblongifolia* seedlings were shorter within the drier intermediate habitat (see Figure 4.2) of the Appin Road site ( $11.83 \pm 1.05\text{cm}$ ) than the wetter intermediate habitat ( $17.63 \pm 1.12\text{cm}$ ) and *B. robur* habitat ( $26.00 \pm 2.87\text{cm}$ ) and shorter in *B. oblongifolia* habitat at Darkes Forest ( $10.76 \pm 1.10\text{cm}$ ) than intermediate ( $19.58 \pm 1.17\text{cm}$ ) and *B. robur* habitat ( $38.50 \pm 5.73\text{cm}$ ). *B. robur* seedlings were shorter in the drier intermediate habitat ( $13.72 \pm 0.89\text{cm}$ ) than in the wetter intermediate habitat ( $17.98 \pm 1.08\text{cm}$ ) and in *B. robur* habitat ( $27.40 \pm 2.56\text{cm}$ ) at Appin Road and

shorter in intermediate habitat ( $19.02 \pm 1.17$ ) than *B. robur* habitat ( $24.36 \pm 1.35$ ) at the Darkes Forest site (Figure 4.10).

(A)



(B).



(C).

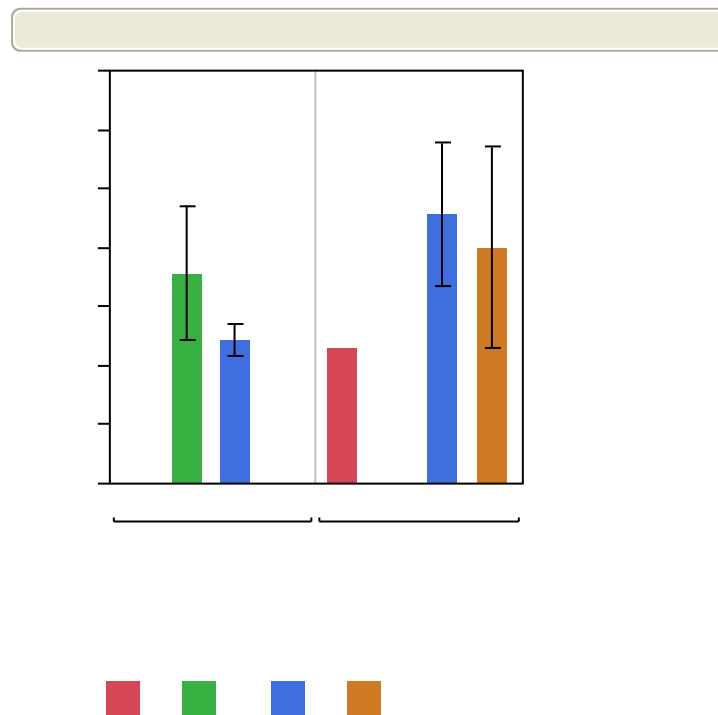


Figure 4.10 Average height of (A)*B. oblongifolia* (O), (B) *B. robur* (R) and (C) hybrid (H) seedlings two years after transplantation as seed into the two hybrid zones, Appin Road (site1) and Darkes Forest (site 2). Zones of transplant within sites include *B. oblongifolia* (1), intermediate (1a, 2) and *B. robur* (3) habitats.

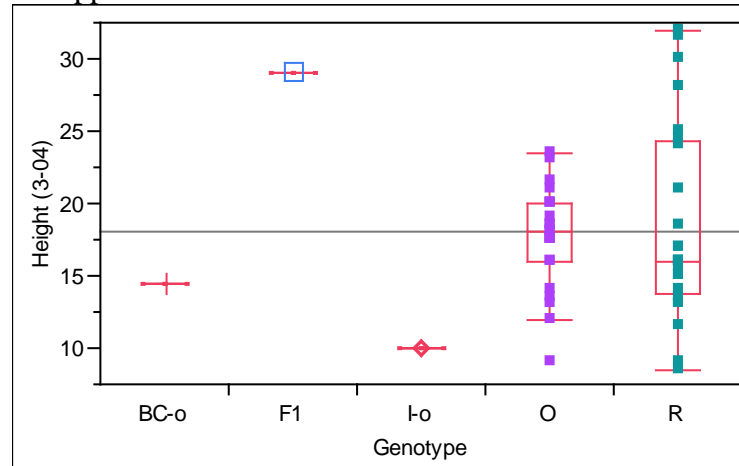
As there were differences between habitats and sites, the heights of individual hybrid seedlings were compared within sites and habitats with the 95%

confidence limits (CL) of the mean heights of the species present. If both species were present in substantial numbers then the highest and lowest 95% CLs were used regardless of species. There were no hybrids in *B. robur* habitat at Appin Road. Hybrid seedling performance was variable and not predictable according to genotype, habitat or the types of measurements. However, there were few hybrids (15) and if there were patterns they were not discernable.

The heights of individual hybrids were compared with the 95% CLs of the averages for the two species within habitats and sites. Heights varied considerably between hybrids even within the same broad genotypic classes. Three of the fifteen hybrids had heights within the 95% CLs of the two species (Figure 4.11). A first generation (11.5cm) in intermediate habitat (95% CL = 10.48 – 15.15cm) at Appin Road and at Darkes Forest a backcrossed hybrid (11.5cm) in *B. oblongifolia* habitat (95% CL = 9.69 -12.90cm) and an introgressed hybrid (18.5cm) in intermediate habitat (95% CL = 13.95 – 24.59cm) had average heights. Seven hybrid heights were below the lower 95% CL for the two species. They included, two introgressed hybrids, one in each intermediate habitat (10cm, lower 95% CL = 15.3cm and 8.5cm, lower 95% CL = 10.48cm), (Figure 4.11A and B); and three backcrossed hybrids one in the first intermediate habitat (14.5cm, CL = 15.3cm) (Figure 4.11A) and two in the other intermediate habitat (10cm each, lower 95% CL = 10.48cm)

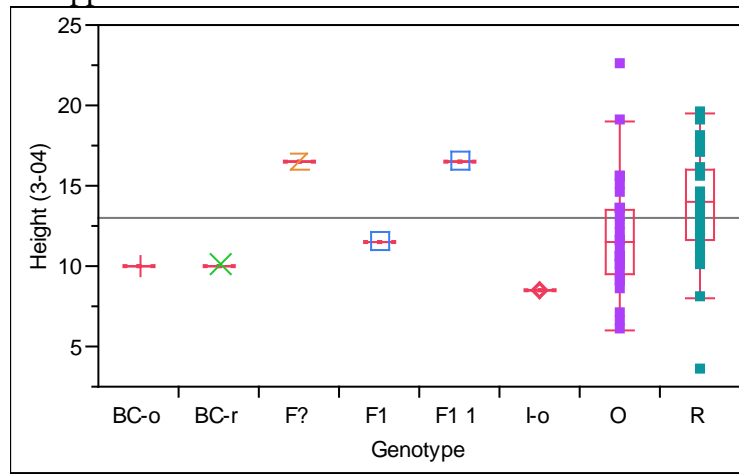
(Figure 4.11B) were below average at the Appin Road site. At Darkes Forest an introgressed in intermediate habitat (11.5cm, lower 95% CL = 13.95cm), (Figure 4.11D) and a later generation in *B. robur* habitat (11.5cm, lower 95% CL = 20.10cm) were below average (Figure 4.11E). Four hybrids had above average heights. Two first generation hybrids (29cm, upper 95% CL = 20.62cm; 16.5cm, upper 95% CL = 15.19cm) in the two intermediate habitats along with a later generation hybrid (16.5 cm) at Appin Road (Figure 4.11A and B) were above average in height. At Darkes Forest in intermediate habitat (upper 95% CL = 24.59cm) a backcrossed hybrid (40cm) and in *B. robur* habitat (95% CL = 20.10 – 27.59) (Figure 4.11D) a backcrossed hybrid (28.5cm), (Figure 4.11E) were above average.

A. Appin Road site - intermediate 1 habitat

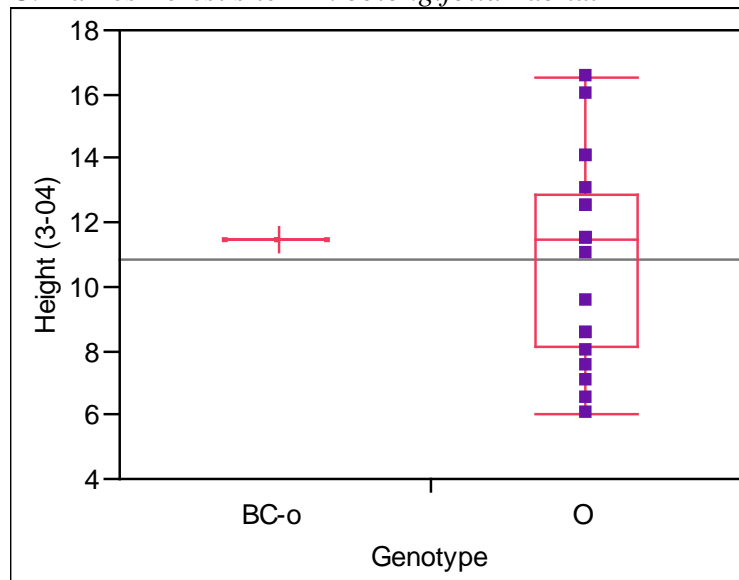




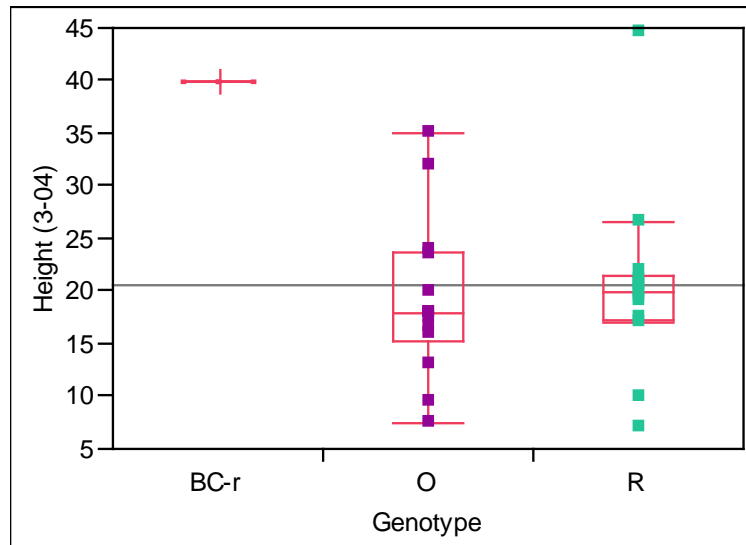
B. Appin Road site - intermediate 2 habitat



C. Darkes Forest site – *B. oblongifolia* habitat



D. Darkes Forest site – Intermediate habitat



E. Darkes Forest site – *B. robur* habitat

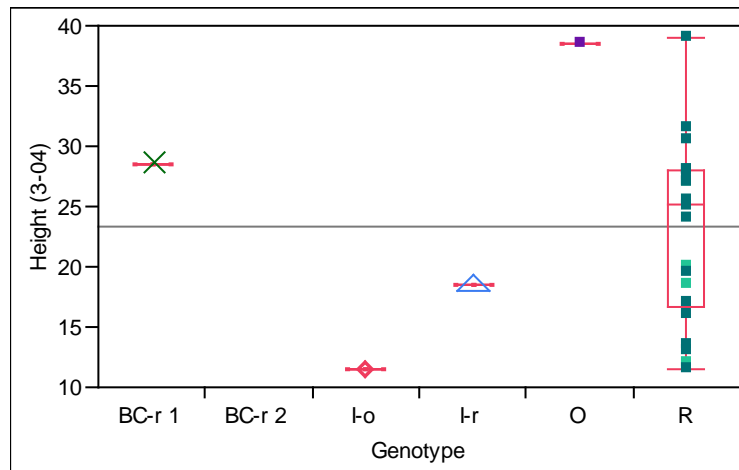


Figure 4.11 Heights of individual seedlings transplanted into the hybrid zones as seed and after two years of growth. Genotypic groups include *B. oblongifolia* (O), *B. robur* (R), and hybrid classes (first generation, F1; backcross to *B. oblongifolia*, BC-o; backcross to *B. robur*, BC-r; Later

generation F?; *B. oblongifolia* with one *B. robur* allele, I-o; *B. robur* with one *B. oblongifolia* allele, I-r).

There were habitat and site differences in the number of leaves on the transplanted seedlings ( $F_{15,230} = 11.20$   $P < 0.0001$ ). There was no statistical difference in the number of leaves on each species. There were more leaves on hybrid seedlings at Darkes Forest ( $13.00 \pm 1.89$  s.e) than at the Appin Road site ( $6.67 \pm 1.51$ ). Hybrid seedlings had fewer leaves in the intermediate habitat ( $7.50 \pm 2.14$ ) at Darkes Forest than in *B. robur* ( $14.50 \pm 3.03$ ) and *B. oblongifolia* habitats ( $17.00 \pm 4.28$ ). *B. oblongifolia* seedlings, as well as being shorter, had fewer leaves in the drier intermediate habitat at Appin Road ( $7.33 \pm 0.78$ ) than the wetter intermediate habitat ( $10.08 \pm 0.84$ ), but more leaves in intermediate habitat at Darkes Forest ( $15.46 \pm 0.84$ ) than in *B. oblongifolia* ( $12.30 \pm 0.82$ ) and *B. robur* habitat ( $7.00 \pm 4.28$ ). *B. robur* seedlings had fewer leaves in the dryer intermediate habitat ( $4.46 \pm 0.67$ ) than the wetter intermediate habitat ( $5.96 \pm 0.81$ ) and *B. robur* habitat ( $10.20 \pm 1.92$ ) at Appin Road and fewer leaves in the intermediate habitat ( $5.67 \pm 0.87$ ) than the *B. robur* habitat ( $9.44 \pm 1.01$ ) at Darkes Forest (Figure 4.12).

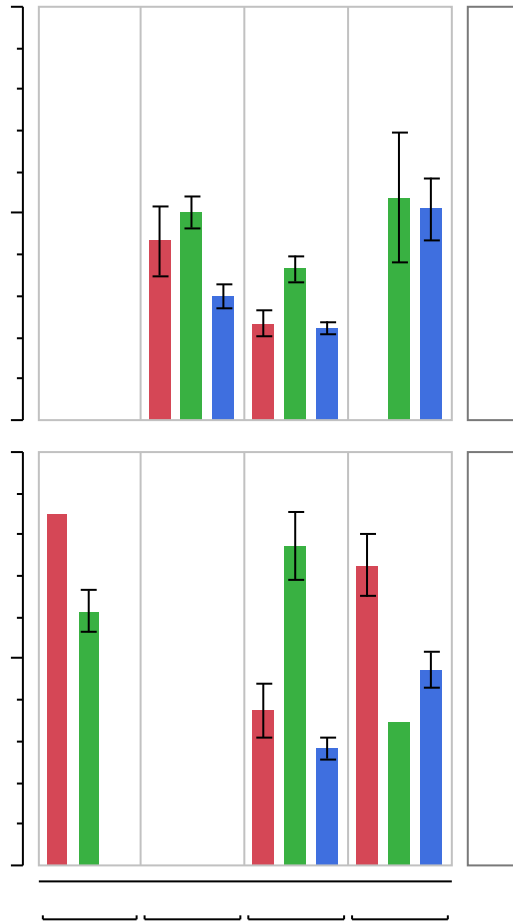
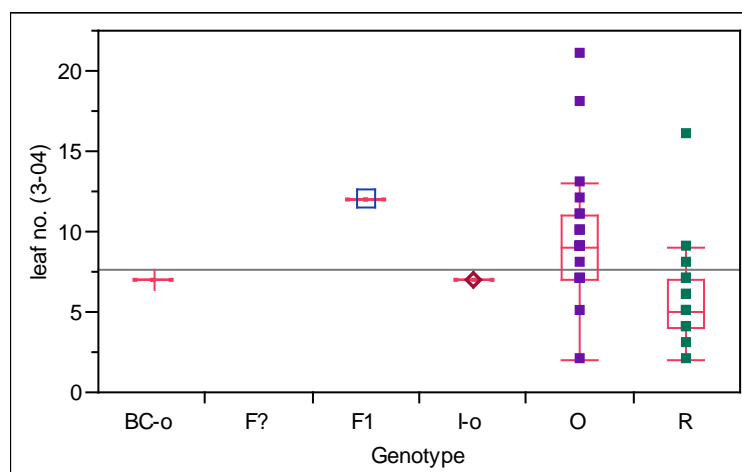


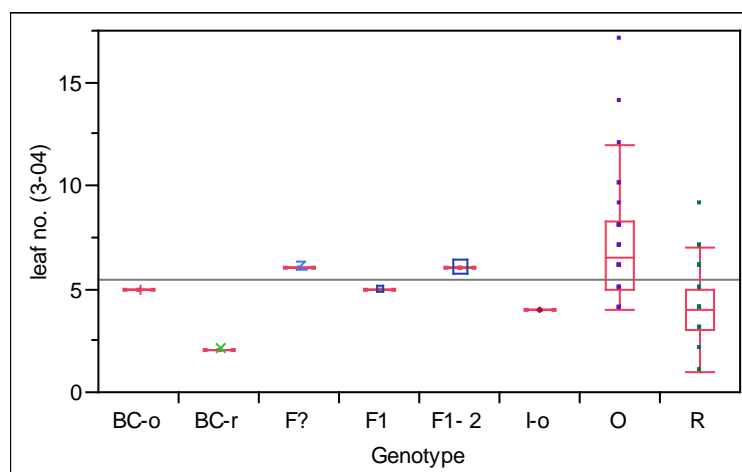
Figure 4.12 Number of leaves on seedlings in the three intermediate habitats two years after transplantation of seed.

The numbers of leaves on individual hybrids were compared with the 95% CLs of averages for the parental species within habitats and sites. As for heights, the numbers of leaves per plant also varied greatly between hybrid seedlings, although most (10) were intermediate (between 95% CLs for the two species in each habitat within each site). In the first intermediate habitat at Appin Road (Figure 4.13A) a first generation plant had above (12 leaves) the upper 95% CL (11.04 leaves). This seedling was large also having above average in height. A backcrossed seedling (17 leaves) in *B. oblongifolia* habitat at Darkes Forest (Figure 4.13C) (upper 95% CL = 16.72) and a later generation hybrid (16 leaves) in *B. robur* habitat (upper 95% CL = 15.18) (Figure 4.13E), also had above average numbers of leaves, but average height. A backcrossed plant in intermediate habitat at Appin Road (lower 95% CL = 3.42) (Figure 4.13B) was a small seedling (2 leaves) and below average height.

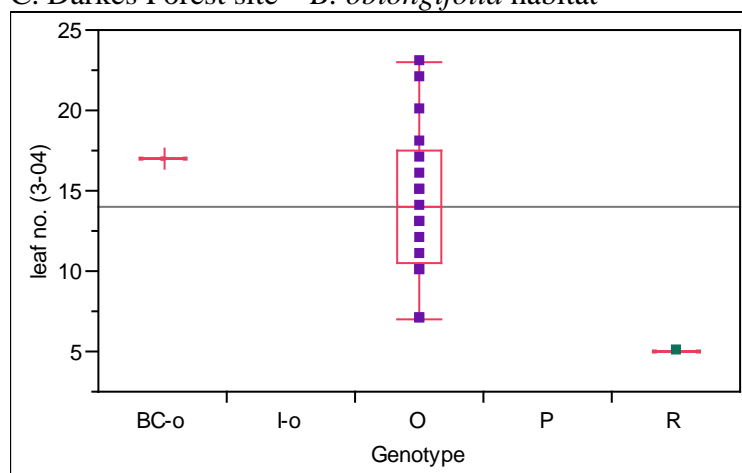
A. Appin Road site - intermediate 1 habitat



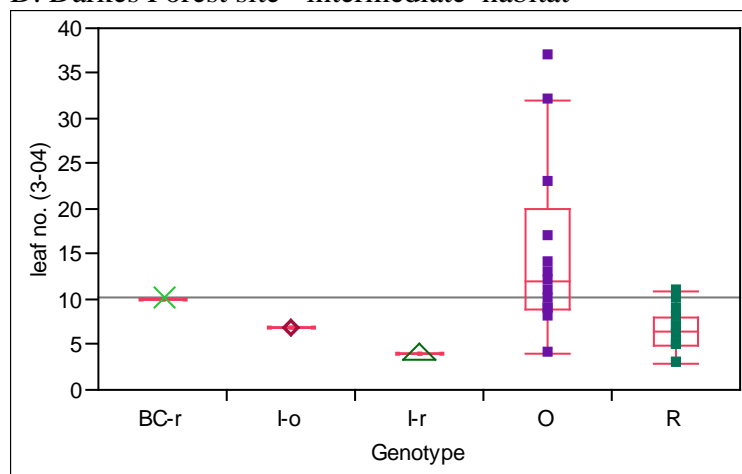
B. Appin Road site - intermediate 2 habitat



C. Darkes Forest site – *B. oblongifolia* habitat



D. Darkes Forest site - intermediate habitat



E. Darkes Forest site – *B. oblongifolia* habitat

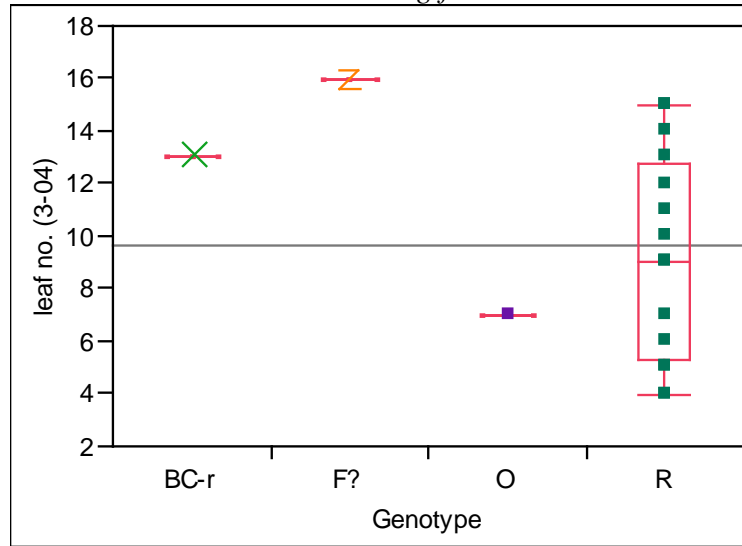


Figure 4.13 Numbers of leaves on individual transplanted seedlings after two years of growth. O = *B. oblongifolia*, R = *B. robur* and hybrid classes (first generation, F1; later generation F?; backcross to *B. oblongifolia*, BC-o; backcross to *B. robur*, BC-r; *B. oblongifolia* with one *B. robur* allele, I-o; *B. robur* with one *B. oblongifolia* allele, I-r).

#### 4.3.7 Herbivore damage

There was very little difference in herbivore damage across the two hybrid zones ( $F_{15,230} = 1.86$ ,  $P = 0.028$ ). *B. robur* seedlings had higher herbivore damage at Appin Road ( $27.56 \pm 3.09\%$ ) than at Darkes Forest ( $15.71 \pm 2.83\%$ ). Hybrid seedlings incurred greater herbivore damage in the wetter



intermediate habitat at Appin Road ( $36.67 \pm 10.50$ ), (Figure 4.14).

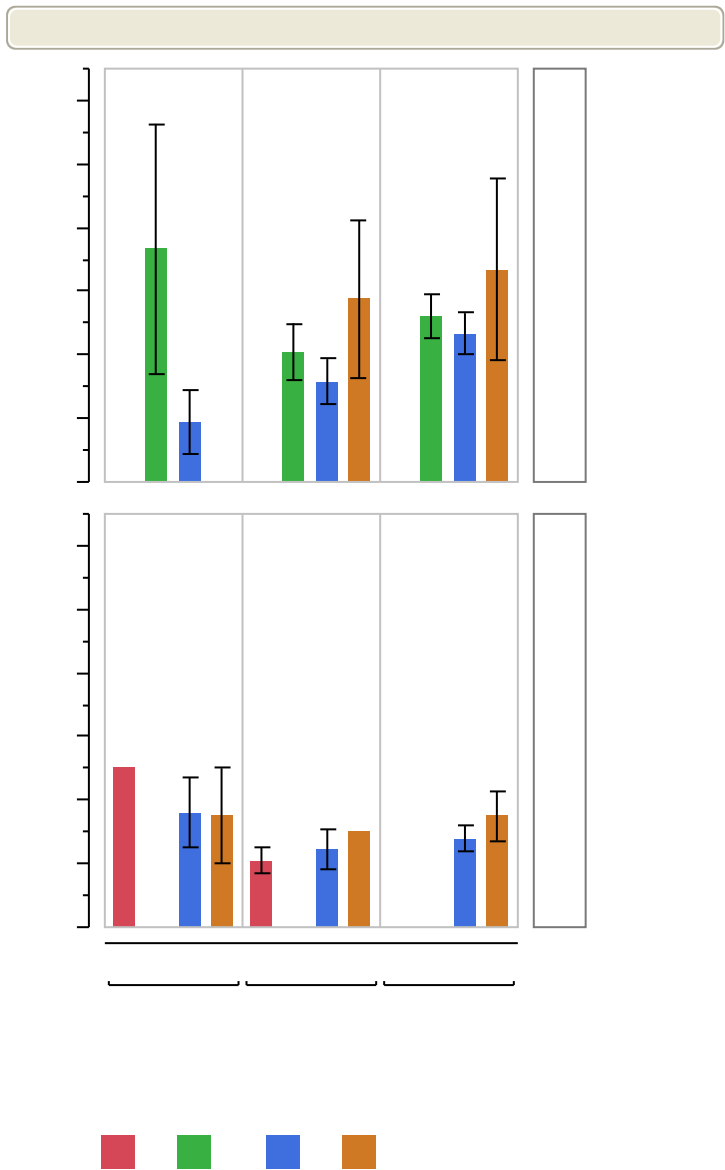


Figure 4.14 Mean percentage herbivore damage on transplanted seedlings after two years of growth within sites, Appin Road (site 1) and Darkes Forest (site

2) and within habitats, *B. oblongifolia* (zone 1), intermediate (zone 1a and 2), and *B. robur* (zone 3).

All three types of leaf damage, edge, hole and miner damage, were found on seedlings of both species and hybrids in all three habitat types, *B. oblongifolia*, *B. robur* and intermediate habitats, and at both sites (Figure 4.17 A and B). There was no difference in damage to the edge of leaves found between the three genotypes (*B. oblongifolia*, hybrid and *B. robur*), or between genotypes within sites, or between genotypes within habitats within sites ( $F_{15,229} = 1.64$ ,  $P = 0.064$ ). There was little difference in the amount of holes found in leaves ( $F_{15,230} = 2.07$ ,  $P = 0.012$ ). Fewer holes were found in leaves on *B. oblongifolia* seedlings growing in the drier intermediate zone ( $0.71 \pm 0.21$ ) at Appin Road than the wetter intermediate zone ( $1.43 \pm 0.28$ ) and *B. robur* habitat ( $1.90 \pm 0.45$ ), although this could have been due to the seedlings having fewer leaves. Miner damage was also variable ( $F_{15,232} = 2.37$ ,  $P = 0.0034$ ), but only amongst hybrid genotypes and not the two species. On average, hybrid plants had more leaves damaged by miners ( $3.79 \pm 0.66$ ) than *B. oblongifolia* ( $2.45 \pm 0.46$ ) and *B. robur* seedlings ( $1.61 \pm 0.27$ ) particularly in the wetter intermediate habitat

of Appin Road ( $6.67 \pm 1.35$ ), (Figure 4.15).

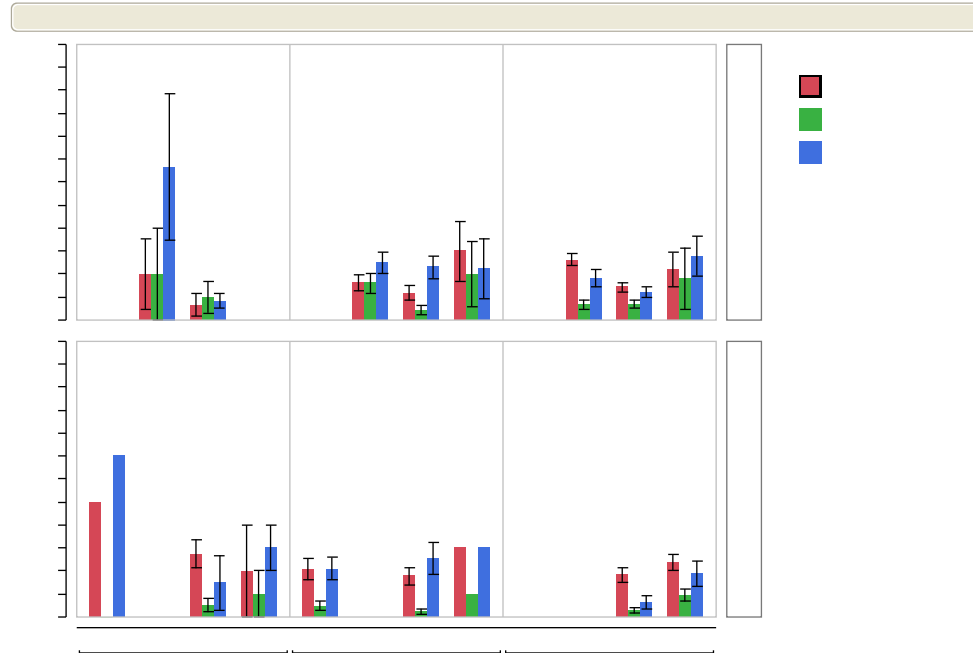
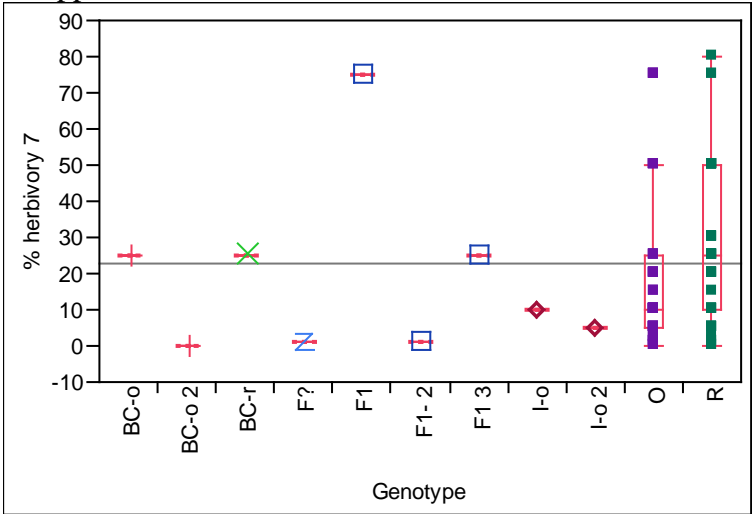


Figure 4.15 The average number of leaves on *B. oblongifolia* (O) and *B. robur* (R) and hybrid (H) seedlings affected by three types of herbivore damage within *B. oblongifolia* (1), intermediate (1a, 2), and *B. robur* (3) habitats across the two contact zones at Appin road site (1) and at Darkes Forest site (2).

Individual hybrids at Appin Road all had low (0 to 10%) or average (95% CL = 13.59 to 31.67%) herbivore damage, except for one first generation hybrid that had above average (75%) herbivore damage (Figure 4.16A). At Darkes

Forest, three hybrids, two backcrossed and one introgressed hybrid had above average (25% each), (95% CL = 7.37 to 19.94%) herbivore damage. A later generation hybrid had average herbivore damage (10%) and an introgressed hybrid, had below average herbivore damage (2%), (Figure 4.16B).

A Appin Road site



B. Darkes Forest site

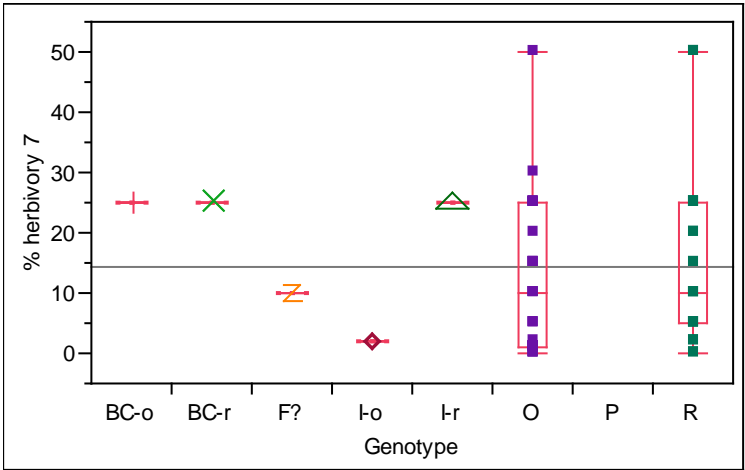


Figure 4.16 Percentage herbivore damage on individual transplanted seedlings after two years of growth. O = *B. oblongifolia*, R = *B. robur* and hybrid classes (first generation, F1; later generation F<sub>2</sub>; backcross to *B. oblongifolia*, BC-o; backcross to *B. robur*, BC-r; *B. oblongifolia* with one *B. robur* allele, I-o; *B. robur* with one *B. oblongifolia* allele, I-r).

#### 4.4 Discussion

##### 4.4.1 Mature plants and seed

Amongst mature plants, as expected, *B. oblongifolia* plants were predominant in the drier soils designated as *B. oblongifolia* habitat and *B. robur* plants were predominant in the wetter soils designated as *B. robur* habitat. However, in intermediate habitat where the water content of the soil was intermediate between the two parental species habitats, similar numbers of each species were found. Possibly divergent selection was operating in parental habitats but absent in intermediate habitats. Campbell *et al.* (2008) found a “relaxation of selection” within intermediate habitat of *Ipomopsis* hybrid zones, where there was no difference in fitness between the two parental species or their hybrids. An alternative explanation could be that there is a graduation of selective forces across hybrid zones from *B. oblongifolia* to *B. robur* habitat related to the graduation of soil moisture (Keith and Myerscough, 1993) where *B.*

*oblongifolia* plants are fitter in habitat closer *B. oblongifolia* habitat and *B. robur* plants are fitter in habitat closer to *B. robur* habitat. Given the patchiness of the hybrid zones, further investigation into these two theories would require very fine scale soil measurements.

Interestingly, amongst this sample of mature plants, only hybrids introgressed with one *B. robur* allele were found within *B. oblongifolia* and intermediate habitats at Appin Road; and no hybrids were found within *B. oblongifolia* and intermediate habitats at Darkes Forest. However, in *B. robur* habitat at both sites, there was a range of hybrid genotypic classes. This suggests either selection against *B. robur* genotypes in habitats outside *B. robur* habitats or a greater flow of pollen from *B. oblongifolia* plants to *B. robur* plants, or a combination of both. Seeds are winged and wind dispersed and pollen is mainly dispersed by honeyeaters (Schibeci 1994). Although both seed and pollen appear to disperse over short distances, pollen dispersal has the potential to occur over larger distances (Schibeci 1994). Schibeci (1994) found some evidence that gene flow was asymmetrical with pollen movement most likely from *B. robur* to *B. oblongifolia*, as evidenced by a low frequency of *B. robur* alleles in pure *B. oblongifolia* populations. Conversely, in this study, in pure stands (Usher *et al.* 2010) one hybrid with a *B. oblongifolia* allele was found in a *B. robur* population suggesting introgression of *B. oblongifolia*

genes into *B. robur* populations. Furthermore, seedling and maternal plants genotypes (Chapter 5) revealed that gene flow is occurring in both directions mainly facilitated by existing hybrids. Schibeci (1994) also found gene flow in both directions. Nevertheless, in the study area, *B. oblongifolia* populations are larger and more extensive than *B. robur* populations. Consequently, *B. robur* populations are more likely to be swamped with pollen from *B. oblongifolia* plants. In the transplant experiment, evidence for selection against *B. robur* alleles was found in *B. oblongifolia* habitat at Darkes Forest, where no *B. robur* seeds germinated, but there was no evidence for selection against *B. robur* seedlings in any of the intermediate habitats.

There was no evidence for selection against hybrid plants as they re-sprouted 10 months after fire. Hybrid plants and their parental species were similar in their ability to re-sprout. Interestingly, apart from four introgressed plants, all first generation and later generation plants, including backcrosses, were found in *B. robur* habitat. Furthermore, the hybrid plants in *B. robur* habitat had average performance measurements similar to *B. robur* plants, but not similar to *B. oblongifolia* plants. Individual hybrid performances varied from below to above average for parental species. Individual hybrids were too few to make generalizations amongst hybrid classes and no predictable patterns were observed. Re-sprouting hybrid plants were no more susceptible to herbivore

attack which was variable amongst all genotypes. Although herbivore damage was similar across the different habitats within each site, between sites there was a large difference. Appin Road site plants were attacked on average almost three times as much as Darkes Forest site plants. It is unclear why herbivore damage was different between sites. Mature *Banksia* plant densities were similar between the two sites. There were on average twice as many seedlings over the same distance at Appin Road than at Darkes Forest. There may have been greater densities of unrelated plants at Darkes Forest but the densities of other plants were not assessed in this study.

Although the average reproductive output of hybrid plants was not statistically different to the parental species, there were half as many healthy seed collected from hybrid plants. Only plants with fruits were surveyed, so it is unknown whether there were infertile hybrids among the plants within the hybrid zones. Nine of the twelve hybrid plants had below average numbers of seed per plant. This needs to be investigated further because Schibeci (1994) found that hybrid plants between *B. oblongifolia* and *B. robur* did not appear to produce less seed than parental species. There was no difference in fungal attack on seeds or otherwise damaged seed between hybrids and parental species.

There was no difference in seed production per plant between habitats and sites. There were differences in the number of fruits between sites. Plants at



Appin Road produced more healthy seeds per fruit than Darkes Forest plants, although the same numbers of healthy seed were collected per plant. It is possible that Darkes Forest plants had a larger number of older fruits than the Appin Road plants. *Banksia* plants retain aerial seed banks until fire, often for several years and seeds from older fruit can have reduced viability (K. Ottewell pers. com.). Furthermore, seeds from Darkes Forest, although they looked healthy, were found to be less viable than seeds from Appin Road (Chapter 5).

#### 4.4.2 *Transplant Experiment.*

There were some shortcomings in relation to the transplant experiment and, because of low numbers in some habitats and genotypic groups, inferences need to be made with caution. First, when seeds were collected, I mixed collections from the two sites together. Even though the two sites were relatively close, within 5km, acclimation to site could confound habitat differences. In chapter 5, I considered acclimation of seed (and subsequent seedlings) collected from maternal plants within the two sites and three habitats and placed in pots. Although the average performance measurements from a number of plants within *B. oblongifolia* genotypes and *B. robur* genotypes were considered, there could have been some genetic differences that affect performance between the two sites. I found no acclimation (or

genetic effects on seedlings) of maternal plants to site passed on to seedlings in any of the measurements for performance. There were, however, some maternal effects due to habitat, which were passed on to seedlings (see chapter 5). Nevertheless seeds from the Darkes Forest site were less viable. As these were spread across sites and habitats, any differences in germination observed should have been due to habitat differences.

Second, during transplantation of seed it was impossible to keep track of individual seed from each maternal plant (as it was in the potted seedling experiment, see Chapter 5). Correlated performance of sibling seed could affect the interpretation of the results. Nevertheless, seeds were mixed thoroughly before transplantation and most of the seedlings were genotyped so comparisons between genotypes were possible. Furthermore, measurements between seedlings, even within maternal lineages, were variable. Therefore affects from correlation between siblings may have been minimal. The maternity of seed under each cage was unknown. Only the proportion of seed from each maternal genotype planted into each of the three habitats was known. Therefore, it was not possible to use each cage as a unit of replication and environmental variation between cages could not be factored into the analyses. Even though seeds of different genotypes were randomly distributed among the cages, results need to be interpreted with caution.

Third, there was no replication for *B. oblongifolia* habitat (see ‘*Characterization of habitats*’). Nevertheless, seed taken from the same mixture of maternal genotypes were placed into intermediate habitat and *B. oblongifolia* habitat, which allowed an excellent comparison between these two habitat types, but unfortunately without replication and at different sites. Finally, heavy rain washed away much of the seed, mainly in *B. robur* habitat, so inferences about *B. robur* habitat were not possible.

#### 4.4.3 Seedling performance

Germination of field seedlings (36%) was about half that of seedlings placed in seed trays (70%), (Chapter 5). Many of the seeds, mostly from the two *B. robur* habitats, were washed away by heavy rain. When seed groups that were badly affected by heavy rain were excluded, the germination rate was calculated to be 50%. Schibeci (1994) found only 4% of seeds had established in the field over twelve months. The difference between the two studies could have been attributed to good rain after seed transplants at the time of this study. Enright *et.al.* (1998) reported that seedling recruitment was greatly enhanced during wet summers after fire as opposed to dry summers. Different recruitment events appear to produce very different numbers of seedlings.

Amongst seedlings there was a range of hybrid genotypic classes germinating and surviving in all three habitat types within the hybrid zones. Campbell *et al.* (2008) also demonstrated in F<sub>1</sub> and F<sub>2</sub> hybrids that hybrids were as fit as parental species within hybrid zones between two *Ipomopsis* species. In this study also there was no evidence for selection acting against hybrid seedlings when compared to parental seedlings. The percentage of hybrid seedlings germinating (5.8%) was similar to the percentage of hybrids within the maternal population (6.6%). Hybrid seedlings were similar to the parental species seedlings in all aspects of fitness and performance measured within the three habitats associated with the hybrid zones. Individual hybrid performances ranged from below to above average in all measures of performance. There was no pattern within habitats or genotypes in performance measures amongst hybrids, but again hybrid numbers were extremely small within genotypic classes. Schibeci (1994) found some seeds from both species and their hybrids grew in all three habitat types, also indicating hybrid fitness across the hybrid zones.

Even though hybrid numbers were small and given that results so far are incomplete, preliminary data suggests that hybrids are not less fit than the parental species at least for the recruitment stage of development. There was no support for the 'tension zone' model (Barton and Hewitt 1985). *Banksia*

hybrid zones do not appear to be maintained by hybrid inferiority and gene flow into the hybrid zone. Although the ‘mosaic’ model, in which a patchy distribution of habitat gives rise to a patchy distribution of parental forms (Rand and Harrison 1989), describes the *Banksia* hybrid zones, hybrids are not less fit or restricted to unoccupied regions. Neither is the *Banksia* hybrid zone maintained by hybrid superiority in intermediate habitats (the ‘bounded superiority’ model, Moore 1977) as both species and their hybrids germinated and performed equally well in intermediate habitats. Some hybrid performance measures were below average for the two species, others were average and above average. Therefore the ‘evolutionary novelty’ model (Arnold 1997) best describes the *Banksia* hybrid zone because in this model hybrids can be as fit, or fitter, than the parental species even in parental habitats.

Data from parental habitats were disappointing for the reasons outlined above; however there were some interesting outcomes. Even though there was no repetition for *B. oblongifolia* habitat, there was evidence to suggest selection against immigrant alleles into *B. oblongifolia* habitat. There was a difference in the genotypes of seed (from the same mixture of maternal genotypes) that germinated in *B. oblongifolia* habitat at Darkes Forest and intermediate habitat at Appin Road. No *B. robur* seed germinated in *B. oblongifolia* habitat at the Darkes Forest site, but in intermediate habitat at the Appin Road site 30 *B.*

*robur* seeds germinated (43%). Further investigation is needed to provide evidence for the role of seed inviability within certain habitats in the maintenance of species boundaries between *B. oblongifolia* and *B. robur*.

Within *B. robur* habitat few seeds remained after heavy rain. It was impossible to draw conclusions about *B. robur* habitat in the transplant experiment. Schibeci (1994) found that most of the seed that germinated (4% germination of 900 seed), amongst both species and hybrids, germinated in *B. robur* habitat. In fact, Schibeci (1994) found that more *B. oblongifolia* seeds germinated in *B. robur* habitat than in *B. oblongifolia* habitat which demonstrated that there may be temporal differences in seedling establishment, possibly dependant on soil moisture content. In this study, some *B. oblongifolia* seedlings and plants, found in moist soils, seemed to be badly affected by spots, which could have been a fungal infection. It is unknown what effect these spots have on plants, and needs to be investigated further. (see Chapter 6 - *Natural seedling transects*). Selection against *B. oblongifolia* plants in *B. robur* habitat could occur later in development. There were only a few seeds collected from *B. oblongifolia* plants in *B. robur* habitat, but those that were did not, however, have high levels of fungus infection.

Hybrid seedlings showed no evidence for disruption of gene complexes involved in defense mechanisms through hybridization. Most of the individual

hybrid seedlings (11 of 15) had average or below average herbivore damage for the two species. Although herbivore damage on plants was similar between habitats (with the exception of greater average herbivore damage on hybrids in one habitat), between sites (see re-sprouting plants) there was considerable difference amongst *B. robur* seedlings. The Appin Road site *B. robur* seedlings were attacked by herbivores more than Darkes Forest seedlings, possibly due to the greater density of seedlings (see Chapter 6 for seedling densities and for a comparison of herbivore damage amongst seedlings appearing naturally).

Despite limitations to this study and limited power due to low numbers of hybrids, some inferences could be made. Mature plants of each species were found predominantly within their own habitats. There appears to be selection against immigrant alleles in *B. oblongifolia* habitat which needs further investigation. In intermediate habitat, almost equal numbers of each species were found amongst mature plants and seedling establishment of each species was in proportion to the seed transplanted. This indicates either a lack of selection against any of the genotypes within intermediate habitat or a gradient of selection across intermediate habitat associated with the drainage gradient. There was no evidence for selection against established hybrids during re-growth or seed production, however production of hybrids was low it was not

statistically different. Hybrid seedlings of many genotypic classes were found in all three habitats, although hybrid mature plants were mainly found in *B. robur* habitat in this study. Nevertheless, no direct evidence for selection against hybrid seedlings was found in this study.



## *Chapter 5*

### **Extent of hybridization, performance of potted seedlings, interactions of traits and effects of maternal plants on seedling performance.**

#### *5.1 Introduction*

The processes involved in forming and maintaining hybrid zones are complex (Rand and Harrison 1989). In particular, hybrid fitness can be influenced by a combination of factors including ecological interactions with both nuclear and cytoplasmic genomes (Campbell *et al.* 2008, van der Sluijs *et al.* 2008). It can be difficult to separate the effects of exogenous (environmental) and endogenous (genetic) selection.

In chapter 4, there was some evidence for environmental selection acting on the parental species within the *Banksia* hybrid zones. It was demonstrated that the different habitats of the hybrid zones can influence seedling germination and growth. Parental species were predominant in their native habitats, and no *B. robur* seeds germinated in *B. oblongifolia* habitat (Chapter 4), although there was no replication of this result. In two of the three intermediate habitats

there was no evidence for environmental selection in the performance of either species or hybrids. There was no evidence for hybrid inferiority (endogenous selection) amongst hybrid seedlings during recruitment and the first two years of growth.

There is the potential for hybrid fitness to differ between field and glasshouse studies (see Campbell *et al.* 2001). For example, in Usher *et al.* (2010), it was demonstrated that *Banksia oblongifolia* and *B. robur* field seedlings were significantly smaller and less well developed, and also morphologically more similar to one another, than potted seedlings. Studies away from the natural environment can lead to the misinterpretation of hybrid zone dynamics (Campbell *et al.* 2001). However, comparisons between field and glasshouse performance can elucidate and separate the types of selection acting on field seedlings. For example, if there is endogenous selection acting against hybrids then hybrids are expected to fare poorly compared to the parental species in both glasshouse and field situations.

In this chapter, I describe an experiment in which seeds collected from across the hybrid zones are germinated and raised in pots in a relatively uniform environment compared to the hybrid zones, in an attempt to reduce the effects of exogenous selection and determine the genetic effects of hybridization on seed germination and growth of seedlings. Nevertheless, if performance is to

be measured within uniform conditions, there is the danger that the particular conditions chosen for the experiment will suit one or other of the species. Dudley (2004) pointed out that even single factors can affect plants in complex ways. Because *B. robur* and *B. oblongifolia* plants appear to be adapted to two environments that are very different in soil moisture and drainage (see Chapter 4), it is expected that a constant supply of water will advantage *B. robur*, but good drainage is expected to be an advantage to *B. oblongifolia*. Therefore, I set up three different watering regimes and measured the health of the plants by monitoring photosynthetic yields.

The same performance measurements used in the transplant experiment (Chapter 4) were employed in this study of potted seedlings with the addition of seed weight, leaf area and proteoid root mass. As already mentioned, leaf area is related to fitness through its effect on photosynthetic capacity. Seed weight (Farris and Lechowicz 1990) can be indirectly fitness-related (Kahmen and Poschlod 2000). Farris and Lechowicz (1990) pointed out that there is evidence to suggest that larger seed weight can lead to larger plants, better survival and increased reproductive success. Here, I test whether seed weight is correlated with germination, survival and growth performance.

Plants of the Proteaceae family have proteoid roots (Figure 5.1) in all but one genus, *Persoonia* (Purnell 1960). Proteoid roots have discrete clusters of

closely spaced rootlets and in *Banksia* species are seen as compound mat-like structures (Watt and Evans 1999). Proteoid roots allow plants to grow in soils with nutrients, such as phosphorus, limited in availability because they are bound to metal cations (Watt and Evans 1999). Therefore the amount of proteoid root matter could be fitness-related in regard to growth and hence reproductive potential. Here, I tested whether the mass of proteoid roots was related to seedling performance.



Figure 5.1 Exposed proteoid roots of a *B. robur* seedling raised in a pot.

Farris and Lechowicz (1990) found that no one particular plant trait contributed exclusively to reproductive success (also see Dudley 2004). Seed characters, physiological factors and growth of architectural characters

interacted to contribute to plant size, and hence reproductive output (Farris and Lechowicz 1990). Furthermore, multiple traits such as leaves, branches, height and root mass, may respond together (Dudley 2004). Therefore, I use multivariate correlations to determine relationships between traits for the parental species and to determine whether the interaction of traits affects the overall performance of seedlings. Nevertheless, I also compare seedling performance in each of the separate traits because *B. oblongifolia* and *B. robur* have different growth forms. The growth form of hybrids may affect their performance. For example, does intermediate leaf size result in reduced leaf area on hybrids compared to parental seedlings?

Seedling performance can be affected, not only by the environment in which they grow, but also by the maternal plant environment (Galloway and Etterson 2009, Galloway and Etterson 2007). Maternal plants from different sites could also have genetic differences (Galloway *et al.* 2009) related to performance which are passed on to seedlings (even though Usher *et al.* 2005 found that the microsatellite data were not significantly different between sites). To determine whether seedling performance differs between sites and between habitats due to maternal environmental or genetic effects, the performances of glasshouse seedlings from plants within the two sites and within the three habitats were compared.

As maternal plant genotypes and the genotypes of their seedlings were known, the extent and direction of hybridization could be determined for this recruitment event. Schibeci (1994) found high levels of outcrossing which, together with overlapping flowering periods, created the opportunity for hybridization. Nevertheless, numbers of first generation hybrids were very small (0.05 – 0.14%) and the majority of crosses were intra-specific (Schibeci 1994). Hybridization occurred in both directions (Schibeci 1994).

The following questions are examined, to directly determine the extent of hybridization by comparison of seedling and maternal plant genotypes; to assess the comparative importance of exogenous and endogenous selection on hybrid seedlings, during germination and growth; and to assess whether maternal acclimation has an affect on seedling fitness and performance.

1. What is the extent and direction of hybridization between *B. robur* and *B. oblongifolia* during this recruitment event?
2. Does altering the water supply of seedlings raised in pots affect the performance of *B. oblongifolia*, *B. robur* and hybrid seedlings as measured by photosynthetic yields?
3. Are there interactions among performance measurements that affect the overall size of hybrid and parental seedlings?

4. How does the performance of hybrid seedlings compare with parental species seedlings within individual traits?
5. Is the performance of seedlings affected by the environment inhabited by the maternal plant?

## *5.2 Methods*

### *5.2.1 Extent and direction of hybridization*

To determine if hybridization was constant between generations, the proportion of hybrids found amongst seedlings was compared with the proportion of hybrids amongst the maternal plants. To determine the types of hybridization occurring within the hybrid zones, individual seedling genotypes were scrutinized and compared with their maternal genotypes. The numbers of seedlings that appeared to be produced by inter- and intra-specific cross pollination were recorded. Seedlings that had no new alleles other than those found in the maternal genotype were recorded as produced by possible self-pollination. Seedlings with different alleles from the maternal plant, but all alleles from a single species were recorded as intra-specific crosses. Seedlings having alleles from the other species which were not found within the maternal



genotype were recorded as inter-specific crosses. Types of crosses involving hybrid plants were also recorded.

#### 5.2.2 Seed and seedling preparation

Seeds were collected as described in Chapter 4 and 840 of the seeds (Table 4.1) were weighed to the nearest tenth of a milligram and placed in seedling trays to germinate. After 2 months seedlings were potted into 6cm pots and kept in a glasshouse for 4 months. The seedlings were then re-potted into 12.5cm pots and placed in the open for the next 10 months. The same amount (one teaspoon) of low-phosphorus fertilizer (Osmocoat for Australian native plants) was added to each pot.

Since there is a contrast in the amount of water available to *B. robur* and *B. oblongifolia* seedlings in their natural environment, seedlings were subjected to three different water regimes, after potting into the 12.5cm pots. The amount of water applied to seedlings in pots could influence the performance of seedlings within the different genotypic groups. Therefore, seedlings from the same plant (where seeds were greater than two) or the same species were distributed amongst three watering treatments. Two trays of different depths were set up to control the amount of soil moisture. In the first watering treatment seedling pots were placed in a tray with a depth of 2cm. In the

second treatment seedling pots were placed in a tray with a depth of 1cm. In the third treatment pots were without trays. All of the plants were watered only when the water had evaporated from the majority of the deepest trays (2cm), hence keeping seedlings in the 2cm trays permanently moist, but allowing drainage in the other treatments. To test for seedling stress caused by insufficient water, or over watering, photosynthetic yields were measured on the 17 August 2003 (a cold winter night) in a random sample of 119 seedlings, 47 *B. oblongifolia*, 63 *B. robur* and 9 hybrid seedlings, 14 months after planting, using a chlorophyll fluorescence monitor ('MINI-PAM'). The 'MINI-PAM' is easy to use and portable. A high intensity short-duration flash of light is given to a section of leaf and the chlorophyll fluorescence measured. A disadvantage is that measurements must be made at night or in the dark in order to reduce the effects of photosynthesis and heat loss that work in competition with the re-emitted light (Maxwell and Johnson 2000). Three chlorophyll fluorescence yield measurements were taken for each plant and the average calculated.

### *5.2.3 Seedling performance measurements*

Several parameters were used to determine seedling performance. Seed weight (Farris and Lechowicz 1990), germination success and survival were determined as each can be fitness related (Kahmen and Poschlod 2000). To

determine whether hybrid superiority (Baack and Rieseberg 2007) or hybrid inferiority was evident in hybrid seedlings, the height from ground to leaf tip was measured. To determine whether hybrids had less photosynthetic capacity in regards to leaf number and area than parental species seedlings, the leaves of 77 seedlings (26 *B. oblongifolia*, 37 *B. robur* and 14 hybrid seedlings) were removed and scanned while still green and then the total leaf area was measured using the NIH Image software (<http://rsb.info.nih.gov/niimage/Default.html>). The leaves per plant were counted. To determine whether hybrid seedlings have the same potential to take up nutrients as the parental species seedlings, nutrient absorption capacity was assessed by counting proteoid root nodules which are specialized roots that enable the uptake of principally phosphorus in nutrient poor soils (Watt and Evans, 1999). These were categorized into three groups according to size, 1–2, 2–3 and 3–4 cm. All seedlings were initially monitored for germination every 3 days for 1 month, and then seedlings were measured and leaves counted once a month for 3 months, and again after 3 months, and then after 6 months to 16 months. At 16 months the seedling roots began to protrude from the pots so the seedlings were removed for leaf area and root measurements. To determine the relative performance between each species and hybrids, 77 seedlings, 26 *B. oblongifolia* and 37 *B. robur*, were selected randomly. However, because hybrid seedling numbers were small, more hybrid

seedlings were added to the random sample to increase numbers to 14 hybrids.

#### 5.2.4 Statistical Analyses

##### **Water regime**

To determine whether *B. oblongifolia* and *B. robur* seedlings were advantaged or disadvantaged by any one of the three different water regimes, the health of the seedlings was determined by measuring photosynthetic yields. A simple nested ANOVA analysis (SAS - JMP 5.1) was performed, comparing the photosynthetic yields (dependant variable) within the three water regimes (2cm tray, 1cm tray and no tray) amongst *B. oblongifolia* and *B. robur* seedlings.

##### **Germination and survival**

To determine whether there was selection for or against *B. robur* and *B. oblongifolia* and hybrid seedlings in germination and survival, contingency analyses (SAS - JMP 5.1) were performed comparing the proportion of seeds that germinated with the proportion of seeds that were originally planted and the proportion of seedlings that survived to 16 months with the proportion of seeds that germinated within the three genotypic groups.

To determine whether seed weight was related to germination and survival, one way ANOVA analyses (SAS - JMP 5.1) were performed comparing seed weight with germination and survival of the parental species and hybrid seed.

### **Seedling performance resulting from multiple traits**

To determine which performance measurements were interacting with one another to affect overall performance, a multivariate analysis (SAS - JMP 5.1) was performed on seed weight, seedling height, leaf area, leaf number and numbers of proteoid root clusters per seedling.

To determine the overall growth performance of each species and hybrids, a discriminant analysis (SAS - JMP 5.1) was performed on four performance measurements seedling height, leaf number and area and proteoid root development. Performance of hybrids was deemed to be intermediate if the probability of assignment to either parental species was less than 50%. If the probability of assignment to either parental species was greater than 50%, then hybrid performance was deemed to be more similar to one of the two species.

### **Seedling performance within individual traits**

One way ANOVA analyses (SAS - JMP 5.1) were used to compare individual trait measurement (seedling height, leaf area, leaf number and numbers of

proteoid root clusters per seedling) between the genotypic groups, *B. oblongifolia*, *B. robur* and their hybrids.

To determine whether there was evidence for hybrid superiority or hybrid inferiority among individual hybrids, seedling height, leaf area, leaf number and numbers of proteoid root clusters per seedling measurements of individual hybrids were compared with the 95% confidence limits for the means of each parental species (SAS - JMP 5.1). Hybrids were labeled with the broad genotypic classes as described in ‘Genetic Techniques’ in Chapter 4 (first generation, F1; back crossed to *B. oblongifolia*, BC-o; back crossed to *B. robur*, BC-r; *B. oblongifolia* with one *B. robur* allele, I-o; and *B. robur* with one *B. oblongifolia* allele, I-r),

### **Effects of maternal site and habitat on seedling performance**

To determine whether the site and habitat of maternal plants affects germination and survival success, a simple nested ANOVA (SAS - JMP 5.1) was performed by site and by habitat within site. To determine whether the genotype, site and habitat of maternal plants affects each of the growth performance of seedlings, compound nested ANOVA analyses (SAS - JMP 5.1) were performed.

### 5.3 Results

#### 5.3.1 Extent of hybridization

Amongst 106 maternal plants across the two hybrid zones, 6.6% were detectable hybrids and, similarly, amongst 169 seedlings produced by these plants, and germinated in pots, 6.2% were detectable hybrids. Scrutiny of seedling genotypes revealed that most seedlings were the same species as their maternal parent (Table 5.1). Nevertheless, there was evidence for continuing hybridization between the two species. A variety of hybrid genotypes were found amongst seedlings, including what appeared to be first and later generation hybrids and seedlings backcrossed to both species (Table 5.1). For example, a *B. robur* plant produced three hybrid seedlings that had one allele from each species at all distinguishing loci, genotypes consistent with first generation hybrids (3.3% of seedlings in pots that were genotyped). A hybrid plant backcrossed to *B. robur* (with one *B. oblongifolia* allele at some loci) produced offspring that could have been self pollinated (no new alleles observed other than those seen in the maternal plant); and other offspring that appeared to be backcrossed to each species (having alleles not present in the maternal plant). Undoubtedly some hybrid ancestry was not revealed by the number of genetic markers used in this study. For

example, a *B. oblongifolia* plant with one *B. robur* allele produced seed with *B. oblongifolia* genotypes.

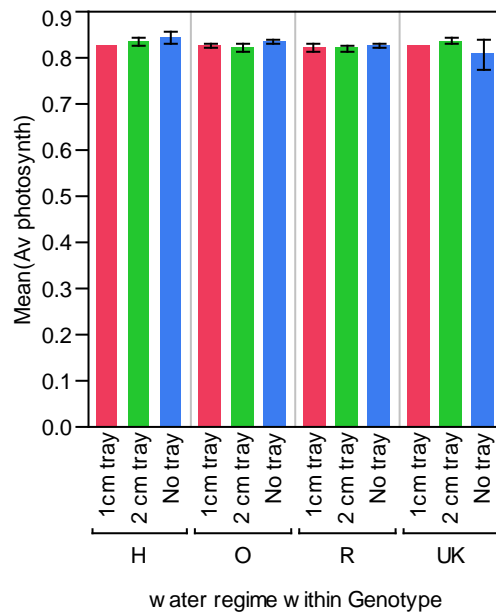
Table 5.1 Genotypes of maternal plants and their seedlings using seven microsatellite markers reveal possible types of crosses (% within each species and within hybrids). Because of the low number of markers used self pollination could be overestimated, particularly among *B. robur* plants.

	Possible types of crosses as revealed by maternal and seedling genotypes				
	Self pollination	Intra- specific outcrosses	Inter-specific outcrosses		
Maternal Genotypes			<i>B. oblongifolia</i> cross	<i>B. robur</i> cross	Hybrid cross
<i>B. oblongifolia</i>	6 (9%)	55 (83%)			5 (8%)
<i>B. robur</i>	25 (27%)	60 (66%)	3 (3.3%)		3 (3.3%)
Hybrid	2 (16.7%)		8 (66.7%)	2 (16.7%)	



### 5.3.2 Water regime

None of the seedlings appeared to be stressed by varying the water supply to the extent varied here. The average photosynthetic yields were high for all plants regardless of the watering regime. There was no difference in photosynthetic yields of seedlings among genotypes or genotypes within watering regimes ( $F_{11,119} = 0.74$ ,  $P = 0.70$ ) Average photosynthetic yields ranged between 0.82 and 0.84 with hybrids also high at 0.84 (Figure 5.2).

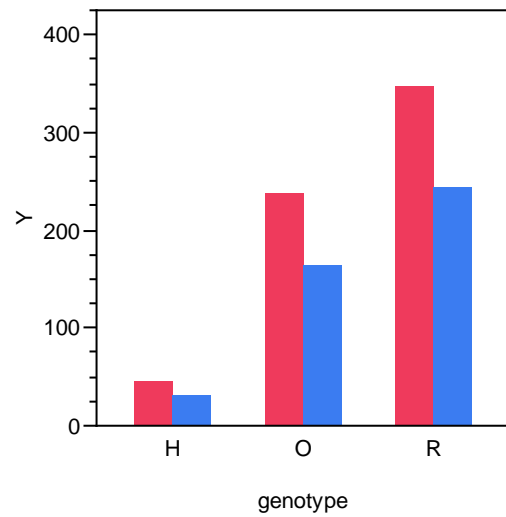


water regime ■ 1cm tray ■ 2 cm tray ■ No tray

Figure 5.2 Mean photosynthetic yields of hybrid (H), *B. oblongifolia* (O), and *B. robur* (R) seedlings and seedlings of unknown genotypes (UK) grown in pots, under three different water regimes (1cm deep trays, 2cm deep trays and no trays). Error bars = standard error.

### Germination and survival

There was no evidence for selection against *B. robur*, *B. oblongifolia* or hybrid seed during germination. Approximately 70% of seeds germinated in the seed trays. Of the seeds that were genotyped, seeds from hybrid plants germinated at a rate of 67% (31 of 46 seeds); seeds from *B. oblongifolia* plants germinated at a rate of 68% (162 of 239 seeds); and seeds from *B. robur* plants germinated at a rate of 70% (245 of 348 seeds). There was no difference between the proportion of seeds within the two species or their hybrids planted into the seed trays and the seeds that germinated ( $\chi^2 = 0.53$ ,  $DF = 2$ ,  $P = 0.77$ ), (Figure 5.3).



Y

■ Number planted

■ Number germinating

Figure 5.3 Number of seeds that were originally planted into seed trays and number of seeds that germinated in each genotypic group (H = hybrid, O = *B. oblongifolia*, R = *B. robur*).

After seedlings germinated, few died over the 16 month period of growth. Survival rates for all groups, *B. oblongifolia*, hybrids and *B. robur*, were 90 to 95%.

Seed weight was related to germination in *B. robur* and hybrid seeds, but not in *B. oblongifolia* seeds. The mean weight of seeds that germinated was heavier than those that did not germinate for *B. robur* ( $36.16 \pm 0.61\text{mg}$  and  $32.72 \pm 0.94\text{mg}$  *s.e.*;  $F_{1,352} = 9.42$ ,  $P = 0.002$  and hybrids seeds ( $28.72 \pm 1.74\text{mg}$  and  $21.58 \pm 2.50\text{mg}$  *s.e.*;  $F_{1,44} = 5.51$ ,  $P = 0.024$ ), but not for *B. oblongifolia* seeds ( $21.95 \pm 0.63\text{mg}$  and  $21.93 \pm 0.44\text{mg}$  *s.e.*;  $F_{1,243} = 0.0007$ ,  $P = 0.98$ ), (Figure 5.4).

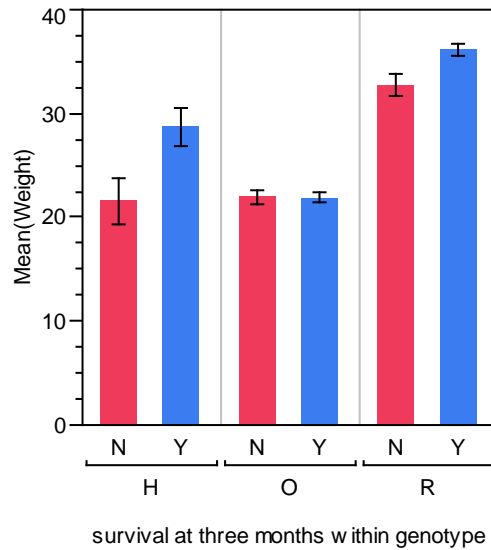


Figure 5.4 Average weights of *B. oblongifolia* (O), *B. robur* (R) and hybrid seeds (H) that germinated (Y) and those that did not germinate (N) after 3 months. Error bars = standard error.

There was no difference in the average weight of seeds between seedlings that survived to 16 months and seedlings that died in either *B. oblongifolia* ( $22.16 \pm 1.33$  vs  $21.71 \pm 0.37$ mg s.e), ( $F_{1,243} = 0.22$ ,  $P = 0.64$ ) or in *B. robur* ( $33.41 \pm 1.85$  vs  $35.29 \pm 0.53$ mg s.e), ( $F_{1,352} = 0.95$ ,  $P = 0.33$ ) (Figure 5.5). Only one seedling from a hybrid plant died.

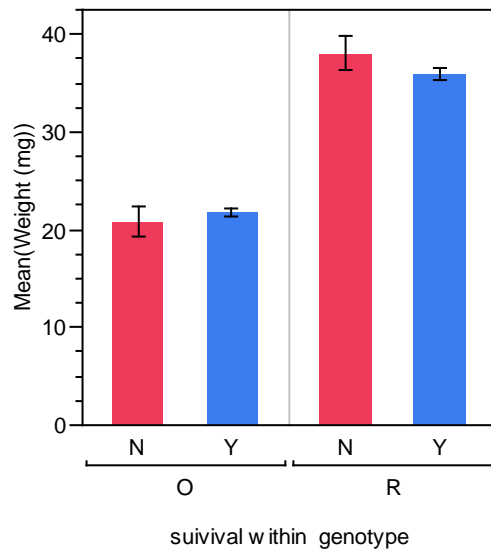


Figure 5.5 Average weights of *B. oblongifolia* seeds (O) and *B. robur* seeds (R) amongst seedlings that survived to 16 months after germination (Y) and those that did not survive (N). Error bars = standard error.

### 5.3.3 Performance correlations

Not surprisingly, there were strong pairwise correlations between most of the growth traits measured. Leaf area and leaf number correlated with each other among *B. oblongifolia* ( $P = 0.0006$ ), *B. robur* ( $P = 0.0007$ ) and hybrid ( $P = 0.003$ ) seedlings (Table 5.2). There were also pairwise correlations between leaf area and seedling height among *B. oblongifolia* ( $P = 0.02$ ), *B. robur* ( $P = 0.02$ ) and hybrid ( $P = 0.03$ ) seedlings. Correlation between leaf number and seedling height was strong for both species ( $P < 0.0001$  each), (Table 5.2A and B) but not hybrids ( $P = 0.22$ ), (Table 5.2C). The average number of leaves for each species was different, and hybrid leaf morphology was either similar to one of the parental species or intermediate (see individual trait measurements below). Therefore, average leaf numbers for hybrids, including both species traits and intermediate traits, would have confounded correlations between leaf number and height. This was not the case for leaf area and height because leaf area was the same in both species.

Proteoid root nodule development differed between the two species, with *B. oblongifolia* having greater numbers of smaller nodules (see individual trait measurements below). There was a negative correlation between small and large nodules found among *B. robur* seedlings ( $P < 0.0001$ ), (Table 5.2B). However, *B. oblongifolia* seedling root nodules of 2-3 cm was positively correlated with leaf number ( $P = 0.005$ ), leaf area ( $P = 0.0008$ ) and seedling height ( $P = 0.01$ ), (Figure 5.2A). Likewise, for *B. robur* seedlings, root nodules of 3-4 cm was positively correlated with leaf number ( $P = 0.002$ ), leaf area ( $P = 0.03$ ) and seedling height ( $P = 0.001$ ), (Table 5.2B).

Seed weight was not related to seedling performance after germination. Seed weight was only correlated with root nodules of 2-3 cm ( $P = 0.02$ ) amongst *B. robur* seedlings and no other measurements (Table 5.2B).

Table 5.2 Multivariate pairwise correlations of the five performance measurements in parental species (A and B) and hybrids (C) \* denotes statistical differences ( $P < 0.05$ ).

*A. B. oblongifolia* (26 seedlings)

	Leaf area	leaf no.	seed weight	proteoid 1-2	proteoid 2-3	proteoid 3-4	Height
Leaf area	1.0000						
leaf no.	0.6069*	1.0000					
seed weight	-0.1697	-0.0358	1.0000				
proteoid 1-2	0.0205	-0.3797	-0.2378	1.0000			
proteoid 2-3	0.5461*	0.4454*	-0.1392	-0.1641	1.0000		
proteoid 3-4	-0.0811	0.1742	0.1263	-0.4519	-0.1491	1.0000	
Height	0.4684*	0.7416*	0.0902	-0.3437	0.4467*	0.3332	1.0000

*B. B. robur* (37 seedlings)

	Leaf area	leaf no.	seed weight	proteoid 1-2	proteoid 2-3	proteoid 3-4	Height
Leaf area	1.0000						
leaf no.	0.5346*	1.0000					
seed weight	-0.0246	0.0822	1.0000				
proteoid 1-2	0.0115	-0.2672	-0.1842	1.0000			
proteoid 2-3	0.2819	0.1986	0.3044*	-0.1067	1.0000		
proteoid 3-4	0.4345*	0.6011*	0.0469	-0.6278*	0.0732	1.0000	
Height	0.3919	0.6578*	-0.0848	-0.1346	0.0000	0.5084*	1.0000



### C. Hybrids (12 seedlings)

	Leaf area	leaf no.	seed weight	proteoid 1-2	proteoid 2-3	proteoid 3-4	Height
Leaf area	1.0000						
leaf no.	0.7489*	1.0000					
seed weight	0.2907	0.1017	1.0000				
proteoid 1-2	-0.1596	-0.2280	-0.3818	1.0000			
proteoid 2-3	0.2147	0.4094	-0.0864	-0.0399	1.0000		
proteoid 3-4	0.3387	0.2785	0.7675*	-0.6737*	0.0702	1.0000	
Height	0.6310*	0.3813	0.5405*	-0.5703	0.2010	0.8252*	1.0000

When all of the growth measurements were analyzed together, the performances of the two species were distinct from one another due to the different growth forms of leaves and roots (Figure 5.6). Ten of the fourteen hybrid seedlings were intermediate in performance between the two species ( $P > 0.5$ ). Hybrid seedlings that were not intermediate included, a large seedling backcrossed to *B. oblongifolia* that had a performance exceeding most *B. oblongifolia* seedlings. A seedling introgressed with a *B. robur* allele had a performance similar to *B. oblongifolia* seedlings. A seedling backcrossed to *B. robur* had performance similar to *B. robur*. Interestingly, one of the *B. oblongifolia* seedlings introgressed with a *B. robur* allele had performance exceeding most *B. robur* seedlings. Three *B. robur* and five *B. oblongifolia*

seedlings with parental genotypes had performance similar to intermediate hybrids ( $P > 0.5$ ).

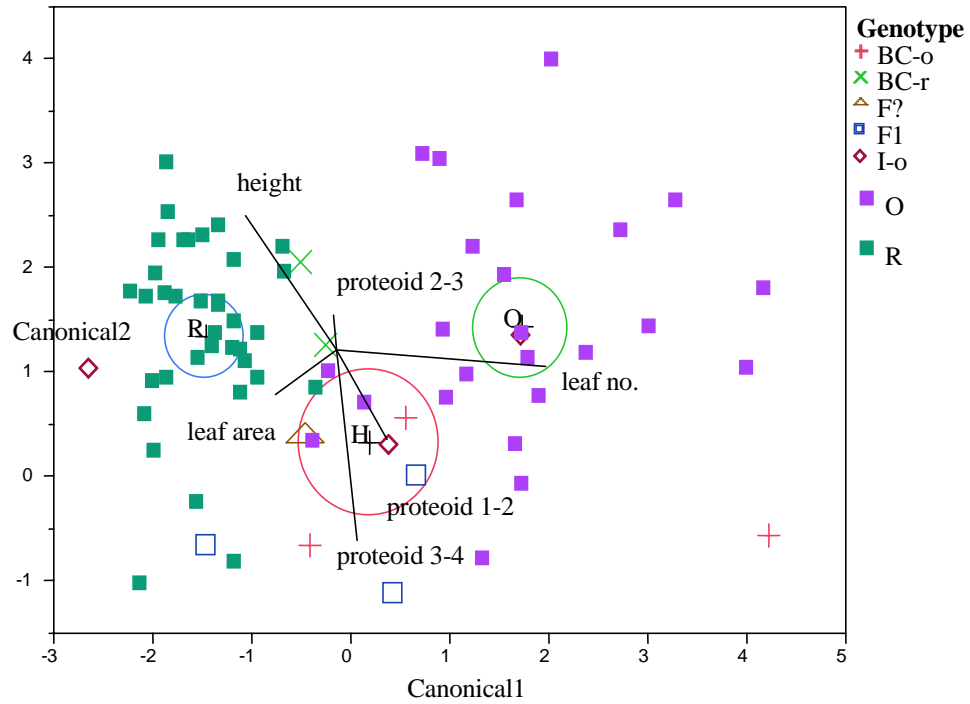


Figure 5.6 Overall growth performance of parental (R = *B. robur*, O = *B. oblongifolia*) and hybrid (H) seedlings (BC-r = backcross to *B. robur*, BC-o = backcross to *B. oblongifolia*, I-o = *B. oblongifolia* with one *B. robur* allele, F1 = first generation, F? = later generation). Circles depict means 95% confidence limits.

## Seedling Performance within single traits

### Seed Weight

The weights of 840 seeds collected and planted into seed trays were compared between species and hybrids using maternal genotypes (see Table 4.2). In some cases hybrid seeds taken from pure species plants were detected when the seedlings were genotyped. These seeds were sired by either the other species or hybrid plants and were included in the hybrid category. Other hybrid seed could have been missed and included in parental species; nevertheless their numbers are likely to be small (see section on ‘extent of hybridization’ above).

*B. robur* plants (427 seeds collected from 53 plants) had heavier seeds than *B. oblongifolia* plants (308 seeds collected from 43 plants) and the average weight of hybrid seed (53 seeds collected from 7 plants) was intermediate between the two species. The average weight of seeds from each genotypic group, *B. oblongifolia* ( $21.76 \pm 0.54\text{mg}$ ), *B. robur* ( $35.15 \pm 0.45\text{mg}$ ), and hybrid ( $26.39 \pm 1.24\text{mg}$ ), differed significantly from one another ( $F_{2,642} = 188.09$ ,  $P < 0.0001$ ), (Figure 5.7).

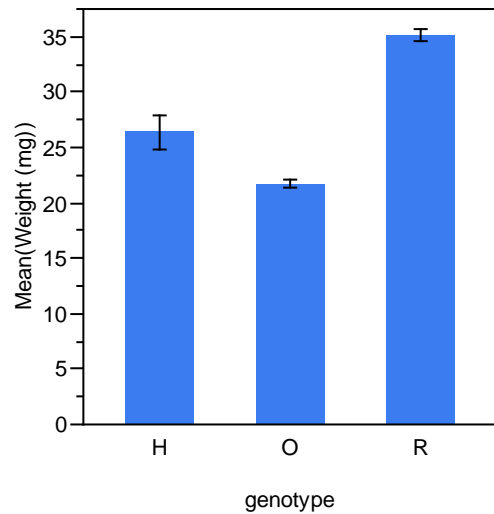


Figure 5.7 Average weights of seeds collected from hybrid plants (H), *B. oblongifolia* plants (O) and *B. robur* plants (R) growing across the hybrid zones. Error bars = standard error.

The weights of individual hybrid seeds varied between one another but none were below average for the two species. Ten of the twelve hybrid seed (from surviving hybrid seedlings) had weights within the 95% CL for the two species (16.14 – 41.93mg). Two first generation hybrids (46.3 and 42.1mg) had above the upper 95% CL for *B. robur* seed weights (Figure 5.8).

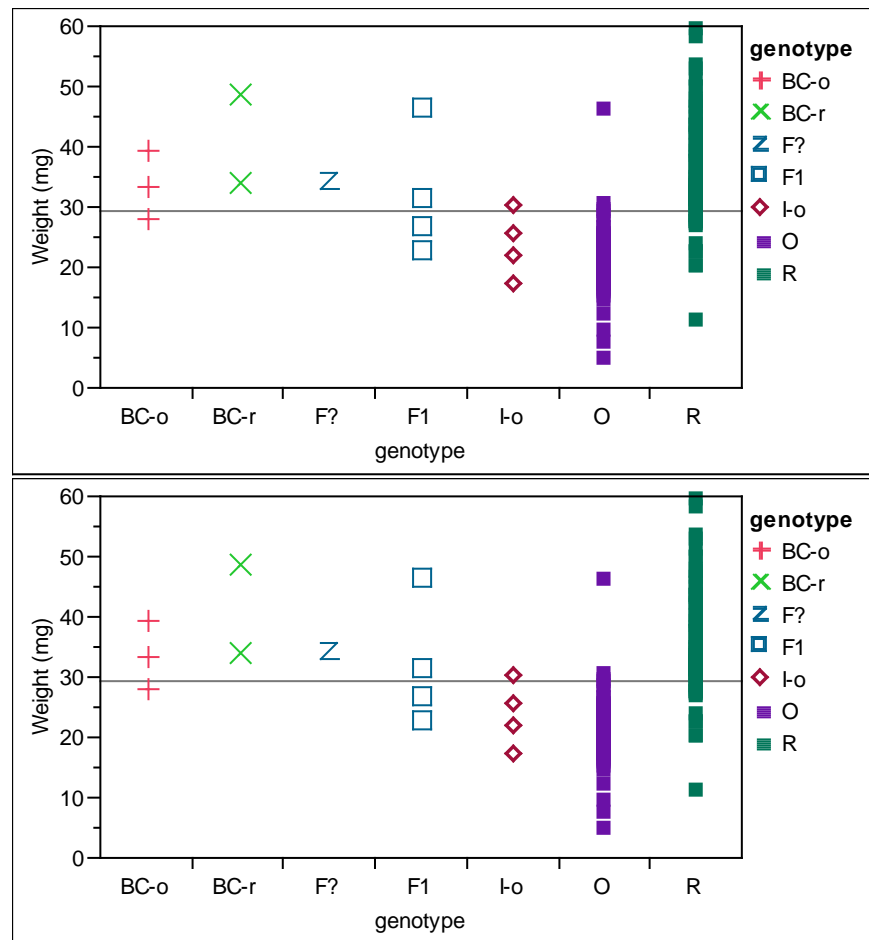


Figure 5.8 Weights of individual seed collected from plants growing across the hybrids zones (R = *B. robur*, O = *B. oblongifolia*) and hybrid seedlings (BC-r = backcross to *B. robur*, BC-o = backcross to *B. oblongifolia*, I-o = *B. oblongifolia* with one *B. robur* allele, F1 = first generation, F? = later generation).

Hybrid seedlings showed no sign of either heterosis or hybrid inferiority in growth after 16 months. There was no difference in the average heights of *B.*

*oblongifolia* ( $25.08 \pm 0.79\text{cm s.e}$ ), *B. robur* ( $26.78 \pm 0.64\text{cm s.e}$ ), and hybrids ( $25.46 \pm 1.65\text{cm s.e}$ ), ( $F_{2,143} = 1.48$ ,  $P = 0.23$ ), (Figure 5.9).

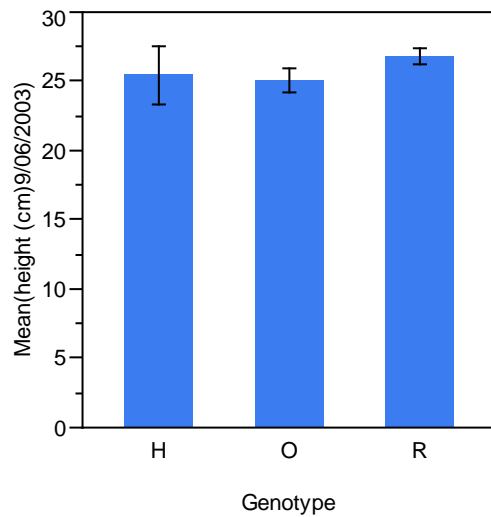


Figure 5.9 Height of seedlings within hybrid (H), *B. oblongifolia* (O) and *B. robur* (R) genotypic groups, collected as seed from maternal plants growing across the hybrids zones and grown in pots for 16 months. Error bars = standard error.

Heights of individual hybrids varied between one another even within hybrid classes (Figure 5.7). Backcrossed and first generation hybrids both had heights that were above, below and within the 95% CLs (23.57 – 28.01cm) for the two species. Introgressed seedlings had heights above and below the

95% CLs and a later generation hybrid had a height within the 95% CLs of the means for the two species (Figure 5.10).

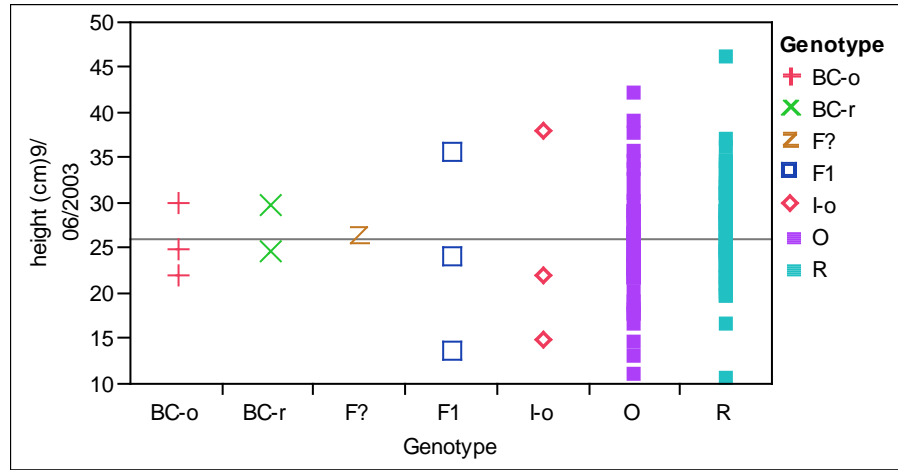


Figure 5.10 Height of individual seedlings collected as seed, from maternal plants growing across the hybrids zones, and grown in pots for 16 months. (R = *B. robur*, O = *B. oblongifolia*) and hybrid seedlings (BC-r = backcross to *B. robur*, BC-o = backcross to *B. oblongifolia*, I-o = *B. oblongifolia* with one *B. robur* allele, F1 = first generation, F? = later generation).

Hybrid seedlings were similar to the parental species in photosynthetic capacity regarding leaf number and area. There was no difference in average leaf area per seedling between the *B. oblongifolia* ( $55536 \pm 6063 \text{ mm}^2 \text{ s.e}$ ) *B. robur* ( $39545 \pm 5082 \text{ mm}^2 \text{ s.e}$ ) or their hybrids ( $49744 \pm 8262 \text{ mm}^2 \text{ s.e}$ ) ( $F_{2,74} = 2.12$ ,  $P = 0.13$ ) (Figure 5.11A) even though leaf numbers were characteristically different between the two species (Figure 5.11B).

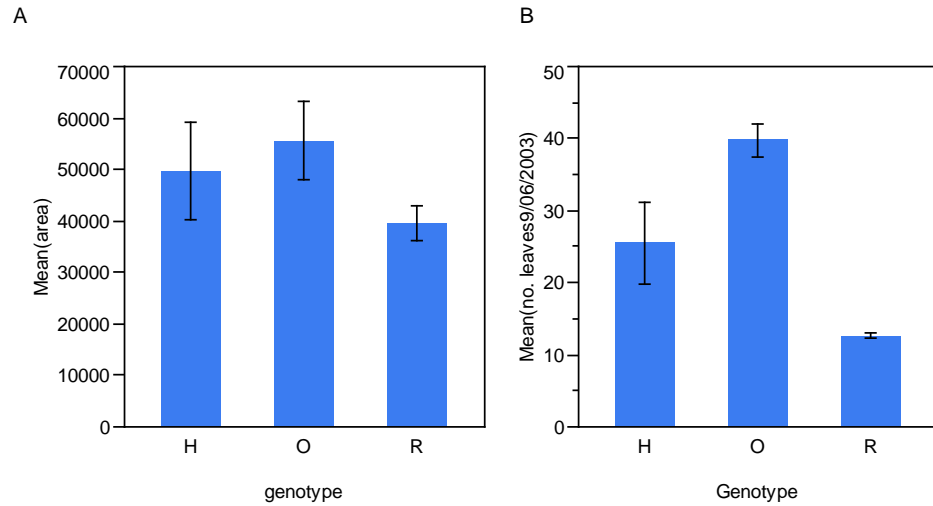


Figure 5.11 Mean Area (A) and leaf number (B) of hybrid (H), *B. oblongifolia* (O) and *B. robur* (R) seedlings collected as seed, from maternal plants growing across hybrids zones, and grown in pots for 16 months. Error bars = standard error.

The leaf area varied between individual hybrids (Figure 5.12). Six of the twelve hybrids (four backcrossed and two first generation) had leaf areas between the 95% CLs (29764 -67203mm<sup>2</sup>). A later generation, a first generation and two introgressed hybrids had below the lower 95% CLs for the mean leaf area of the two species. An introgressed hybrid had above average leaf area and a backcrossed hybrid had well above average leaf area (148497mm<sup>2</sup>).



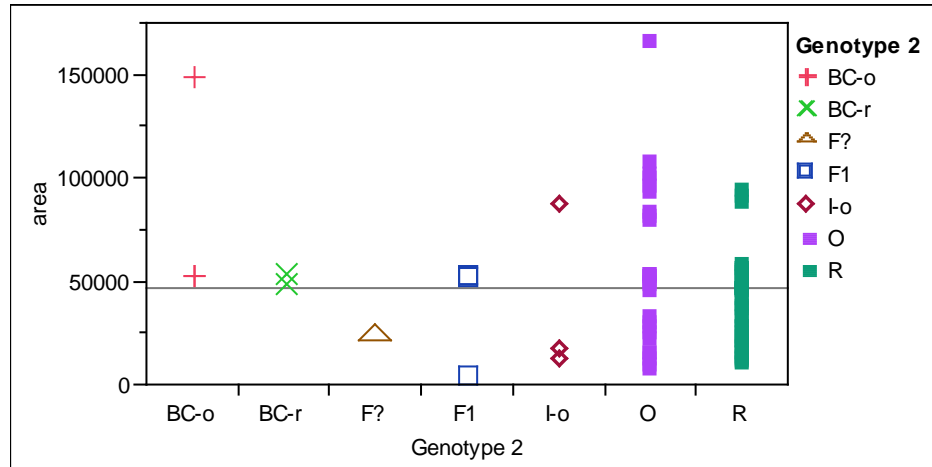


Figure 5.12 Leaf areas of individual seedlings collected as seed, from maternal plants growing across the hybrids zones, and grown in pots for 16 months. (R = *B. robur*, O = *B. oblongifolia*) and hybrid seedlings (BC-r = backcross to *B. robur*, BC-o = backcross to *B. oblongifolia*, I-o = *B. oblongifolia* with one *B. robur* allele, F1 = first generation, F? = later generation).

The mean number of leaves on *B. oblongifolia* seedlings ( $39.75 \pm 1.64 \text{ s.e}$ ) was greater than *B. robur* seedlings ( $12.64 \pm 1.33 \text{ s.e}$ ); and the mean number of leaves on hybrid seedlings ( $25.50 \pm 3.45 \text{ s.e}$ ) was intermediate between the two species ( $F_{2,143} = 84.47, P < 0.0001$ ) (Figure 5.11B).

The leaf numbers of individual hybrid seedlings were all between the 95% CLs (10.20 – 42.78) for the two species with the exception of three hybrids. A first generation and introgressed hybrid were below the lower 95% CLs. The same backcrossed seedling that had above average leaf area also had well above average number of leaves (82 or twice the average for *B. oblongifolia* seedlings), (Figure 5.13).

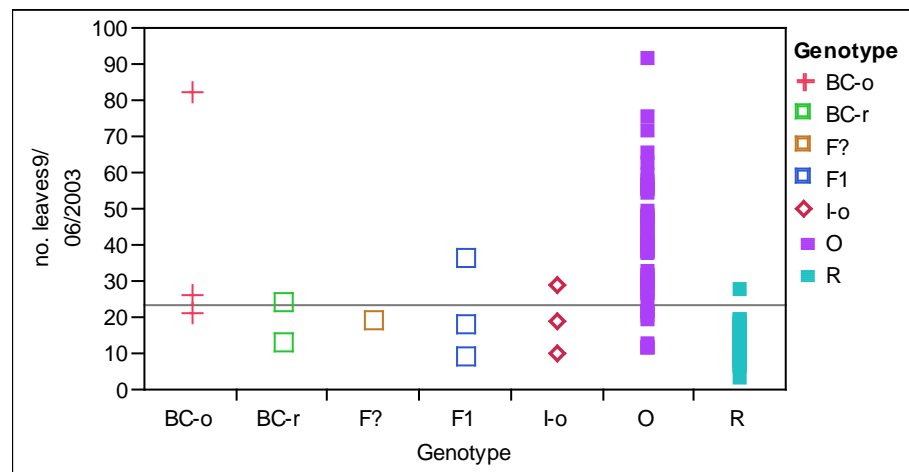
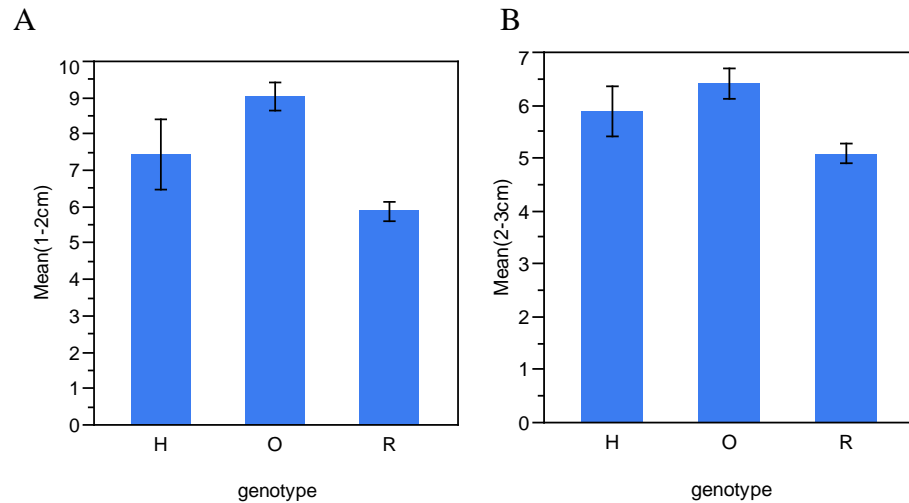


Figure 5.13 Number of leaves on individual seedlings collected as seed, from maternal plants growing across the hybrids zones, and grown in pots for 16 months. (R = *B. robur*, O = *B. oblongifolia*) and hybrid seedlings (BC-r = backcross to *B. robur*, BC-o = backcross to *B. oblongifolia*, I-o = *B. oblongifolia* with one *B. robur* allele, F1 = first generation, F? = later generation).

Hybrid seedlings had a similar potential to the parental species seedlings in the absorption of nutrients from the soil. *Banksia oblongifolia* had a greater number of smaller proteoid root nodules (1-2 cm;  $9.1 \pm 0.37$  and 2-3 cm;  $6.42 \pm 0.26$  s.e) than *B. robur* (1-2 cm;  $5.83 \pm 0.30$  and 2-3 cm;  $5.10 \pm 0.21$  s.e), and hybrids were intermediate between the two species (1-2 cm;  $7.48 \pm 0.88$  and 2-3 cm;  $5.93 \pm 0.62$  s.e), (1-2 cm;  $F_{2,421} = 24.12$ ,  $P < 0.0001$ ), (2-3 cm;  $F_{2,421} = 8.08$ ,  $P = 0.0004$ ) (Figure 5.14A and B). However, *B. robur* had a greater number of large nodules ( $10.40 \pm 0.38$  s.e) than hybrids ( $9.00 \pm 1.15$  s.e) and *B. oblongifolia* seedlings ( $7.46 \pm 0.48$  s.e), ( $F_{2,420} = 11.54$ ,  $P < 0.0001$ ), (Figure 5.14C).



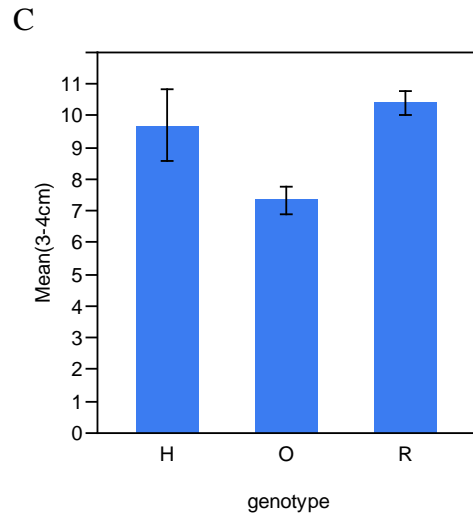
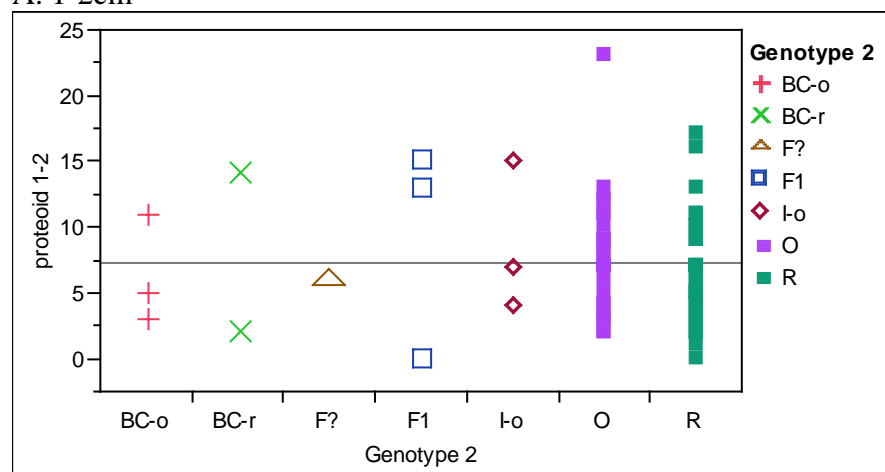


Figure 5.14 Proteoid root mass (mean number of nodules within three size groupings A, B and C) of hybrid (H), *B. oblongifolia* (O) and *B. robur* (R) seedlings collected as seed, from maternal plants growing across the hybrids zones, and grown in pots for 16 months. Error bars = standard error.

Individual hybrid plants varied widely in the number of proteoid roots within each of the size categories (Figure 5.15A, B and C) however no hybrids were below average for all three size categories. The backcrossed individual that demonstrated a greater leaf number and area, also had above average numbers of proteoid roots in all three categories, 11 in 1-2cm (upper 95% CI

= 9.80), 9 in 2-3cm (upper 95% CI = 6.93) and 12 in 3-4cm (upper 95% CI = 11.15). A backcrossed (19), first generation (17.5), a later generation (16) and an introgressed hybrids (18) all had above average numbers of 3-4cm proteoid roots (upper 95% CL = 11.16). Two backcrossed hybrids and an introgressed hybrid had average 3-4cm proteoid roots (95% CL = 6.53 - 11.16), (Figure 5.15C). A first generation (15) and an introgressed hybrid (15) had above average numbers of 1-2cm proteoid roots (upper 95% CL = 9.80) (Figure 5.15A).

A. 1-2cm



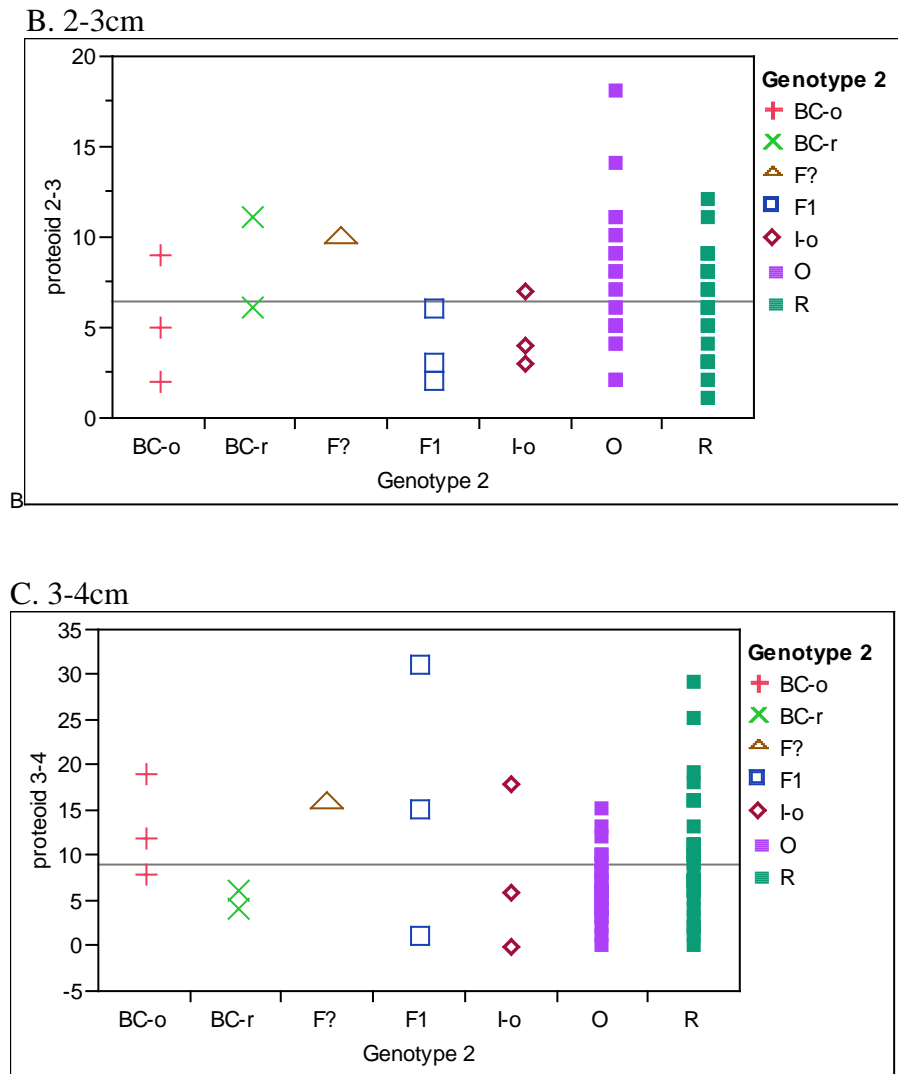


Figure 5.15 Number of proteoid root nodules within three different size groups (A, B and C) on individual seedlings collected as seed, from maternal plants growing across the hybrids zones, and grown in pots for 16 months. (R = *B. robur*, O = *B. oblongifolia*) and hybrid seedlings (BC-r = backcross to *B.*

*robur*, BC-o = backcross to *B. oblongifolia*, I-o = *B. oblongifolia* with one *B. robur* allele, F1 = first generation, F? = later generation).

#### 5.3.4 Effects of maternal site and habitat on seedling performance

##### Effects of maternal genotype, site and habitat on seedling performance

The site but not the habitat of the maternal plant (from which the seeds were collected) affected the germination rates of the seeds ( $\chi^2 = 18.84$ ,  $DF = 1$ ,  $P < 0.0001$ ). Seeds produced by plants in the Appin Road site germinated at a rate of 77%, but seeds produced in the Darkes Forest site germinated at a rate of 56%. There was no difference in seedling survival to 16 months ( $\chi^2 = 14.89$ ,  $DF = 14$ ,  $P = 0.38$ ) between the seeds from different maternal plant genotypes, sites or habitats.

There were some genotype and habitat differences in the average height of seedlings grown from seed taken from plants in the two contact zones ( $F_{12,342} = 2.47$ ,  $P = 0.0042$ ). Seedlings grown from seed collected from *B. oblongifolia* plants were shorter (least sq mean  $24.57 \pm 0.64\text{cm s.e}$ ) than seedlings grown from seed collected from *B. robur* ( $26.59 \pm 0.45\text{cm}$ ) and hybrid plants ( $26.19 \pm 1.19\text{cm}$ ). Seedlings grown from seeds collected from *B. oblongifolia* plants at the Darkes Forest site were progressively shorter

when taken from *B. robur* habitat ( $28.65 \pm 1.90\text{cm}$ ), intermediate ( $23.64 \pm 1.81\text{cm}$ ) and *B. oblongifolia* habitats ( $20.32 \pm 1.60\text{cm}$ ).

Differences found in the numbers leaves and leaf areas between sites and habitats occurred amongst *B. oblongifolia* seedlings. As expected by morphology, seedlings raised from seed collected from *B. oblongifolia* plants had greater numbers of leaves (least sq mean  $38.97 \pm 0.93 \text{ s.e}$ ) than seedlings from hybrid ( $24.66 \pm 2.25$ ) and *B. robur* plants ( $13.01 \pm 0.85$ ) ( $F_{12,424} = 45.37$ ,  $P < 0.0001$ ). Furthermore, seedlings grown from seed collected from *B. oblongifolia* plants in *B. oblongifolia* habitat at Darkes Forest have fewer leaves ( $30.12 \pm 2.77$ ) than seedlings from *B. oblongifolia* plants in intermediate ( $40.65 \pm 2.56$ ) and *B. robur* habitats ( $41.65 \pm 2.34$ ). Seedlings raised from seeds taken from *B. oblongifolia* plants at the Darkes Forest site had a larger average surface area (least sq mean  $63710 \pm 6402\text{sq cm s.e}$ ) than seedlings from *B. oblongifolia* plants at the Appin Road site ( $40249 \pm 9513\text{sq cm}$ ), ( $F_{11,65} = 3.48$ ,  $P = 0.0007$ ). Seedlings grown from seeds collected from *B. oblongifolia* plants in *B. oblongifolia* habitat at Darkes Forest had smaller leaf areas ( $28170 \pm 10984\text{sq cm}$ ) than seedlings grown from seeds collected from *B. oblongifolia* plants in intermediate ( $93905 \pm 12033\text{sq cm}$ ) and *B. robur* habitats ( $69054 \pm 10169\text{sq cm}$ ).



Proteoid roots of size 1-2cm were different ( $F_{12,424} = 5.01$ ,  $P < 0.0001$ ) between seedlings grown from seed taken from *B. oblongifolia* plants (least sq mean  $9.12 \pm 0.37$  s.e) and seedlings grown from seed taken from *B. robur* ( $5.78 \pm 0.34$  and hybrid plants ( $6.77 \pm 0.86$ ). Seedlings grown from seed collected from *B. oblongifolia* plants in *B. robur* habitat at Darkes Forest had less proteoid roots ( $7.43 \pm 0.95$ ) than seedlings from seed collected from *B. oblongifolia* plants in intermediate ( $11.10 \pm 1.02$ ) and *B. oblongifolia* habitat ( $9.28 \pm 1.07$ ). The opposite trend was seen in numbers of 2-3cm proteoid roots ( $F_{12,424} = 2.11$ ,  $P = 0.016$ ). Seedlings grown from seed collected from *B. oblongifolia* plants in *B. robur* habitat at Darkes Forest had more proteoid roots ( $7.22 \pm 0.67$ ) than seedlings from seed collected from *B. oblongifolia* plants in intermediate ( $6.20 \pm 0.72$ ) and *B. oblongifolia* habitat ( $4.83 \pm 0.76$ ). Seedlings from *B. oblongifolia* plants had fewer ( $7.17 \pm 0.47$ ) 3-4cm proteoid roots ( $F_{12,423} = 3.84$ ,  $P < 0.0001$ ) than seedlings from plants of hybrid ( $11.15 \pm 1.11$ ) and *B. robur* genotypes ( $10.49 \pm 0.43$ ).

Seeds from *B. oblongifolia* plants weighed less (least sq mean  $21.71 \pm 0.53$ mg s.e) ( $F_{14,630} = 37.26$ ,  $P < 0.0001$ ) than seeds from hybrid ( $31.24 \pm 1.48$ mg) and *B. robur* plants ( $34.66 \pm 0.67$ mg). Seeds from *B. robur* plants were heavier from Darkes Forest ( $36.11 \pm 1.16$ mg) than Appin Road ( $33.21$

$\pm 0.66\text{mg}$ ). The weight of seeds from *B. oblongifolia* plants varied across habitats at Darkes Forest, from *B. oblongifolia* ( $18.18 \pm 1.19\text{mg}$ ), intermediate ( $24.29 \pm 1.11\text{mg}$ ) and *B. robur* habitats ( $20.27 \pm 1.68\text{mg}$ ). Seeds from *B. robur* plants in intermediate habitat at Appin Road were lighter ( $30.12 \pm 1.15\text{mg}$ ) than seeds from *B. robur* plants in *B. robur* habitat ( $36.30 \pm 0.65\text{mg}$ ).

## 5.4 Discussion

### 5.4.1 Extent of hybridization

The presence of first generation hybrids amongst mature plants and seedlings within the contact zones is evidence for past and continuing hybridization between the two species. The complex nature of hybrid genotypes amongst mature plants and seedlings suggests hybrid fertility which was confirmed by viable seed production by hybrid maternal plants. Introgression of genes appeared to have occurred in both directions. However, there was evidence that introgressed genes were removed by backcrossing to parental species, a process which has probably restricted introgression to contact zones. However, low variation and too few genetic markers may have masked some

hybrid ancestry, making a comparison between parental species and hybrids problematic.

Eight of the eleven seedlings identified as inter-specific crosses came from hybrid plants and ten of twelve seedlings from hybrid plants were sired by one of the parental species. These findings highlight the importance of hybrid plants in continuing hybridization and introgression. It is also evidence that perhaps flower phenology restricts hybridization as hybrid plant flowering times overlap more with the parental species than the parentals overlap with one another (Schibeci 1994). Hybridization could be restricted first by floral phenology and second by the restrictions that intermediacy places on adaptation to parental habitats in traits such as leaf size and proteoid root development. The latter will be explored more in the next chapter.

#### *5.4.2 Individual seedling performance*

It is likely that none of the potted seedlings in the genotypic groups, *B. oblongifolia*, *B. robur* and hybrid seedlings were advantaged or disadvantaged by the environment of the pots and water regimes. None of the groups appeared to be stressed as shown by the high average

photosynthetic yields of the three genotypic groups amongst the three water regimes.

Although hybrid seedling numbers were small, seeds and seedlings from hybrid plants germinated and survived to 16 months almost in the same proportion as parental species (5.8% vs 6.6%). Individual hybrid seedlings displayed a wide range of performances amongst the different traits measured. Nevertheless, when growth traits were analyzed together, individual hybrids were mainly intermediate with some exceptions.

There were two hybrid seedlings that demonstrated greater than average growth for the two species. One hybrid seedling, backcrossed to *B. oblongifolia*, had leaf numbers and area superior to all but one parental seedling and above average proteoid root development. Another seedling with a first generation genotype from a hybrid maternal plant, had a greater number of large proteoid root nodules than all but one parental seedling and was also was taller and had a heavier seed weight than most other seedlings. In contrast, a first generation hybrid from a *B. robur* maternal plant was the shortest seedling and had the least leaf area, fewest leaves, intermediate seed weight, and low numbers of medium to large proteoid roots. The differences between hybrid seedlings may have been caused by environmental differences between pots. Alternatively, these examples may support the

findings that individual hybrids can be very different in fitness (Rieseberg and Ellstrand, 1993) and that some hybrid genotypes can be as fit, or fitter, than parental genotypes (Rieseberg and Carney 1998) at least in some characters.

Proteoid root sizes, like leaves, were characteristically different between the two species. *B. oblongifolia* seedlings generally had a greater number of small nodules and *B. robur* had a greater number of large nodules. If larger leaves are an adaptation to wet areas and proteoid root nodules are an adaptation to the water-logged soils and poor nutrient accessibility in *B. robur* habitat, then there would be implications for hybrids that have intermediate proteoid root development. Neither *Banksia oblongifolia* nor hybrids would be expected to grow as well in *B. robur* habitats. Conversely if smaller leaves (Givnish 1979, Dudley 1996) and proteoid root nodules are an adaptation to drier conditions then *B. robur* and hybrids are not expected to grow as well in *B. oblongifolia* habitats. Adaptation within the *Banksia* hybrid zones is another area of interest for study in the future.

Even though the two species have different leaf numbers and sizes, the average leaf area did not vary between the two species or their hybrids. The two species have different adaptation strategies to different habitats without compromising their photosynthetic capacity. *B. robur* seedlings had a

smaller number of large leaves and in *B. oblongifolia* seedlings had larger number of small leaves. Although some hybrids had intermediate leaf sizes (Usher *et al.* 2010), the mean leaf area was the same as the parental species, indicating that hybrids are not compromised in photosynthetic potential by having intermediate leaf sizes.

#### 5.4.3 Performance correlations

Multiple traits appear to work together to produce larger seedlings. For the two parental species and their hybrids, as seedlings became taller they generally had more leaves, greater leaf area per plant and greater numbers of proteoid root nodules. All of the growth measurements correlated with one another with few exceptions. There was strong correlation between leaf area, the number of leaves and the height of seedlings in each species. There was also strong correlation between proteoid root number (medium sized amongst *B. oblongifolia* and large amongst *B. robur* seedlings) and leaf number, leaf area and seedling height in each species. Like many plant traits, it appears that these multiple traits are linked and respond to the environment together (see Dudley 2004). If plant size and reproductive success are linked (Farris and Lechowicz 1990, Dudley 2004) and seedling size predicts its mature size, then the correlation between plant size and reproductive success has implications in this study. Although hybrids seedlings were intermediate

in many characteristics, the average leaf area and height of seedlings was the same as the parental species, indicating that the overall size of many of the hybrid seedlings was not compromised by their intermediacy. This has important implications only if the seedling size is related to its eventual size and reproductive output as a mature plant.

There was no evidence to suggest that seed weight contributes to performance in the parental species seedlings after germination, because none of the performance measures correlated with seed weight. Because hybrid seedlings had a mixture of parental and intermediate phenotypes, correlations between larger proteoid root numbers and heavier seed weight was likely reflect the differences between the two species. It is unclear why there was a positive correlation between seed weight and seedling height amongst hybrid seedlings. Although seed weight was not performance related in regard to seedling growth, it was found in chapter 4 that selection could have been acting against germination of *B. robur* seed in *B. oblongifolia* habitat. In this study mature hybrid plants found outside *B. robur* habitat had *B. oblongifolia* genotypes introgressed with only one *B. robur* allele. Therefore, the weights of hybrid seeds could give an indication of their viability in certain habitats. This needs to be tested further.

When all seven of the measured characteristics were analyzed together, 9 of the 12 hybrids were intermediate between the two species. Intermediacy has implications for hybrid survival outside of intermediate habitats if the characteristics of the parental species are strongly selected for in their own habitats.

#### *5.4.4 Effects of maternal site and habitat on seedling performance*

When the performance of seedlings was compared between the sites and habitats of origin of their maternal plants, there appeared to be some acclimation to habitat (likely due to habitat differences and not maternal genetic effects) and to site (leaf area amongst *B. oblongifolia* seedlings and seed weight amongst *B. robur* seedlings). There was, however, a 20% difference between sites in the germination rate of seeds collected from each site. This could have had implications for the transplant experiment (see Chapter 4, 4.2 Methods section 4.2.3 *Transplant Experiment*) in that, although seeds were pooled between sites, performance differences were more likely due to habitat effects and not confounded by maternal differences between sites. Nevertheless, differences between habitats may have confounded comparisons between parental habitat and intermediate habitat. It is unknown why seeds from plants at the Darkes Forest site were less viable. There was a similar density of mature plants occupying both sites



however almost three times the number of seedlings at the Appin Road (Chapter 6). Perhaps Darkes Forest populations were older or perhaps there was more competition from other species at Darkes Forest. This would need to be investigated further.

Seed weight was influenced by the site and habitat of the maternal plant from which seed was collected. *B. robur* seeds collected from the Darkes Forest site were heavier than those from the Appin Road site. Seed from *B. oblongifolia* (Darkes Forest) and *B. robur* plants (Appin Road) increased in size when taken from habitats of increasing moisture. Interestingly, seed weight was not correlated with other performance measures (Table 5.2) indicating that seed weights did not affect the performance of the next generation seedlings. An exception was found in leaf numbers and area among seedlings raised from seed collected from *B. oblongifolia* plants at Darkes Forest. Leaf numbers and area increased among seedlings originating from the drier habitats to the wetter habitats.

Mean seed weights of hybrid plants were more similar to parentals in parental habitats and intermediate in intermediate habitats. This could be further evidence for selection constraints on seed size and germination in particular habitats (see chapter 4 and selection against the germination of *B. robur* seed in *B. oblongifolia* habitat), or simply the product of backcrossing

in parental habitats. In the next chapter I will explore whether hybrids found in parental habitats are more likely to have parental rather than intermediate characteristics within all groups of plants and seedlings (transplant and naturally emerging seedlings).

Although hybrid seedlings were few, there was no evidence for endogenous selection against hybrids in a relatively uniform environment. Hybrid seedlings performances varied widely between individual seedlings but most were similar to seedlings with parental genotypes. Generally, hybrid traits and performances were intermediate between traits and performances of the two parental species with a few exceptions.

## *Chapter 6*

### **Distribution and performance of seedlings emerging naturally across the *Banksia* hybrid zones**

#### *6.1 Introduction*

The distribution of seedlings can be governed by limited seed dispersal. However, in the *Banksia* hybrid zones, pollen dispersal and the unique mosaic nature of hybrid zones, allow different genotypes to occupy more diverse habitats. This system provides an excellent opportunity to monitor the relative survival and performance of seedlings in different habitats. In this chapter, I explore the natural distribution of *Banksia oblongifolia*, *B. robur* and hybrid seedlings emerging across the two hybrid zones after fire and determine how they are performing in the different habitats associated with the hybrid zones. To look at temporal differences between the distribution of seedlings after a recent recruitment and the distribution of past accumulated recruitment events, I will also compare the distribution of the mature plants with the distribution of seedlings within the hybrid zones.

*B. robur* and *B. oblongifolia* have differential fitness within the various habitats of the hybrid zones which is likely to be a result of their different morphology. They have dissimilar leaf size and number. Although leaf morphology is complex (Poorter *et al.* 2009), leaf size and number are important factors in determining the fitness of plants in their environment (Geber and Griffen 2003). In this chapter, I look at indirect evidence for selection within parental habitats by determining the predominant leaf morphology of hybrids within the parental habitats. Considering that hybrid seedling morphology was either intermediate or similar to the parental species (chapter 3), I will examine the morphology of hybrids to determine whether leaf morphology could be a constraining factor in the development of hybrids in parental habitats. I will examine whether hybrid plant and seedling morphology in parental habitats resembles the parental species predominant in those habitats. As there was no obvious selection against any of the genotypes in intermediate habitats, I will determine whether hybrids of intermediate morphology are restricted to intermediate habitat. I will determine whether hybrids found in parental habitats have parental leaf morphology in emerging seedlings, transplant seedlings and mature plants.

The distribution of emerging seedlings when compared with the distribution of mature plants (consisting of many generations) across the hybrid zones can answer some important questions about continuing hybridization

The following questions will be examined in this chapter.

1. What is the natural distribution of seedlings emerging after fire? How does this compare with the distribution of mature plants? What is the proportion of hybrids?
2. What is the relative performance of seedlings after two years of growth?
3. How does performance of naturally emerging seedlings compare with performance of seedlings transplanted as seed?
4. Is selection operating, either directly or indirectly on leaf morphology (as a result of selection on another trait) amongst seedlings emerging naturally, transplant seedlings and mature plants within the three habitats associated with the hybrid zones?

## *6.2 Materials and Methods*

To determine the natural distribution, performance, morphology and genotypes of seedlings within three habitats types (see 4.2.4. *Habitat Types*)

associated with the two contact zones, the Appin Road and Darkes Forest sites, 870 seedlings emerging after fire were tagged within one meter wide transects across the hybrid zones. Three transects at the Appin Road site (130m, 150m, 70m) and four at the Darkes Forest site (76m, 100m, 100m, 150m,) were monitored and measured over a two year period (see 4.2.6 *Seedling Fitness and Performance Measurements*). The adult plants and seedlings were counted along each transect and the average density calculated over the range of each transect.

Within transects soil samples were taken every six meters or where there were obvious changes in habitat or floristic composition to determine habitats types (see 4.2.1 *Habitat Types*). After two years and after fine scale habitat types had been determined along transects, 174 seedlings, 42 from the Darkes Forest site and 132 from the Appin Road site (seedlings were more abundant at the Appin Road site) were genotyped. Seedlings were randomly selected from *B. oblongifolia* and *B. robur* habitats, however all seedlings from intermediate habitat were genotyped. Intermediate habitats were very restricted and the numbers of seedlings within them were limited. From the Darkes Forest site 14 seedlings were from *B. robur* habitat, 14 from *B. oblongifolia* habitat and 14 from intermediate habitat. From the Appin Road site 44 seedlings were from *B. robur* habitat, 44 from *B. oblongifolia* habitat and 44 from

intermediate habitat. The 174 seedlings were genotyped using microsatellite markers (see 4.2.5 *Genetic Techniques*). To determine whether there was selection against hybrids during the first two years of growth, the number of surviving seedlings after two years was recorded and compared with those originally emerging. To determine the comparative fitness and performance of seedlings, measurements as described in chapter 4 (4.2.6 *Seedling Fitness and Performance Measurements*) were used. The height of each seedling from the ground to leaf tip was measured and the number of leaves counted. Percentage herbivore damage was estimated by counting the numbers of leaves damaged and estimating the amount of damage on each leaf.

#### 6.2.1 Statistical analyses

##### **Survival**

A contingency analysis (SAS - JMP 5.1) was conducted on seedlings between the three genotypic groups, *B. oblongifolia*, *B. robur* and hybrids to determine whether there was evidence for selection against the survival of hybrid seedlings by comparing the genotypes of seedlings that emerged with those that survived for the first two years of growth and development.

### **Individual seedling fitness and performance**

Compound nested ANOVA analyses (SAS - JMP 5.1) were used to compare the mean of performance measurements between each genotypic group, *B. oblongifolia*, *B. robur* and their hybrids; between the two sites, Appin Road and Darkes Forest; and between the three habitats, *B. oblongifolia*, *B. robur* and intermediate.

To determine whether there was evidence for hybrid heterosis or hybrid inferiority, the performance measurements of individual hybrids, labeled by their broad genotypic classes (first generation, F1; back crossed to *B. oblongifolia*, BC-o; back crossed to *B. robur*, BC-r; *B. oblongifolia* with one *B. robur* allele, I-o; and *B. robur* with one *B. oblongifolia* allele, I-r), were compared with the 95% confidence limits for the two species using a one way ANOVA analysis (SAS - JMP 5.1).

### **Comparison between naturally emerging seedlings and transplant seedlings**

A contingency analysis (SAS - JMP 5.1) was conducted comparing survival to two years between the transplant experiment seedlings (Chapter 5) and the naturally emerging seedlings. One way ANOVA analyses (SAS - JMP 5.1) were performed, comparing seedling height and number of leaves (within



species) among naturally emerging seedlings and transplant seedlings, to determine whether the process of transplanting seeds has an affect on seedling performance. A one way ANOVA analysis (SAS - JMP 5.1) was performed, comparing herbivore damage among naturally emerging seedlings, transplant seedlings and re-sprouting plants, to determine whether the process of transplanting seeds has an affect on the amount of herbivore damage on seedlings; and to determine whether there is a difference in herbivore damage between mature plants and seedlings.

#### **Leaf morphology within the different habitats of the hybrid zones**

Leaf morphology of natural and transplant seedlings, and mature plants, was compared between habitats, to determine whether or not hybrid seedlings growing within the parental species habitats were phenotypically more like the parental species than in intermediate habitats. To determine the morphology of seedlings within the different habitats, discriminant analyses (SAS - JMP 5.1) were performed on the leaf width and length of the largest leaf from each seedling, the ratio of leaf width and leaf length (used as a surrogate for leaf shape) and the number of leaves per seedling. For mature plants the same procedure and measurements were followed as in chapter 3 but within habitats rather than populations. A probability of less than 90% (see Chapter 3) was

deemed to have intermediate (hybrid) morphology in both seedling and mature plants.

### 6.3 Results

#### 6.3.1 Distribution of seedlings

Seedlings were more numerous at the Appin Road site, however mature plants were less abundant than at the Darkes Forest site. Seedlings emerging after fire in the contact zones were much more numerous (2.3 to 2.8 seedlings per meter) on the northern facing aspect of the Appin Road site than the southern aspect (0.3 seedlings per meter) or the Darkes Forest Site (0.2 to 0.7 seedlings per meter). Adult plants averaged 0.5 plants per meter at Appin Road, but 0.5 to 1 plant per meter at Darkes Forest. Indeed, the transect with the most adult plants at Darkes Forest had the least number of seedlings (0.2 seedlings per meter).

A greater percentage of hybrids were found amongst seedlings than mature plants ( $\chi^2 = 12.63$ ,  $DF = 3$ ,  $P = 0.006$ ). Amongst the sample of 174 seedlings, 16.6% (29) had hybrid genotypes, 44.3% (77) *B. oblongifolia* and 39.1% (68) *B. robur* (Figure 4.3). A sample of 84 maternal plants, 28 from each of the three habitats, produced 8.3% (7) hybrids, 46.5% (39) *B. oblongifolia*, 45.2% (38) *B. robur* (Table 6.1).

At the Appin Road site hybrid plants and seedlings with a range of hybrid genotypic classes were found within all habitat types. At the Darkes Forest site, amongst seedlings, few hybrids were detected. Only three hybrids backcrossed to *B. oblongifolia* in *B. oblongifolia* habitat were found (Table 6.1). Amongst seedlings most hybrids were backcrossed to parental species, 15 backcrossed to *B. oblongifolia* and 7 backcrossed to *B. robur*. There was also one first generation (0.6% of seedlings genotyped), a later generation and five introgressed seedlings, 4 with a *B. oblongifolia* allele and one with a *B. robur* allele. Surprisingly the different hybrid classes were not restricted to specific habitats and backcrosses appeared in opposing parental habitats (Table 6.1).

Parental species were predominant in their native habitats amongst mature plants and seedlings. For mature plants only one *B. robur* plant (3.7%) was found in *B. oblongifolia* habitat and for seedlings only four *B. robur* plants (5.2%) were found. In *B. robur* habitat only 4 *B. oblongifolia* plants (13.8%) and only 9 (15.5%) *B. oblongifolia* seedlings were found. In intermediate habitat similar numbers of the two parental species were found for both mature plants and seedlings (Table 6.1).

Table 6.1 Genotypes within habitats and sites of mature plants and seedlings emerging after fire sampled across the two hybrid zones. Hybrids are divided into their broad genotypic classes (first generation, F1; later generation F?;

backcross to *B. oblongifolia*, BC-o; backcross to *B. robur*, BC-r; *B. oblongifolia* with one *B. robur* allele, I-o *B. robur* with one allele *B. oblongifolia*, I-r).

Site	Habitat	Genotypic groups	No. of plants	No. of seedlings
Appin Road	<i>B. oblongifolia</i>	<i>B. oblongifolia</i> <i>B. robur</i> hybrid	13 0 2 (I-o)	36 3 5 (2 BC-o, 2 BC-r, I-o)
	<i>B. robur</i>	<i>B. oblongifolia</i> <i>B. robur</i> hybrid	0 12 1 (F <sub>1</sub> )	8 26 10 (4 BC-o, 3 BC-r, F <sub>1</sub> , I-o, I-r)
	Intermediate	<i>B. oblongifolia</i> <i>B. robur</i> hybrid	6 8 2 (I-o)	17 16 11 (6 BC-o, 2 BC-r, F <sub>2</sub> , 2 I-o)
Darkes Forest	<i>B. oblongifolia</i>	<i>B. oblongifolia</i> <i>B. robur</i> hybrid	11 1 0	11 0 3 (BC-o)
	<i>B. robur</i>	<i>B. oblongifolia</i> <i>B. robur</i> hybrid	4 10 2 (F <sub>1</sub> , BC-r)	1 13 0
	Intermediate	<i>B. oblongifolia</i> <i>B. robur</i> hybrid	5 7 0	4 10 0

### 6.3.2 Survival and growth

Hybrid seedling survival was similar to parental species. After two years 70% of all seedlings had survived. There was no significant difference in survival between the three genotypic groups, *B. oblongifolia* (70%), *B. robur* (67%) and hybrids (80%) ( $\chi^2 = 1.84$ ,  $DF = 2$ ,  $P = 0.40$ ).

There were genotype, site and habitat differences in the average heights of seedlings growing naturally in the field ( $F_{13,133} = 3.75$ ,  $P < 0.0001$ ). Hybrid seedlings were on average shorter (least sq mean  $16.36 \pm 2.3\text{cm s.e}$ ) than *B. oblongifolia* ( $20.47 \pm 1.61\text{cm}$ ) and *B. robur* seedlings ( $23.53 \pm 1.24\text{cm}$ ). *B. robur* and *B. oblongifolia* seedlings were both shorter at the Appin Road site ( $20.65 \pm 1.82$  and  $16.72 \pm 1.27\text{cm}$ ) than the Darkes Forest site ( $26.42 \pm 1.69$  and  $24.23 \pm 2.97\text{cm}$ ). There was no difference between habitats (Figure 6.1).

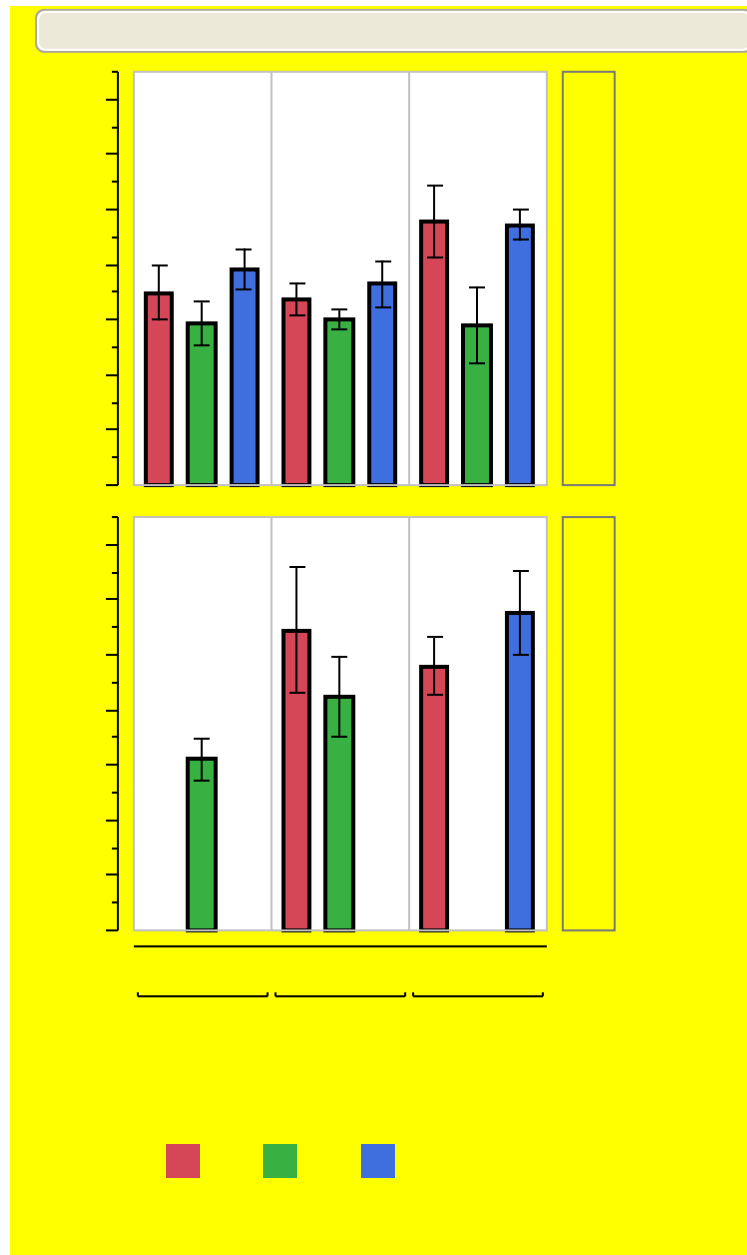


Figure 6.1 Mean heights of seedlings recruited naturally after fire and after two years of growth across the two hybrid zones, A. within the three

genotypic groups (H = hybrids, O = *B. oblongifolia*, R = *B. robur*), B. within the two sites, C. within the three habitats (HE = intermediate, OE = *B. oblongifolia* and RE = *B. robur* habitat). Error bars = standard error.

Among the 24 surviving hybrid seedlings, heights of individual seedlings varied between one another, but there were only two seedlings with above average heights (Figure 6.2). Two hybrid seedlings backcrossed to *B. oblongifolia* had heights (31 & 27cm) above the 95% CI for both species (15.17 – 26.28cm). Ten hybrids had below the 95% CI for both species and twelve had average heights (Figure 6.2).

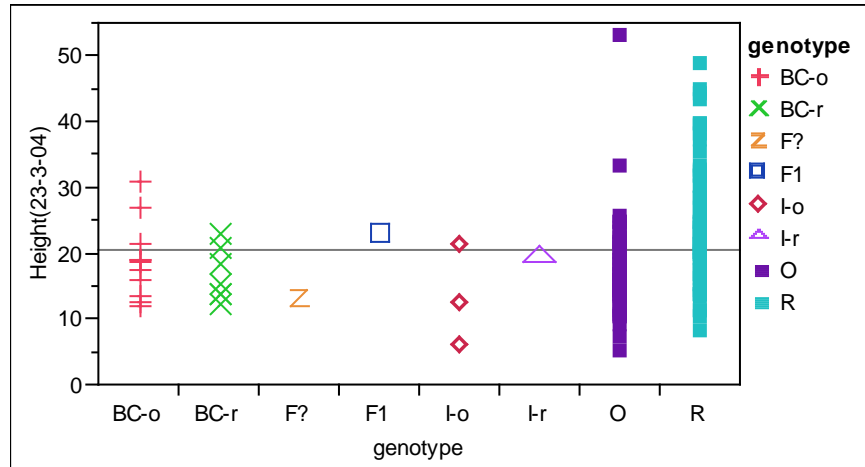


Figure 6.2 Heights of individual seedlings recruited naturally after fire and after two years of growth across the two hybrid zones (*B. oblongifolia* (O) and *B. robur* (R) and hybrid classes (first generation, F1; later generation F?; backcross to *B. oblongifolia*, BC-o; backcross to *B. robur*, BC-r; *B. oblongifolia* with one *B. robur* allele, I-o *B. robur* with one allele *B. oblongifolia*, I-r).

The average number of leaves on *B. robur* seedlings (least sq mean  $7.08 \pm 0.74$  s.e), was less than *B. oblongifolia* ( $13.11 \pm 0.97$ ) and hybrids ( $10.03 \pm 1.42$ ) were intermediate ( $F_{13,133} = 3.63$ ,  $P < 0.0001$ ). *B. oblongifolia* seedlings and *B. Robur* had more leaves at Darkes Forest ( $15.70 \pm 1.87$  and  $8.57 \pm 1.01$ ) than at the Appin Road site ( $10.53 \pm 0.76$  and  $5.60 \pm 1.09$ ), (Figure 6.3).



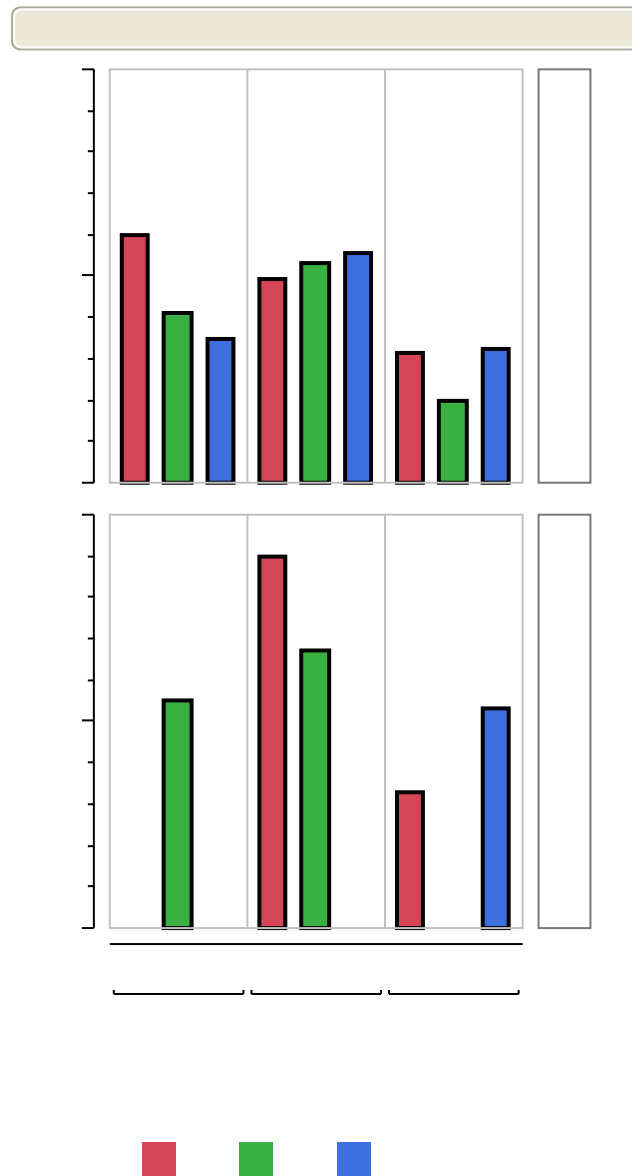


Figure 6.3 Mean numbers of leaves on seedlings recruited naturally after fire and after two years of growth across the two hybrid zones, A. within the three genotypic groups (H = hybrids, O = *B. oblongifolia*, R = *B. robur*), B.

within the two sites, C. within the three habitats (HE = intermediate, OE = *B. oblongifolia* and RE = *B. robur* habitat). Error bars = standard error.

The numbers of leaves on individual hybrids were mainly within the 95% CI (5.96 - 12.49) for the means of the two species. Sixteen hybrids had average leaf numbers, four were above the upper 95% CI and four were below the lower 95% CI (Figure 6.4).

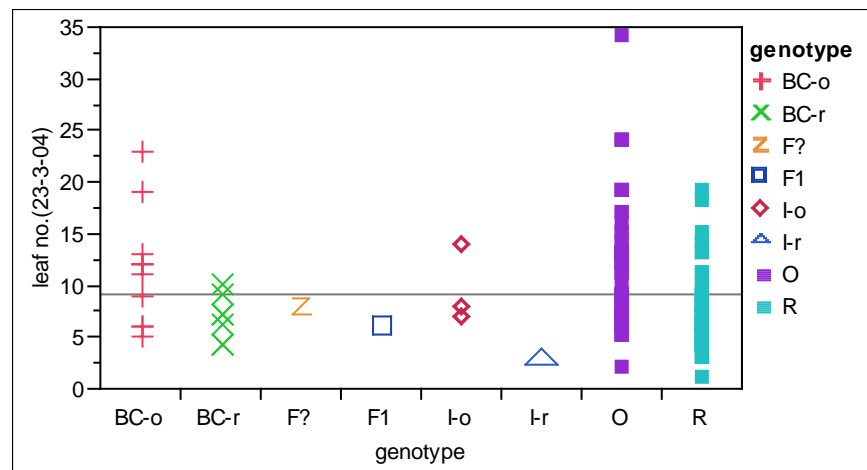
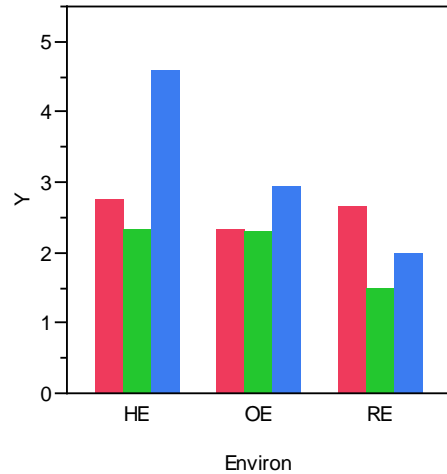


Figure 6.4 Numbers of leaves on individual seedlings recruited naturally after fire and after two years of growth across the two hybrid zones (*B. oblongifolia* (O) and *B. robur* (R) and hybrid classes (first generation, F1; later generation F?; backcross to *B. oblongifolia*, BC-o; backcross to *B. robur*, BC-r; *B. oblongifolia* with one *B. robur* allele, I-o *B. robur* with one allele *B. oblongifolia*, I-r).

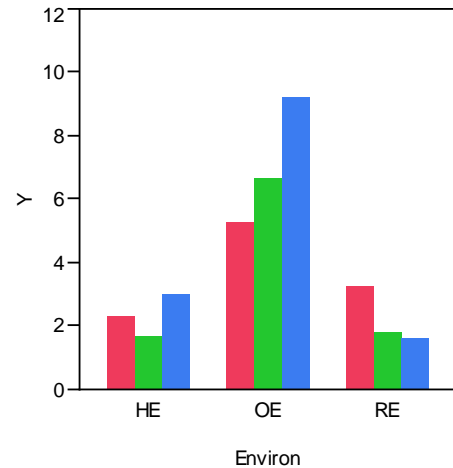
### 6.3.3 Herbivory

There was no difference in herbivore damage between genotypes or genotypes within sites or habitats. All three types of leaf damage, edge, hole and miner damage, were found on seedlings in both species and on hybrids, and in all three habitat types, *B. oblongifolia*, *B. robur* and intermediate habitats, at both sites (Figure 6.5). Only miner damage was different between genotypes, genotypes within sites and habitats ( $F_{12,65} = 5.14$ ,  $P < 0.0001$ ). The leaves of *B. oblongifolia* seedlings were attacked by miners more ( $6.71 \pm 0.60$ ) than hybrid seedlings ( $4.96 \pm 1.02$ ) and *B. robur* seedlings ( $2.01 \pm 0.69$ ), particularly in the Darkes Forest site ( $10.29 \pm 1.02$ ). Hybrid plants in intermediate habitat at Appin Road also had more miner damage ( $6.33 \pm 1.11$ ) than hybrids in the other habitats ( $4.25 \pm 1.35$  and  $2.67 \pm 1.11$ ).

A. Appin Road



B. Darkes Forest



Y  
■ Mean(edge(23-3-04))  
■ Mean(holes(23-3-04))  
■ Mean(miners(23-3-04))

Y  
■ Mean(edge(23-3-04))  
■ Mean(holes(23-3-04))  
■ Mean(miners(23-3-04))

Figure 6.5 Mean number of leaves with the three types of herbivore damage on seedlings recruited naturally after fire and after two years of growth across the two hybrid zones. HE = intermediate habitat, OE = *B. oblongifolia* habitat, and RE = *B. robur* habitat. Error bars = standard error.

Herbivore damage of individual hybrids varied among one another. Seven hybrid seedlings had percentage herbivore damage within 95% CI for the parental species (9.02 - 17.07). Twelve hybrid seedlings had percentage herbivore damage above the upper 95% CI for the parental species and five

hybrid seedlings had percentage herbivore damage below 95% CI for the parental species (Figure 6.6).

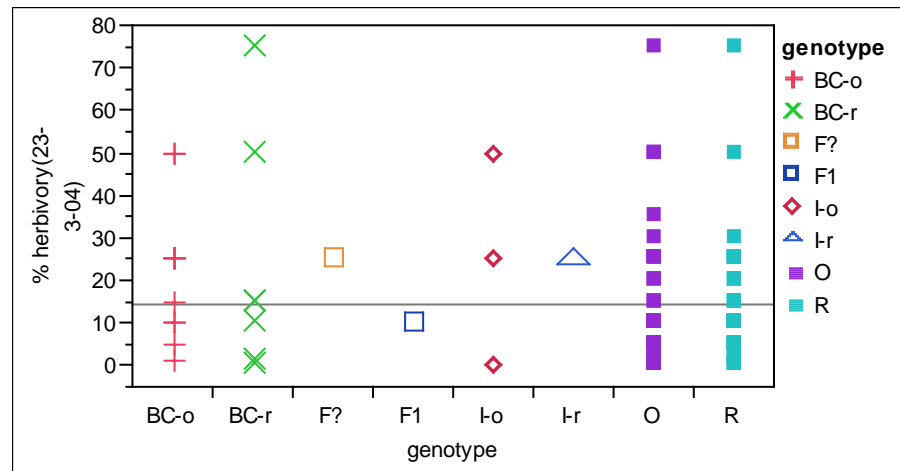


Figure 6.6 Percentage herbivore damage on individual seedlings recruited naturally after fire and after two years of growth across the two hybrid zones (*B. oblongifolia* (O) and *B. robur* (R) and hybrid classes (first generation, F1; later generation F?; backcross to *B. oblongifolia*, BC-o; backcross to *B. robur*, BC-r; *B. oblongifolia* with one *B. robur* allele, I-o *B. robur* with one allele *B. oblongifolia*, I-r).

#### 6.3.4 Comparison between naturally emerging seedlings and transplanted seedlings

Surprisingly, survival was greater amongst transplant seedlings (80%) than natural seedlings (69%), ( $\chi^2 = 7.24$ ,  $DF = 1$ ,  $P = 0.007$ ). *B. robur* seedlings were taller among the natural seedlings ( $24.35 \pm 1.03\text{cm s.e}$ ) than the transplanted seedlings ( $18.18 \pm 0.83\text{cm s.e}$ ), ( $F_{1,178} = 21.07$ ,  $P < 0.0001$ ) and had more leaves ( $7.14 \pm 0.42$  vs  $6.09 \pm 0.13$ ), ( $F_{1,178} = 3.92$ ,  $P = 0.49$ ). *B. oblongifolia* seedlings were the same heights between treatments ( $17.31 \pm 0.91$  vs  $15.49 \pm 0.76\text{cm s.e}$ ), ( $F_{1,171} = 2.33$ ,  $P = 0.13$ ) and had the same number of leaves ( $11.25 \pm 0.79$  and  $11.11 \pm 0.57$ ), ( $F_{1,171} = 0.23$ ,  $P = 0.88$ ).

When percentage herbivore damage on transplant seedlings was compared with percentage herbivore damage on re-sprouting adult plants (Chapter 4) and the seedlings emerging after fire, *B. robur* transplant seedlings had more damage ( $21.44 \pm 1.80\%$  vs  $14.03 \pm 2.32\%$  and  $12.62 \pm 2.26\%$  s.e), ( $F_{2,227} = 5.74$ ,  $P = 0.0037$ ).

#### 6.3.5 Morphology within habitats

In *B. oblongifolia* habitat, seven of the nine hybrid seedlings and the one hybrid plant had *B. oblongifolia* morphology. In *B. robur* habitat only five of the nineteen hybrids had *B. robur* morphology. In intermediate habitat, nine of

the twenty four hybrids had hybrid (intermediate) morphology. However, all but one of the seedlings in *B. oblongifolia* habitat had genotypes more similar to *B. oblongifolia* than *B. robur*. Amongst the emerging seedlings 19 of the 28 hybrid seedlings had morphology resembling *B. oblongifolia* (Table 6.2). This was not surprising as most seedlings (18) were backcrossed or introgressed to *B. oblongifolia* (that is hybrids had genotypes more similar to *B. oblongifolia* than *B. robur*). In *B. robur* and intermediate habitats, there appeared to be a range of hybrid genotypes and morphologies (Table 6.2). Nevertheless, hybrid numbers were small and inferences need to be made with caution.

Table 6.2 Morphological classifications of hybrid seedlings and plants (genetically determined) within the three habitats of the hybrid zones. Hybrids were placed in their broad genotypic classes (first generation, F1; later generation F<sub>2</sub>; backcross to *B. oblongifolia*, BC-o; backcross to *B. robur*, BC-r; *B. oblongifolia* with one *B. robur* allele, I-o *B. robur* with one allele *B. oblongifolia*, I-r) and their morphological classification determined. ( $P < 0.90^*$  is the probability of having intermediate leaf morphology and hence classified as a morphological hybrid).

		Broad hybrid genotypic classification					
Habitat	Classification by leaf morphology.	Emerging hybrid seedlings	<i>P</i>	Transplant hybrid seedlings	<i>P</i>	Mature hybrid plants	<i>P</i>
<i>B. oblongifolia</i>	<i>B. oblongifolia</i>	I-o BC-o BC-o BC-o BC-o BC-r	0.97 0.96 0.90 0.96 0.99 0.99	BC-o	0.97	I-o	1.00
	<i>B. robur</i>	BC-r	0.98				
	hybrid	BC-o	0.63*				
<i>B. robur</i>	<i>B. oblongifolia</i>	BC-o BC-o BC-o F <sub>1</sub>	0.96 0.99 0.99 0.98			F <sub>1</sub>	0.97
	<i>B. robur</i>	BC-r BC-r I-r	0.95 0.90 0.95			BC-r I-r	1.00 1.00
	hybrid	I-o BC-r BC-r BC-o	0.82* 0.64* 0.58* 0.77*	F <sub>7</sub> BC-r BC-r	0.60* 0.76* 0.75*	BC-o BC-o	0.81* 0.78*
Intermediate	<i>B. oblongifolia</i>	BC-o BC-o BC-o BC-o BC-o I-o I-o BC-r F <sub>7</sub>	1.00 1.00 1.00 1.00 0.98 1.00 0.95 0.96 0.99	BC-o I-o I-o	0.97 0.92 0.99	I-o I-o	1.00 1.00



	<i>B. robur</i>			I-o BC-r	0.96 0.98		
	Hybrid	BC-r	0.84*	F <sub>1</sub> F <sub>1</sub> F <sub>1</sub> F <sub>2</sub> F <sub>2</sub> BC-o BC-r I-r	0.65* 0.62* 0.74* 0.57* 0.67* 0.64* 0.52* 0.73*		

## 6.4 Discussion

### 6.4.1 Seedling distribution

The distribution of seedlings was different from the distribution of mature plants between sites. Even though there were more mature plants at the Darkes Forest site, seedlings were more abundant at the Appin Road site. In chapter 5 it was found that seeds from Darkes Forest were less viable than seeds from Appin Road, which would explain why fewer seedlings emerged at the Darkes Forest site. The Darkes Forest population could have been older than the Appin Road population, hence bearing older seed which could have been less viable (K. Ottewell, pers. com.). Alternatively, competition from other species could have been greater at the Darkes Forest site than Appin Road. Competition can be a dominating factor in an individual's environment

(Dudley 2004). However, competition was not assessed in this study. Nevertheless the two sites appear to have different dynamics which would be interesting to investigate further.

The percentage of hybrids amongst emerging seedlings was greater than amongst mature plants. This could mean that selection against hybrids occurs after 2 years. Alternatively, there could have been more hybridization within this recruitment event due to greater in the overlap in flowering times. Flowering times can differ from year to year (Taylor and Hopper 1988). However, variation in the spatial distribution of hybrids (hence variation between samples) was most likely, as hybrids between the two generations appeared to remain constant (chapter 4 and 5).

Between habitats mature plants and seedlings were similar in distribution. Parental species seedlings predominate in their own habitats, and almost equal numbers of the two species were found in intermediate habitat. Hybrid seedlings were found in all three habitat types at Appin Road. Backcrossed hybrids (hybrids with more loci from one species than the other), particularly to *B. oblongifolia*, were the main hybrid genotypic classes detected. Backcrossing to both species indicated pollination of hybrid plants by both species and hence introgression in both directions. However, more hybrids are needed to observe clear trends. Hybrids backcrossed to each species were

found in all three habitats, not only within the habitat of the backcrossing species. This is not surprising as the species were found in close proximity to one another within the mosaic hybrid zones. More hybrids were detected from the Appin Road site than from the Darkes Forest site. This could be related to the low viability of seeds from the Darkes Forest site generally and needs further investigation.

#### 6.4.2 *Survival, growth and herbivore damage*

As for the maternal plants and the transplant seedlings, there was no evidence for selection either for, or against, hybrid seedlings emerging naturally across the hybrid zones. An exception was in the height of naturally occurring hybrid seedlings which were shorter on average. All individual hybrids were either intermediate or like the parental species.

Interestingly, leaf numbers were not statistically different between habitats. In Chapter 3, it was found that phenotypic plasticity appeared to occur in response to water stress (Sultan and Bazzaz 1993b, Dudley 2004). *B. oblongifolia* seedlings were probably stressed due to drought conditions, which resulted in their smaller size and fewer leaves. *B. robur* seedlings were less constrained by lack of water so were able to grow taller and produce relatively more leaves. Therefore, the two species appeared to be more similar

to one another in the field than seedlings of each species raised in pots (Chapter 5). Despite the drought conditions during this study, there was no difference in survival between the two species or their hybrids.

#### *6.4.3 Comparison between naturally emerging seedlings and transplanted seedlings*

There were differences in performance between seedlings emerging naturally within the hybrid zones and seedlings sprouting from transplanted seed. Interestingly, transplanted seedlings suffered more herbivore damage, among all genotypes, than naturally emerging seedlings and re-sprouting plants (Chapter 4). Higher herbivore damage on transplanted seedlings could have resulted from transplantation into regions where some genotypes were untested against certain herbivores, although the different types of herbivore damage were never exclusive to any one habitat. There were also some differences in height and number of leaves, within species, between transplanted and naturally emerging seedlings. Differences between transplanted seedlings and seedlings emerging naturally across the hybrid zones have implications for transplant experiments generally. Studies of hybrid zones using transplant experiments may not fully reflect the natural situation. A comprehensive approach to hybrid zone studies perhaps needs to include measurements of plants occurring naturally in the field.

#### 6.4.4 Morphology in parental habitats

Only in *B. oblongifolia* habitat did there appear to be a bias toward parental morphology amongst hybrids. The majority of hybrid seedlings and the one hybrid plant in *B. oblongifolia* habitat had leaf morphology similar to *B. oblongifolia* morphology, however not exclusively. Also, in *B. oblongifolia* habitat, hybrid genotypes were more similar to *B. oblongifolia* than *B. robur*. There appears to be, either selection against *B. robur* morphology (and genes) in *B. oblongifolia* habitat or a greater exchange of pollen between *B. oblongifolia* and hybrid plants than between *B. robur* and hybrid plants. If there was selection against *B. robur* seed germination in *B. oblongifolia* habitat, as suggested in the transplant experiment (Chapter 4), then *B. robur* leaf morphology could be a pleiotropic effect of selection against *B. robur* seed germination. Generally there appears to be little evidence for selection of specific leaf morphology in *B. robur* or intermediate habitats, however these results need to be investigated further using larger numbers of hybrids.

## Chapter 7

### General Discussion

The heath-land *Banksia* communities of the Illawarra region are a mosaic of almost pure species stands and complex hybrid swarms (Schibeci 1994). In this study, I found that mixed populations between *B. robur* and *B. oblongifolia* are indeed zones of continuing hybridization. Genotypes of plants from the hybrid zones and their seedlings revealed small numbers of interspecific crosses and introgression facilitated by fertile hybrid plants. Hybrid zones are restricted to very narrow bands, outside of which the two species are genetically and morphologically distinct. Isolating mechanisms are sufficient to prevent widespread introgression of genes, implying that hybrid genotypes are less fit outside environmentally intermediate hybrid zones (also see Schibeci 1994). Nevertheless, in this study, hybrid plants were as fit as the parental species. Within pure stands, only one *B. oblongifolia* allele was found within *B. robur* populations. This could indicate asymmetry in gene flow from *B. oblongifolia* to *B. robur*; however Schibeci (1994) found the opposite, a *B. robur* allele in *B. oblongifolia* populations. Very small amounts of introgression appear to be occurring in both directions. It is possible that these are rare alleles within the genomes of the species. The fact that the two species are largely genetically and

morphologically distinct, and yet interbreed readily, also suggests strong divergent selection between the two species.

#### *7.1 Distribution of hybrid zone plants and seedlings*

*B. oblongifolia* and *B. robur* plants and seedlings were found in a patchy distribution closely linked to soil moisture content. Schibeci (1994) also found that there were frequent transitions from high frequencies of one species to high frequencies of the other over short distances within the hybrid zones. The close linkage of each species with different habitats within very close proximity suggested that the hybrid zones were more likely to be restricted by habitat type rather than geneflow. Krauss *et al.* 2009 found that the movement of pollen within populations of *Banksia hookeriana* was extensive due to bird pollination.

Although parental species dominated different habitats, some plants and seedlings of the other species were found in opposing habitats. This could have been due to greater plasticity of some plants, undetected hybrids, or undetected microenvironments. Soil moisture was found to vary over short distances (less than 2m) and differences in fitness could be associated with cryptic variation within the environment (Dudley 2004).

In intermediate habitat, plants and seedlings of both species were found in similar numbers, along with hybrids (12.5% amongst mature plants and 25% amongst seedlings). It is possible that there is a 'relaxation of selection' within intermediate habitats (see Campbell *et al.* 2008) allowing both species to occupy the same habitat. Alternatively, the gradient of soil moisture from *B. oblongifolia* habitat to *B. robur* habitat may support *B. oblongifolia* plants closer to *B. oblongifolia* habitat and *B. robur* plants closer to *B. robur* habitat. Nevertheless, the former seems most likely, as equal numbers of the two species grew in intermediate habitat in soil with relatively consistent water content, in the transplant experiment at the Appin Road site (Figure 4.2). Furthermore, the performance of seedlings from all three genotypic groups (*B. oblongifolia*, *B. robur* and hybrids) appeared to be similar in intermediate habitats. Campbell *et al.* (2008), also, found no difference in fitness between two *Ipomopsis* species and their hybrids within hybrid habitat. One explanation for a lack of selection in intermediate habitat is that intermediate habitat and the hybrid genotype were ancestral states before divergence into separate habitats. Therefore, hybrids and parentals should remain as fit as one another in intermediate habitat. Alternatively, the hybrid habitat is benign to both species which are phenotypically plastic enough to survive in intermediate habitat as well as their preferred habitat. However, these two hypotheses would be difficult to separate. Even though both



species and hybrids appear to thrive in intermediate habitats, these habitats are very restricted and confined between the two parental habitats. Moore (1977) suggested 'that stable hybrid zones are often narrow because they tend to occur in ecotones which are them-selves narrow'.

Among mature plants, hybrids were found mainly in *B. robur* habitat. Only *B. oblongifolia* plants introgressed with one *B. robur* allele were found in *B. oblongifolia* and intermediate habitats and only at one site. Contrary to the mature plants, hybrids were found in all three habitats amongst seedlings emerging naturally at the Appin Road site after fire. Furthermore, a greater percentage of hybrids were found among emerging seedlings than mature plants. The difference between generations could have been due to a number of factors. First, hybrids were few so there may have been sampling differences due to a patchy distribution of hybrids within the hybrid zones. This explanation is likely, as the proportion of hybrids between generations amongst maternal plants and their seedlings was similar. Second, temporal variation in conditions between recruitment events could produce different genotypic patterns. Temporal variation occurred between this study and a transplant experiment (Schibeci 1994), completed 10 years prior to current one, in the same study area. Schibeci (1994) reported very low germination rates with seedlings establishing mainly in *B. robur* habitat, nevertheless

hybrids were found in all three habitats. In this study, germination rates were much higher in all three habitats, but the highest germination rates occurred in intermediate habitat. Nevertheless, again hybrids were found in all three habitats indicating hybrid fitness across the hybrid zones. Third, also possible is subsequent selection, after the two years of this study, against certain genotypes as, what appeared to be, a fungal infection was found on leaves amongst *B. oblongifolia* seedlings found in wetter habitats. Whether fungal attack causes future deaths or reduction fecundity in certain genotypes and habitats, needs to be investigated further.

### *7.2 Selection among seedlings*

Generally, no intrinsic genetic incompatibilities amongst hybrid seedlings were evident. Nevertheless, this study confirmed that performances and morphologies of hybrid plants and seedlings can be extremely variable (Rieseberg and Carney 1998). Amongst seedlings raised in pots from seed collected across the hybrid zones, *B. oblongifolia*, *B. robur* and hybrid seedlings, on average, germinated, survived, grew as tall and had the same leaf area as one another. Several plant characteristics appear to work together, in both species, to govern the overall size of seedlings. These include leaf area and number, height of seedling, and proteoid root mass. Seed weight appears to be unrelated to plant performance within the two

*Banksia* species. Farris and Lechowicz (1990) found that no one plant characteristic determined reproductive success, but the proximate correlate to plant reproductive success is plant size. If seedling size is related to mature plant size, then most hybrid plants would be as fit as the parental species. Amongst seedlings raised in pots, most of the hybrids were intermediate between the two species in their overall performance (Chapter 5). Interestingly, hybrid leaf morphology was similar to one or other of the parental species rather than intermediate (Chapter 3). Conversely, most hybrids had intermediate numbers of leaves. Nevertheless, on average, the two species and their hybrids had similar leaf areas per seedling indicating that the photosynthetic potential of hybrid seedlings was not compromised by intermediate numbers of leaves. There were some examples, amongst hybrids, of more extreme performances. Two hybrid seedlings, a backcross to *B. oblongifolia* and a first generation hybrid, demonstrated well above average performance measurements. Whether these examples demonstrate novel phenotypes (Arnold 1997, Rieseberg and Carney 1998) depends on how the seedlings continue to develop. There was also evidence for hybrid inferiority in one first generation hybrid which was smaller than all other seedlings.

Among field seedlings, in the transplant experiment, hybrids germinated at a similar rate to parentals, although hybrid numbers were small. In the transplant experiment and naturally emerging seedlings hybrids survival was also similar to the parental species. Although individual hybrid performance varied considerably between one another, on average, hybrids grew as tall as parental seedlings within habitats and had intermediate numbers of leaves.

Morphologies of hybrid seedlings differed between habitats, although numbers were small so inferences need to be made with caution. There was some evidence among naturally emerging seedlings that hybrid seedlings in *B. oblongifolia* habitat had *B. oblongifolia* morphology, but not exclusively. Conversely, in intermediate and *B. robur* habitats, hybrids had a mix of morphologies that were similar to the two species as well as intermediate between the two species. Nevertheless, there was also some evidence that hybrids with *B. robur* morphology were few outside *B. robur* habitat. However, these results need to be tested with larger sample sizes.

There was no evidence for disruption of genetic combinations for defense mechanisms amongst hybrids. Generally herbivore damage on hybrids was low compared to the parental species, with the exception of the naturally emerging seedlings, which were, nevertheless, not statistically different from the parental species. Furthermore, herbivore damage amongst transplant

seedlings was slightly higher than emerging seedlings and re-sprouting plants, but also not statistically different. There were no differences detected in the type of herbivores between habitats.

### *7.3 Selection among mature plants*

There was no evidence for selection acting on hybrid plants during seed production or re-growth of plants from lignotubers after fire. Hybrid plants produced half as many healthy seed as the parental species, but the means were not statistically different. Schibeci (1994) found no difference in vigour and fecundity between mature hybrid plants and parental plants, as measured by plant height, the total inflorescences produced and mean number of follicles per infructescence. Hybrid plants were, on average, intermediate in characteristics between the two species or similar to one or other of the species in all habitats. Similar to seedlings, individual hybrid performances varied between one another. One hybrid with a *B. robur* genotype introgressed with a *B. oblongifolia* allele demonstrated heterosis with a shoot to lignotuber area well above all other seedlings.

### *7.4 Selection against parental alleles in opposing habitats*

There was some direct and indirect evidence for selection against *B. robur* seed germination in *B. oblongifolia* habitat. *B. robur* seeds did not appear to

germinate in *B. oblongifolia* habitat in the transplant experiment. However, because there was no replication of this result, it needs to be investigated further. Indirect evidence was found in plant and seedling distributions. Few *B. robur* plants (3.7%) and seedlings (5.2%) and few hybrid seedlings with *B. robur* morphology (1 in 8) were found in *B. oblongifolia* habitat providing indirect evidence for selection against *B. robur* phenotypes. There was less evidence for selection against *B. oblongifolia* seedlings in *B. robur* habitat which could not be clarified by the transplant experiment as few seed remained in *B. robur* habitat after heavy rain. Nevertheless, relatively few *B. oblongifolia* plants (13.8%) and seedlings (15.5%) were found in *B. robur* habitat. However, there was a range of hybrid phenotypes found in *B. robur* habitat, indicating less restraint on morphology in *B. robur* habitat than *B. oblongifolia* habitat. Alternatively, there could be greater pollination of *B. robur* plants by *B. oblongifolia* plants than the reverse. There was some evidence for the latter as there were more seedlings backcrossed to *B. oblongifolia* than other hybrid genotypes among naturally emerging seedlings. Finally, mean seed weights of hybrid plants were more similar to parentals in parental habitats and intermediate in intermediate habitats. This could be further evidence for selection constraints on seed size and germination in particular habitats.

Harrison (1986) described the mosaic hybrid zone in terms of a mosaic of selection pressures imposed by the underlying environment. This description applies to the *Banksia* hybrid zones. Nevertheless, if *B. oblongifolia* and hybrid plants are able to compete with in *B. robur* plants in *B. robur* habitats, then *B. robur* plants are at risk of extinction through genetic swamping by pollen from the larger *B. oblongifolia* populations. However, because of the unique nature of mosaic hybrid zones, global extinction will be less likely if the proportion of the two species vary from site to site (Harrison 1986).

#### 7.5 Hybrid zone models

Hybrid inferiority does not appear to be a factor in the maintenance of the *Banksia* hybrid zones and the integrity of the two species, at least after a recruitment event. Contrary to the idea that hybrid breakdown is common in later generation hybrids, I found no evidence for hybrid inferiority or intrinsic nuclear genetic incompatibilities during recruitment within the *Banksia* hybrid zones. Hybrids were not intrinsic less fit than the parental species, as described in the ‘Tension Zone’ model (Barton and Hewitt, 1981) or the ‘Mosaic’ model (Harrison 1986). There was also no evidence for hybrid inferiority in parental habitats and superiority in intermediate habitats as described by the ‘Bounded Hybrid Superiority’ model (Moore, 1977). However, individual hybrids demonstrated characteristics and performances

that were very different from one another with a few unique characteristics, which is consistent with the 'Evolutionary Novelty' model (Rieseberg and Ellstrand, 1993, Arnold 1997).

### 7.6 Phenotypic plasticity

There were some interesting comparisons between the morphology of seedlings growing in pots and those growing in the field within the hybrid zones. In pots, seedlings of parental species were morphologically distinct and hybrids were similar to the parental species. However, amongst field seedlings, the relationship between genotype and phenotype was less clear (Chapter 3). In pots, seedlings from all three genotypic classes, *B. oblongifolia*, *B. robur* and hybrids grew to the same height (Chapter 5). In the field, it was found (Chapter 6) that seedlings were smaller and less well developed than potted seedlings generally, but more so in *B. oblongifolia* and hybrid habitats than *B. robur* habitat. *B. oblongifolia* seedlings were shorter and less well developed due to drought conditions at the time of the study. *B. robur* seedlings were less affected by water stress due to the swampy environment that *B. robur* plants inhabit. Phenotypic plasticity can occur in response to stress (Dudley 2004). As already mentioned water stress can have severe deleterious affects on plant metabolism (Bradford *et al.* 1982, Sultan and Bazzaz 1993b), so growth can be restricted and, also, plants can



be at an earlier ontogenetic stage (Gedroc *et al.* 1996). Therefore, the numbers of leaves on each of the two species were more similar to one another in the field than in pots and, hence, the two species appeared morphologically more similar to one another in the field than in pots.

### 7.7 Conclusion

After fire, during re-growth of plants from lignotubers and recruitment of new seedlings, when selection was likely to be intense, hybrid performance appeared to be, on average, no different to their parental species. The variety of performances among hybrids was best explained by the ‘evolutionary novelty’ model (Arnold 1997). Nevertheless, despite the abundance of hybridization and gene exchange within the *Banksia* hybrid zones, the genetic and morphological integrity of the different species was maintained. A combination of factors probably contributed to the maintenance of the hybrid zones. Schibeci (1994) found that interspecific gene exchange between established plants by common pollinators was restricted by different peak flowering times. However, there was some overlap in flower times particularly between hybrid plants (Schibeci 1994) and parental species. In this study, backcrossing was found to be common but first generation hybrids were rare. Divergent environmental selection in parental species habitats is likely as the two species occupy different habitats, although not

exclusively. There seems to be selection against immigrant alleles, at least in *B. oblongifolia* habitat. Rare and episodic recruitment events, restricted intermediate habitat and the other factors mentioned above, are all likely to affect the structure and maintenance of the *Banksia* hybrid zones.

This study was not comprehensive due to the constraints of working with long-lived species with unpredictable episodic recruitment. Many questions were raised and ideas for further research in adaptation and selection within the *Banksia* hybrid zones. As there is a lack of genetic variation within the two species, many more genetic markers are needed to accurately determine the hybrid classes which were only described in broad terms in this study. To determine whether or not immigrant alleles are selected against in parental habitats and establish clear trends in hybrid fitness, many more hybrid and parental seedlings are needed in transplant experiments within the different habitats and over a longer study period. A study of all stages of the life-cycle would be ideal. Furthermore, it would be interesting to know whether low genetic variation is due to founder effects. A more extensive genetic analysis of the *Banksia* hybrid zones along their east coast range would elucidate more about hybrid zone formation. Future studies on the *Banksia* hybrid zones should include *B. paludosa* which was found on the edge of the Darkes Forest site and could be hybridizing with *B. robur* and *B. oblongifolia*

forming a hybrid complex. Floral phenology was not included in this study however it would be interesting to determine whether flowering time differences between the species are directly or indirectly the result of habitat divergence; whether flowering times are genetically determined; and whether reinforcement of reproductive barriers is likely through temporal isolation of the two species (see Schibeci 1994). Future studies should also include a study of fungal infection on plants and seed, as leaves on *B. oblongifolia* appeared to be severely affected by fungal attack in wetter habitats.

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