GENETIC DIVERSITY AND DIVERGENCE WITHIN AND AMONG NATURAL POPULATIONS OF *ARAUCARIA* IN EASTERN AUSTRALIA.

Thesis submitted by

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ABSTRACT

Historical and contemporary fragmentation of natural populations has resulted in many species being confined to disjunct isolates. An understanding of the underlying distribution of genetic diversity and divergence within species is critical to informed conservation and management practices, yet for many species this information is lacking. While many plant species have been substantially exploited over the past few centuries in Australia, thus potentially exacerbating any genetic effects of historical fragmentation, little is known on the effects of these practices on the genetic diversity and divergence of their extant populations. However, for most plant species, the genetic effects of historical fragmentation prior to exploitation are unknown. Any meaningful interpretation of contemporary genetic impacts must be viewed against an historical background. The genus *Araucaria* (Araucariaceae) has undergone dramatic range reductions and population size fluctuations throughout its history on the Australian continent and is now represented by two extant species: *Araucaria bidwillii* and *Araucaria cunninghamii*. The historical effects of their dynamic histories are examined using two independent neutral molecular markers, RAPDs and ISSRs, and is discussed in terms of extant population size, isolation and historical microfossil records. For both species, these markers proved to be highly congruent in the amounts of diversity they detected and how that diversity was spatially partitioned among populations. High diversity characterised all but one of the populations sampled and was accompanied by high divergence among the populations

sampled. Discrepancies in the amount of variation these markers attributed to regional and populational divergence is discussed and emergent trends noted. The results cast some doubt on some of the earlier, simplistic rainforest refugial hypotheses that have been proposed for eastern Queensland. Furthermore, the results have allowed for a more detailed dissection of the *Araucaria* microfossil record in eastern Australia, which until now could only provide static evidence and indicate the relative regional importance of the genus. When the present results are reviewed in association with the available microfossil evidence, the high diversity and high divergence detected in both species among the populations sampled suggests substantial periods of isolation in the absence of gene flow, yet the maintenance of diversity appears to be the product of the longevity of these species and/or recent population size reductions.

Finally, the implications of these results for both the ongoing conservation and management of extant populations of these economically important and iconic Australian species is discussed with reference to their historical genetic associations. Given the quantification of their historical genetic signals, future research directions are suggested that are imperative for the maintenance of the evolutionary potential of these species.

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CHAPTER ONE

INTRODUCTION

1.1 Fragmentation of plant populations

With the ongoing exploitation of plant populations, the effects of habitat fragmentation on the genetics and demographics of plant species are becoming increasingly essential to quantify. Habitat fragmentation may be defined as the process which produces smaller isolated remnants from a formerly larger continuous habitat or population. Fragmentation may currently be viewed on two differing scales – historical and contemporary. The former is the result of naturally induced changes in habitat suitability due to environmental conditions, while the latter is the result of accelerated anthropogenic use of the landscape (i.e. logging, clearing for agriculture etc.). Research into both of these processes has predominantly focussed on ecological effects, however the genetic consequences of plant fragmentation and its implications for the survival of species has recently gained much attention (Schaal and Leverich 1996). The reasons for this shift of focus have lied within predictions from population genetic theory which suggest small plant populations are at increased risk of genetic erosion and extreme population divergence due to processes such as genetic drift, inbreeding, reduced gene flow and increased probability of localised population extinctions (Young *et al*. 1996). Empirical results gathered to date suggest that plant species are particularly prone to the genetic effects of habitat fragmentation, and that genetic assessment of remnant plant populations should be afforded a high conservation priority. However the genetic effects of habitat fragmentation on plant

populations appears to be more variable than those predicted from population genetic models (Young *et al*. 1996; Epperson 2000; Shapcott 2002; Hobbs and Yates 2003). Furthermore, the majority of this research has focussed on temperate angiosperms, and therefore, the quantification of these effects on tropical tree species remains an important area of research (Alvarez-Buylla *et al*. 1996).

1.2 Conservation of southern conifers

The extant conifers of the world offer a unique opportunity to investigate the effects of historical fragmentation on plant populations. They constitute an ancient assemblage of species, are widely distributed and occur on all continents except Antarctica where they were once abundant (César*i et al*. 2001). Of the seven extant conifer families, three are represented within the forests of the Southern Hemisphere. The Cupressaceae is fairly evenly distributed between Hemispheres, while the Podocarpaceae and Araucariaceae are now largely confined to southern latitudes (Hill 1998). However, this current distribution does not fully represent their previous distributions as fossils from both families occur in the Northern Hemisphere. How historical fragmentation and subsequent reductions in diversity and abundance have affected these two families remains largely unknown. To date, far more is known both ecologically and genetically about the families now confined to the Northern Hemisphere (e.g. Richardson 1998).

Of the 630 conifer species worldwide, the IUCN has identified the approximately 200 species of southern conifers as being at greater risk of

extinction (Farjon and Page 1999), yet, relative to their northern counter-parts, we know virtually nothing about them, ecologically or genetically. This is despite the IUCN listing genetic depletion and genetic drift as major threats to conifer species survival (Farjon and Page 1999). The heightened risk is a consequence of their extant distribution. Southern conifers rarely form monotypic stands, unlike their northern counterparts, and tend to occur as isolated specimens, mainly in mixed angiosperm-conifer forests. This ecological attribute has led many to regard the Southern conifers as relicts (Enright *et al*. 1995). However, the comparatively recent radiations of coniferous taxa may cast doubt on this generalisation. These include the radiation of *Callitris* in arid Australia (Pye *et al*. 2003) and the radiation of *Araucaria* section *Eutacta* on New Caledonia (Jaffré 1995).

In fact it is puzzling how a group of totemic trees, of such substantial ecological and economic importance, has largely escaped the attention of ecologists and geneticists in general. Superimposed on this history of reduction and fragmentation is species-specific exploitation, which has further decimated and fragmented the extant populations of Southern conifers across the Hemisphere. This study focuses on a group that appears to have been particularly affected by historical reductions in diversity and distribution – the Araucariaceae.

1.3 Tools for assessing the genetic effects of historical fragmentation in plant populations

Of the molecular techniques available for investigating plant population genetic structure, anonymous molecular markers, such as random amplified polymorphic DNA (RAPDs) and inter simple sequence repeat units (ISSRs), allow for the

study of genetic diversity and its partitioning without prior knowledge of the genome (Williams *et al*. 1990, Zietkiewicz *et al*. 1994). These techniques are especially suited to situations where there are no genetic studies available from which to derive species-specific markers, such as microsatellites. It is for this reason that RAPDs have been cited as the most widely employed anonymous genetic marker in plant population studies (Nybom and Bartish 2000). Furthermore, a large number of informative loci from across the entire genome can be assessed with either RAPDs or ISSRs at relatively low cost. Preliminary studies of population dynamics of southern conifers have employed anonymous genetic markers, in particular RAPDs, thereby enabling direct comparisons with studies of other southern conifers (e.g. Allnutt *et al.* 1999, Allnutt *et al*. 2001, Bekessy *et al.* 2002, Allnutt *et al*. 2003).

Briefly, RAPDs employ 'arbitrary' primer sequences composed of 10 base pairs (bp) to produce fragments of DNA generated by the polymerase chain reaction (PCR). This reaction, using a single RAPD primer, cuts fragments of DNA between complimentary binding sites, usually within 2 kilobases (kb) of each other (Ritland and Ritland 2000). This generates double-stranded DNA products which are equal to the length of the intervening region. The presence or absence of a product depends on mutations in the primer binding sites. These fragments, which represent RAPD loci, are then separated according to size via electrophoresis on acrylamide or agarose gels.

The underlying principles of ISSR reactions are similar to RAPDs. The primers employed in ISSR reactions are typically longer than RAPD primers. They are comprised of a microsatellite repeat (e.g. CACA…) of usually 16 bp.

These primer sequences are usually anchored at either the 5' or 3' end with 1-2 additional base pairs. The longer primer sequence means that annealing temperatures used in PCR are generally higher than those for RAPDs, which has led to suggestions that this technique is more reliable and more repeatable than RAPDs (Zietkiewicz *et al*. 1994; Nagaoka and Ogihara 1997). However, several studies have reported minimal problems with repeatability of RAPD reactions (e.g. Hollingsworth *et al*. 1998).

Both of these tools have shown great utility in the assessment of the genetic variation in plant populations. ISSRs have more commonly been used in the identification of horticultural cultivars, although they are increasingly being used in the assessment of natural plant populations (e.g. Wolfe *et al*. 1998; Ge and Sun 1999; Clausing *et al*. 2000; Baumel *et al*. 2001; Camacho and Liston 2001; Crawford *et al*. 2001; Ash *et al*. 2003). Further studies comparing and contrasting these molecular tools are now required in order to assess their utility, comparability and congruence in the genetic assessment of natural plant populations.

1.4 Taxonomy of the Araucariaceae

The Araucariaceae contains three extant genera, *Araucaria* A.Juss., *Agathis* Salisb., and the recently described *Wollemia* W.G.Jones, K.D.Hill & J.M.Allen. Although the family is now confined to southern latitudes, the earliest fossil evidence for the existence of the family stems from the Northern Hemisphere (Stockey 1994), which highlights the extensive range reductions this family has experienced.

The genus *Araucaria* is traditionally divided into four extant Sections: *Bunya* Wilde and Eames, *Intermedia* C.T. White, *Columbea* Endl. emend. Wilde and Eames, and *Eutacta* Endl. (Hill and Scriven 1998). Several fossils have also been found which have been difficult to place in extant sections as they are currently circumscribed (Hill and Brodribb 1999). The taxonomic circumscription in this thesis follows that described in the Flora of Australia vol. 48 (Hill 1998).

Araucaria bidwillii Hook. is the last extant member of section *Bunya*; a very distinctive and previously more diverse group of Araucarians, and for that reason is said to be of immense significance in determining evolutionary relationships within the genus (Stockey 1982). Section *Bunya* has several characters in common with section *Columbea* including a cryptogeal germination strategy (Burrows *et al*. 1992) that is thought to allow persistence under herbivory pressures, drought and/or fire. These shared morphological characters suggest a close relationship between these two sections.

Recent analysis of sequence data from the cpDNA *rbc*L gene (Figure 1.1; Setoguchi *et al*. 1998) supports the close relationship between three sections; *Bunya*, *Intermedia* and *Columbea*. In their molecular estimate of the phylogeny of *Araucaria* the immediate sister to *A. bidwillii* is *A. hunsteinii* from section *Intermedia*. Both of these sections also displayed a close association with section *Columbea* (Figure 1.1). These three sections formed a separate clade to section *Eutacta*. This relationship is interesting given the physically closer occurrence of section *Eutacta* in Australia, Norfolk Island, New Caledonia and Papua New

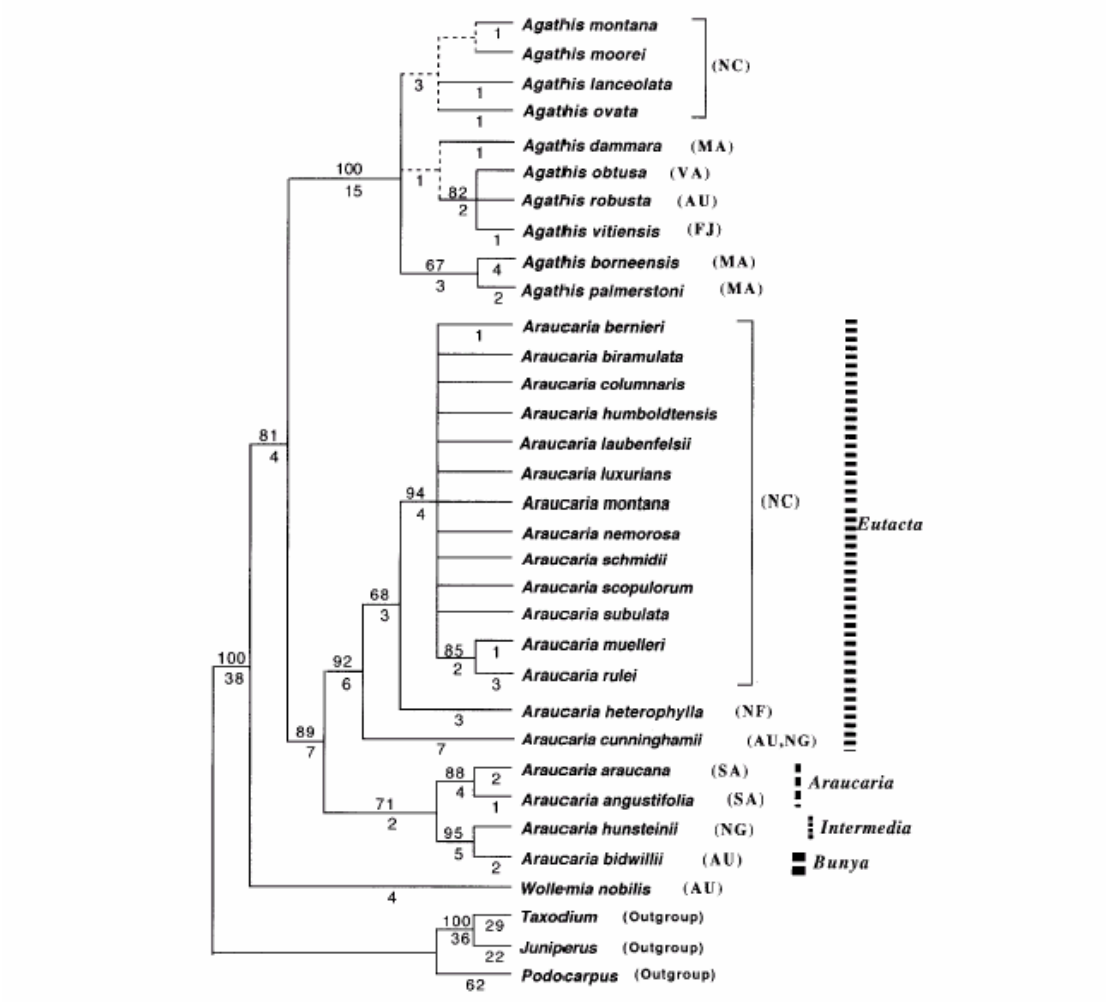

Figure 1.1. Phylogeny of the Araucariaceae, as determined by cpDNA *rbc*L sequence data (from Setoguchi *et al.* 1998).

Guinea, compared with section *Columbea* which is currently restricted to South America. The molecular data show support for a relatively recent radiation of section *Eutacta* on New Caledonia (Setoguchi *et al*. 1998), most likely from an ancestral Hoop pine lineage in Australia.

Araucaria cunninghamii Aiton ex. A.Cunn.., commonly known as Hoop Pine, has been suggested to be the oldest surviving lineage of section *Eutacta*, based on cpDNA *rbc*L sequence data (Setoguchi *et al*. 1998). Section *Eutacta*

contains 15 of the 19 currently recognised species of *Araucaria* and, according to the phylogeny produced from analysis of the cpDNA *rbc*L gene, appears to be the oldest and most divergent of the four extant sections of the genus (Setoguchi *et al*. 1998). This lineage has undergone an impressive radiation in New Caledonia (13 spp.), presumably in response to the ultramafic soils and disturbance regimes associated with that continental island (Jaffré 1995). This adaptive radiation appears to have occurred only relatively recently in geological time (Eocene: Jaffré 1995), as 10 of the 13 New Caledonian endemics show identical *rbc*L sequences (Setoguchi *et al*. 1998).

Several varieties have been proposed for *A. cunninghamii* but only two are recognised in contemporary taxonomic treatments (Hill 1998). These include *A. cunninghamii* var. *papuana* Lauterb. (endemic to Papua New Guinea), and *A. cunninghamii* var. *cunninghamii* (endemic to Australia). An additional variety has previously been proposed (*A. cunninghamii* var. *glauca*) based on the possession of glaucous foliage, however as Hill (1998) notes, glaucous individuals may be found in most populations and there appears to be no taxonomic pattern to their distribution. For the purpose of this study the Australian individuals of Hoop pine sampled are referred to as *A. cunninghamii*.

1.5 Fossil Record of *Araucaria*

Araucarian fossils are amongst the oldest fossils which can be attributed to extant coniferous genera (Stockey 1982), and among these sections *Bunya* and *Eutacta* comprise the earliest records, appearing across both Hemispheres from the Jurassic and possibly earlier (Setoguchi *et al*.1998). The fossil record suggests

section *Bunya* was previously both more speciose and more widespread, occurring in both Hemispheres during the Mesozoic (Stockey 1994). The best known fossil showing affinities to section *Bunya* is *Araucaria mirabilis*, which comes from the Jurassic Cerro Cuadrado Petrified Forest of Patagonia (Stockey 1982; Hill and Brodribb 1999). Other fossils displaying affinities to section *Bunya* have been found in the UK, Japan and India (Hernandez-Castillo and Stockey 2002).

Section *Eutacta* is well represented in the fossil record, with both sections *Bunya* and *Eutacta* being the oldest recorded fossils attributed to the Araucariaceae. Fossils, attributed to section *Eutacta*, have been found in early Cretaceous fossil beds in southern Victoria (Hill and Scriven 1998). Other fossils attributed to section *Eutacta* are widespread across South America, Antarctica, Australia and New Zealand during the Tertiary (Hill and Scriven 1998), but the lineage appears to have been particularly speciose in southeastern Australia (Hill 1995), with fossils extending back to the mid Cretaceous (Pole 2000).

The history of *Araucaria* on the Australian continent has comprised substantial shifts in distribution over the past 200 million years, presumably in response to adverse climatic conditions (Kershaw and Wagstaff 2001). More recently, pollen profiles from North Queensland throughout the Quaternary suggest substantial shifts in levels of local abundance for the genus, which are correlated with changes in climatic conditions (Kershaw and McGlone 1995). However, specific status cannot be ascertained due to generic similarity in pollen morphology. Hence the extent to which historical climatic changes have impacted on species' distributions and abundance remains obscure. Similarly, the future response of members of the genus to accelerated climate change, which is

anticipated to be of an order of magnitude faster than climate changes during the recent geological past (Houghton *et al.* 1996), remains difficult to predict.

1.6 Species descriptions

1.6.1 Araucaria bidwillii

Araucaria bidwillii Hook. (Araucariaceae) is most commonly known as the Bunya pine, but is also referred to by a number of other names with varying degrees of connectivity to, and English interpretation of, the name given to the species in Indigenous languages, e.g. Bunya-Bunya, Bonyi-Bonyi, Bonye, Banza-tunza, Banya-Tunya (Huth 2002).

A. bidwillii conforms to generalised araucarian descriptions, but also displays several unique characteristics which have led to its separate sectional placement. As with all members of *Araucaria*, it is a wind-pollinated, monoecious tree, with whorled, spreading branches. Trees may attain heights of 50m, although mature specimens in sub-optimal drier habitats may be much shorter (e.g. Mt Lewis). The Bunya silhouette is perhaps its most recognisable feature – the unique, emergent dome-shaped canopy and dark green glossy foliage set it apart from the surrounding flora wherever it grows, which ensures its distribution is well known. Leaves are dimorphic, varying from triangular to acicular on orthotropic shoots, to narrowly triangular on plagiotropic shoots, and are 1 - 5 cm long and 3 - 10 mm wide. The bark is extremely thick, and would act to protect mature specimens from fire damage.

Male cones are cylindrical, sessile and amongst the largest in the genus, being 6 - 11 cm long and 10 - 15 mm in diameter. Pollen is not winged and

remarkably uniform within the family, so that species, sectional, and even generic (at least between *Agathis* and *Araucaria*) attribution of microfossils is problematic at best. Pollen grains studied in other *Araucaria* spp. indicate that these are among the largest non-saccate pollen grains found in the conifers (Sousa and Hattemer 2003), which in itself raises questions about effective wind dispersal of large pollen grains in what is essentially a wind-reduced environment where *A. bidwillii* is predominantly found (i.e. rainforest). However, it should be mentioned that the saccate condition of conifer pollen is thought to be more important in aiding orientation on the nucellus rather than being integral for dispersal (Tomlinson *et al*. 1997, Salter *et al*. 2002).

The female cones are also amongst the largest in the family. They are ovoid and can weigh in excess of 10 kg and are 20-30 cm long by 15-20 cm wide (Hill 1998). The immense size of these cones has led to many ornamental specimens being removed due to the threat of falling female cones, which may potentially be lethal and/or damaging to property.

The seeds are unusual for the genus in that they are shed from the cone scale only at maturity, as opposed to other members of the genus where the seeds are free from the cone scale. These seeds, being retained within the cone, are thereby restricted to limited dispersal by gravity (on slopes), water (on watercourses) or potentially small mammals (see below). The majority of the seeds, however, would be expected to germinate in close proximity to the female-cone producing tree, thereby initiating intra-cone competition for establishment. The seeds undergo an unusual cryptogeal germination that leads to the production

of a large desiccation-resistant tuber, which is thought to promote resistance to fire, drought and/or herbivory (Burrows *et al*. 1992).

The female cones are also unusual in that the seeds or 'nuts' they produce have a low fat and high protein content when compared with other edible nuts, as well as being rich in iron and complex carbohydrates (Brand *et al*. 1985). This nutritional content made the seeds a highly sought after food source for early Indigenous peoples and has ensured their continued popularity as contemporary 'Bush Tucker'. The production of female cones occurs annually but every three to four years a much larger crop is produced. This 'masting' strategy is thought to be useful in overwhelming predators thereby providing enough propagules for establishment, at least every few years (Huth 2002). A masting event had occurred prior to my collecting trip to the Bunya Mountains, and it was evident that several entire cones were not predated, and were subsequently germinating, initiating intra-cone competition for seedling establishment. It was during these masting events that the large Indigenous festivals were held (see below), although smaller gatherings were still held in leaner years. This masting phenomenon also occurs in the closely-related Monkey Puzzle tree of South America (*Araucaria araucana*) and has been linked to climatic events associated with ENSO (El Niño – Southern Oscillation). Wet Spring seasons showed a strong negative correlation with seed production 18 months later, possibly due to interference with wind pollination (Sanguinetti *et al*. 2002 in Smith and Butler 2002). Further research is required on the mechanisms that trigger this strategy in *A. bidwillii*.

The harvesting of female cones by Indigenous people has also been used as an explanation for the 'scars' that appear on particularly old specimens. Other

explanations centre around the scars being a residual product of branch drop, but these do not explain the presence of up to 4 scars in close proximity on the trunk, with the number of scars appearing to be correlated with the size of the tree. The most likely explanation for these scars is that notches were cut in the trunk for toeholds and as points of attachment for vines used in climbing the trunks to harvest the cones (Huth 2002). Corroborating this theory is the fact that these scars only occur on very mature specimens and are uniformly absent from younger specimens.

Age estimates have been difficult to ascertain as the mesic habitats in which this species is now found inhibit the formation of distinct growth rings. The Bunya, being a rainforest conifer, is under the same pressures for resources as other rainforest taxa, including competition for light during establishment. Thus, Bunyas may persist for years in a suppressed state before an opportunity for increased light, and therefore increased growth, is presented. This further hinders the opportunity for precise age estimation. The presence versus absence of scars associated with Indigenous utilisation of the trees can perhaps provide a size estimate at least for trees estimated to be around 200 years old. From personal observations these unmarked trees are substantially smaller than some of the 'giant' scarred specimens that are present, so age estimates in excess of 500 years which have been made for its congener *A. cunninghamii* are likely to be conservative, or at least realistic, for *A. bidwillii*.

Bunyas occur predominantly on basalt derived soils, but may also occur on granitic soils as in the case of the Mt Lewis population. The species is found over an altitudinal range of 150 – 1000 m (Boland *et al*. 1985) within 160 kms of the

present coastline. These areas share a reported mean maximum temperature of 28-32° C and a mean minimum of 5-10° C, although frosts are a relatively common occurrence in the southern populations (Smith and Butler 2002) and snowfalls have been reported from the Bunya Mountains. Annual rainfall varies from 900 – 2000 mm over the species' range, although 'cloud-combing' to intercept additional moisture is likely to be an important physiological strategy generally for the genus (Enright 1995), as all Araucarians form an emergent canopy in their respective ecosystems. Anecdotal reports from the Bunya Mountains tell of water running down the trunks of Bunyas in the absence of rainfall and presence of cloud cover, adding further support to this theory.

In keeping with dispersal characteristics of the family, *A. bidwillii* is thought to be wind pollinated and passively/gravity dispersed. Reports of weevils associated with male cones in other species (Kushel 2002) and personal observations of caching of seeds, presumably by rodents, may imply other mechanisms are available for dispersal, but further research is required.

1.6.1.1 Sociology of A. bidwillii

To date, far more is known of the sociological history of the species than its general ecology, which is in itself highly unusual for such an iconic plant species. The history of human attachment to the Bunya is complex and ancient, and appears to be unparalleled in the Australian flora. While the aforementioned nutritious seeds were utilised as an important Indigenous food resource, the Bunya was perhaps more spiritually significant for Indigenous peoples, as it remains so today (Jerome 2002).

The Bunya Mountains were referred to by local tribes as Boobarran Ngummin, or Mother's Breast (Jerome 2002). In masting years the Mountains, being perceived as Mother, nourished the peoples of the surrounding lands during what have been termed 'Festivals'. Message sticks were sent out to inform of these festivals across the landscape, to the adjacent Kabi Kabi people of the coast, and on occasion to as far away as Rockhampton in the north and New South Wales to the south. These Festivals were not simply concerned with the harvesting of nuts. They were spiritual events over which disputes could be settled, young men initiated, trade organised and marriages arranged (Haebich 2002).

In fact the history of the utilisation of the tree closely mirrors the history of colonisation in southern Queensland. This history is a saga of cruelty, torture and devastation. The festivals could attract up to 6,000 people in a given season, and to the European Settlers of the area, this was of immense concern. Atrocities, including mass poisonings of Indigenous people, were frequent, and inextricably linked with the Bunya crop (Evans 2002). Initial estimates of 6,000 people gathering at the approximately triennial events, declined to 2,000 by 1857 (Evans 2002).

The species was initially targeted to inhibit these Indigenous gatherings on settled land (Evans 2002). Ceremonial grounds were also destroyed in an attempt to further control Indigenous use of the landscape; something that had previously persisted for tens of thousands of years. In 1842, a decree was issued prohibiting the felling of Bunya trees, which was as much a reflection of the growing awareness of its importance to Indigenous communities as it was a strategy to

lessen conflict between the tribes and white settlers, as the latter could only perceive its economic importance (Evans 2002). Although never fully honoured, this decree effectively limited further gross commercial exploitation. In 1860 this proclamation was nullified, resulting in widespread logging of the species. The Great Bunya Sawmill was built in 1880 at the foot of the Bunya Mountains but proved unfeasible and was closed by 1892; not before literally thousands of Bunyas were felled (Evans 2002). This was followed in 1908 by the gazetting of Queensland's second National Park, the Bunya Mountains, for the sole purpose of protecting this species, and perhaps with the ulterior motive of lessening potential conflict (Huth 2002). With the declaration of the National Park came the cessation of the traditional Bunya festivals that had brought together tribes from the wider South-East Queensland region. The year 1875 saw the last major Bunya Festival, and by 1880 any reference to the festivals was made in past tense (Evans 2002). This was the social backdrop to which Europeans were introduced to this tree. Plantings on now vanished homesteads can still be seen in its southern range, and indicate the "noble" perception this tree eventually came to hold amongst early settlers.

The declaration of the Bunya Mountains National Park left the coastal populations open for exploitation, and these areas were subsequently heavily logged (Haebich 2002). On the coastal ranges, the Bunya populations were almost completely decimated as transport was not a logistical problem as it was in the distant inland Bunya Mountains (Evans 2002). At the same time, in their seminal work on the Pines of Australia in 1910, Baker and Smith reflected this in their declaration:

> " In Queensland, *A. bidwillii* is still standing in some quantity awaiting the saw-miller, but in New South Wales, *A. cunninghamii* is almost a tree of the past" (Baker and Smith 1910, pp. 316).

The final blow came in 1988, ironically in the Bicentennial Year of Australia's 'discovery'. The ceremonial ground of Baroon was a traditionally important gathering place of the coastal and sub-coastal Aboriginal communities from SE Qld and northern NSW. This site had previously contained one of the largest remaining stands of *A. bidwillii*. However, in 1988 the site was flooded and subsumed as Baroon Pocket Dam, with a substantial piece of Australia's history, going under as well (Haebich 2002). Recently there has been renewed interest in reviving the Bunya festivals, albeit, in a much changed cultural context (Jerome 2002).

The aforementioned activities have most likely had important biological effects on the species. Tribal groups from the Kabi Kabi have been reported to carry stores of nuts back to the coast with them, sometimes burying them in moist riverine localities for later retrieval (Petrie 1904). Upon retrieval many seeds were found to have germinated and in these cases the tubers were eaten (Petrie 1904). This, together with trade during the festivals, and the ability for shed seeds to germinate in the absence of water (Huth 2002) raises the potential for human-mediated dispersal of the species.

1.6.1.2 Contemporary uses of A. bidwillii

Today, 426 ha of Bunya is managed in forestry plantations, predominantly for the production of Bush tucker and timber (Nikles pers. comm.). In comparison to its

congener, *A. cunninghamii*, production for timber is meagre (see section 1.6.2.2). The reasons for this include the persistent sharp-pointed leaves, slow growth rates and the presence of extremely thick bark (Huth 2002). Contemporary uses of the timber are confined to the specialty timber trade. Recently, the Australian guitar manufacturer, Maton, started incorporating Bunya timber for its soundboards (Maton 2004). The timber for this purpose is harvested at 80 years, which makes it a far more sustainable source of soundboards than the currently favoured Sitka Spruce, which may take over 300 years to mature.

Smaller *ex situ* plantings, of one or a few specimens, are frequently encountered, especially in southern Qld, emphasising the reverence this species eventually came to hold among early settlers and their descendants. The Atherton Tablelands, west of Cairns, is also extensively planted with Bunyas, all of which appear to be of southern origin. These *ex situ* plantings have immense contemporary sociological value through their aesthetic appeal. Their huge stature allows them to dominate the skyline wherever they are planted, and many that were planted to mark homesteads over the last two centuries can still be seen today in the absence of the original buildings (e.g. Mary Valley region). The passionate feelings these trees arouse can be evidenced as recently as 2004, when ongoing legal battles continued surrounding development on a site containing a double-trunked Bunya, in what was previously Bunya territory (Obi Obi Creek). Thus, the focus of this species has shifted from a spiritual indigenous perspective, which efficiently managed the natural populations, through European exploitation, to finally rest in a contemporary displaced attachment to the species through aesthetics, and to a much lesser extent utility.

1.6.2 Araucaria cunninghamii

A. cunninghamii is an emergent tree over sub-tropical and tropical rainforests, but is more commonly associated with drier rainforest communities to which it lends its name for classification purposes. Mature trees can reach over 60m in height. In common with other members of the genus, it is a large wind-pollinated and monoecious tree with whorled branches. In contrast to *A. bidwillii* in which the foliage is clustered at the ends of the branches, the foliage of *A. cunninghamii* occurs in clusters along the branches. Male cones are produced terminally, and occur in the lower branches of the tree. These cones are smaller and less robust than those of *A. bidwillii*, with recorded measurements of 20-35 mm long and 5-7 cm in diameter (Hill 1998). As previously mentioned, the pollen is remarkably uniform within the genus.

Female cones are produced terminally in the upper branches of the tree (as for *A. bidwillii*, this is thought to promote outcrossing) but are substantially smaller than those of *A. bidwillii* (5-10 cm long, 5-7 cm in diameter; Hill 1998). Unlike the female cones of *A. bidwillii*, which fall to the ground intact for subsequent dispersal, these cones tend to disintegrate in the canopy when mature, and the seeds, which are not embedded in the cone scale at maturity, are then gravity dispersed. In line with the irregular cone production reported in *A. bidwillii*, *A. cunninghamii* cones are produced annually, however particularly large crops occur every two to seven years (Haines and Nikles 1987).

The bark of the species is characteristic, forming horizontal bands that are later shed in 'hoops', which has lead to its common name of Hoop pine. Age estimates for the species suggest a maximum age of around 500 years, although

Enright (1995) notes that growth within closed forest systems may be slow, and it may take approximately 200 years for individuals to reach above the angiosperm canopy and become reproductively mature.

The species has a broader ecological tolerance than its Australian congener, which is reflected in its broader distribution (see Chapter 4). It occurs on a wide range of soil types, from nutrient-poor, granite-derived soils to highly fertile basalt-derived soils (Enright 1995). Areas of extant occurrence are also characterised by a wide variation in mean annual rainfalls, ranging from 750 to over 1600 mm/yr in Australia and 1600-5800 mm/yr in Papua New Guinea (Dieters *et al*. 2002). The altitudinal distribution of the species is similarly broad, ranging from sea level to 1000 m in Australia, and from 1600 to 2800 m in Papua New Guinea (Dieters *et al*. 2002). Australian populations have been reported to be sensitive to severe frosts and fire, while some PNG provenances are sensitive to drought (Dieters *et al*. 2002).

The species also occupies a broad range of habitats, ranging from ridgetops to gullies in rainforest environments, through drier rainforest types to coastal communities and offshore islands.

1.6.2.1 Sociology and contemporary use of A. cunninghamii

In contrast to the immense sociological importance of *A. bidwillii* for Indigenous Australian peoples, there is no evidence for historical indigenous use of this species (Ian Smith pers. comm.). However, the timber of *A. cunninghamii* has been of immense importance over the past few centuries, and was initially heavily relied upon for the foundation and colonisation of southeastern Queensland,

especially Brisbane and the surrounding Pine Rivers region (the latter deriving its name from the abundant Hoop pine initially encountered in the region). As technology advanced, so did the rate at which this resource was exploited. By the early 1900s, it was realised that the removal of Hoop pine was not sustainable and efforts were made to establish plantations for this purpose (Dieters *et al*. 2002). Plantations were generally located on sites that had previously supported Araucarian forest and seed was initially sourced locally for their establishment (Nikles 1996). By this time, however, Baker and Smith (1910) noted that in NSW, at least, the supply of *A. cunninghamii* was almost exhausted. Similarly, large stands of Hoop pine that dominated Brisbane and the Pine rivers had been removed. Small stands occur today on Mt Glorious and Mt Nebo in the Brisbane area.

Today, Hoop pine is still relied upon as one of Australia's greatest timber assets, with sales of log timber alone exceeding $23 million dollars. The current plantation area of Hoop pine exceeds 45,000 ha (Dieters *et al*. 2002). While native forests are now mostly protected from the threat of logging, obviously this history of exploitation has had a dramatic effect on the distribution and abundance of this species. Although genetic improvement of plantation Hoop pine has been carried out since the 1940s, to date there has been no effort to quantify the direct underlying genetic properties of differing provenances. This is despite gross differences being noted in performance of differing provenances (Dieters *et al*. 2002). For example in the 1980s, forestry trials were begun to investigate notable differences in performance of differing provenances and inter-provenance breeding programs, consisting of North Queensland and South Queensland

parents, were commenced. Growth improvements were noted yet the underlying genetic mechanisms for this remain unknown (Dieters *et al*. 2002).

Information on the genetic integrity of *A. cunninghamii* is now urgently required to facilitate our understanding, not only of the impacts of past regimes of exploitation, but also to inform forestry management of the differences existing within and between provenances selected for plantation timber. Specifically, we need to quantify the historical signal of genetic variation and divergence so that we may interpret the effects of contemporary processes.

1.7 Project aims

The goal of the present study is to assess the genetic variation within and among populations of *Araucaria* in eastern Australia using both RAPD and ISSR molecular markers. Both species are wind pollinated, passively dispersed and extremely long-lived – factors which should generally contribute to a low degree of population differentiation (Nybom and Bartish 2000). Information on the genetic integrity of species has also recently been incorporated into management strategies for the conservation of fragmented populations and to assess which populations should be prioritised for management (e.g. Bekessy *et al.* 2002, Allnutt *et al*. 2003).

The aims of the present study, when placed within the broader context of the thesis, are to,

- assess the utility of both RAPDs and ISSRs for determining genetic variation and its partitioning in Australian *Araucaria* spp., and to assess the utility of these molecular markers in the ongoing assessment of genetic structuring and

the maintenance of genetic diversity in future generations of both native and plantation populations.

Specifically, the following questions were addressed:

1. Given the dynamic history of *Araucaria* in Australia, as revealed by the fossil record, what levels of diversity do extant populations of *A. bidwillii* and *A. cunninghamii* maintain? In other words, has the evolutionary potential of these species, or particular populations, been compromised through historical range reductions and fragmentation?
2. Can the level of genetic diversity within a population be predicted from extant population size? Or has population size, itself, been an influence in the maintenance or loss of genetic diversity through evolutionary processes such as genetic bottlenecks induced, for example, by drift, inbreeding or founder effects?
3. How is the genetic variation distributed among populations? Do these populations form a cohesive unit (metapopulation) or does significant genetic structuring exist?

If significant genetic structuring exists, does it correspond with predictions from conifer population genetic theory? This theory, based on genetic data from northern hemisphere conifers, predicts low levels of population differentiation and high levels of within population variation.

The following chapter details the methodologies chosen to address these questions.

CHAPTER TWO

MOLECULAR METHODOLOGY

2.1 DNA isolation

The isolation of DNA follows a modified CTAB extraction protocol of Doyle and Doyle (1987) which has proven to be a successful methodology for DNA extraction from other Australian conifers (Pye *et al*. 2003). An appropriate amount of leaf material, either fresh or dried in silica gel, was placed in a warmed mortar and pestle, with a pinch of sterilised sand to aid in cellular disruption when grinding. After the addition of 2 mL of warmed (60°C) CTAB solution (2% cetyltrimethylammonium bromide, 1.4M NaCl, 100 mM Tris-HCl (pH 8.0) 1% polyvinyl-pyrrolidone (PVP)), the leaf material was ground to a homogeneous solution, and poured into two 1.5 mL microcentrifuge tubes. The duplicate solutions were then incubated in a water bath at 60°C for 45 min. Following this, 1 mL of chloroform/isopropanol (24:1) was added to the extraction solutions, which were then agitated for 10 min. These solutions were then centrifuged for 10 min to separate phases and to allow removal of the aqueous layers, which were pipetted to fresh 1.5 mL tubes. A volume of 1 mL ice-cold 100% ethanol (EtOH) was then added to each of the tubes, which were placed in a –20°C freezer overnight to precipitate the DNA. The precipitate was then centrifuged to pellet the DNA, which was subsequently washed twice in wash buffer (70% EtOH, 10 mM NH_4O_{ac}) for 5 min each time. The double wash was deemed necessary due to secondary compounds associated with these conifers, which may later interfere

with PCR amplification. Finally the DNA pellet was dried under a vacuum centrifuge, and dissolved in 200 µL of double-distilled H_2O.

Gross extraction success was ascertained by running 5 µL of the DNA extract on a 1% agarose gel, stained with ethidium bromide, in 1X TBE buffer at 100 V for 20 min. Gels were then visualised under UV light (GelDoc 1000 image system, BIORAD, CA). Precise total cellular DNA concentrations were then calculated using a fluorometer (Hoefer™ DyNA Quant™ 200: Amersham Pharmacia Biotech, San Francisco, USA) and all extracts were diluted to approximately 10 ng/µL.

2.2 Primer selection

Forty 10-mer RAPD primers, from primer kits OPB and OPC (Operon Technologies, Alameda, California), were screened with a subset of *A. bidwillii* samples from each population for production of clear and variable RAPD profiles.

For the ISSR analysis, forty-one ISSR primers were screened for variable bands across the same subset of *A. bidwillii* samples. These included UBC (University of British Columbia) primers as well as primers designed by myself from reported microsatellite motifs within *Araucaria* (Scott *et al*. 2003). Primers varied, not only in their microsatellite motif, but also in the composition (A, G, C, T or redundant bases e.g. Y, R), combination, number (1-3) and positioning (5' versus 3') of the anchoring bases.

Twelve 10-mer RAPD primers, from primer kit OPB (Operon Technologies, Alameda, California), which were used to investigate genetic structuring in *A. bidwillii*, were tested for variability and reproducibility in *A.*

cunninghamii. For the ISSR analysis, twelve of the ISSR primers that produced variable bands for *A. bidwillii* were initially tested for variability and reproducibility in *A. cunninghamii*.

2.3 PCR optimisation

Reported problems with the repeatability of RAPD reactions can be overcome with careful optimisation of the protocol before running reactions containing the entire sample set. For this reason, a series of trials was conducted which varied the concentrations of two critical PCR constituents that are known to affect RAPD band reproducibility – $MgCl_2$ and DNA template. The combination that gave the clearest PCR profiles across a series of primers was then selected for the complete RAPD analysis.

The repeatability of ISSR reactions is not thought to be as sensitive to reaction conditions as RAPDs, as longer primers and higher annealing temperatures act to minimise these concerns. However, optimisation of PCR conditions is still required to maximise band resolution. The addition of formamide to PCR reactions, in particular ISSR reactions, has been suggested to improve band resolution through the reduction of background noise and product smearing (Tsumura *et al*. 1996). It achieves this by weakening hydrogen bonding between nucleotides, by assisting in destabilising the DNA in solution, and by preventing the formation of primer-dimers (Ranamukhaarachchi *et al*. 2000). In this study, a concentration of 2% formamide was trialled, following reports of PCR inhibition at higher concentrations (Tsumura *et al*. 1996).

2.4 RAPD reactions

After optimisation of template and $MgCl_2$ concentrations, all RAPD reactions were performed under the following conditions so as to ensure maximum reproducibility. All reactions were performed simultaneously for each primer in two Omn-E Thermal Cyclers (Hybaid Ltd., UK), using 25 µL total reaction volume. Optimised RAPD reactions contained: approx. 50 ng template DNA (5 µL at 10 ng/µL), 1 µL 10 mM primer (Operon), 0.5 µL 40 mM dNTPs (Promega), and 0.25 µL 0.4% bovine serum albumin, and 14.15 µL of double distilled H_2O. These constituents were then subjected to a 30 min soak at 60ºC to remove excess proteins from the reaction. Enzyme mix, containing 2.5 µL 10X PCR buffer (Life Technologies), 1.5 µL 50 mM $MgCl_2$ (Life Technologies), and 0.1 Unit of *Taq* polymerase (Life Technologies), was then added before employing the following thermal cycling conditions: initial denaturation for 5 min at 94ºC, followed by 40 cycles of denaturation for 1 min at 94ºC, annealing for 1 min at 36ºC, and extension for 1 min at 72ºC. The amplification was completed by a final extension step of 2 min at 72ºC, to allow complete extension of the PCR products.

Along with negative controls, which were run to identify contamination or the presence of primer-dimers and/or artifactual bands, a random sample of positive controls were run simultaneously in different thermal cyclers to verify consistent amplification profiles between them.

2.5 ISSR reactions

All reactions were performed simultaneously for each ISSR primer using the same thermal cyclers as for the RAPD reactions. After optimisation, the

following protocol was employed for all ISSR reactions: 40 ng template DNA (4 µL at 10 ng/µL), 1 µL 10 mM primer (UBC, Canada), 1.0 µL 40 mM dNTPs (Promega), and 0.3 µL 0.4% bovine serum albumin, with 14.5 µL of double distilled H_2O. For the formamide trials, 0.5 µL of 100% formamide (Sigma) was added, removing the same amount of H_2O from the reaction. These constituents were then subjected to a 30 min. soak at 60ºC to remove excess proteins from the reaction. Enzyme mix, containing 2.5 µL 10X PCR buffer (Life Technologies), 1.6 µL 50 mM $MgCl_2$ (Life Technologies), and 0.1 Unit of *Taq* polymerase (Life Technologies), was then added, bringing the total reaction volume to 25 µL.

Various cycling regimes were tested, which varied in number of cycles (30, 35, or 40), annealing temperatures (45ºC - 55ºC) and extension times (2 min – 10 min), in order to optimise reaction conditions across a range of primers with different properties (e.g. G/C content, length, 5' or 3' anchoring).

After optimisation of these parameters, the following cycling regime was deemed appropriate for the majority of primers and was subsequently employed for all ISSR reactions: initial denaturation for 3 min at 94ºC, followed by 40 cycles of denaturation for 1 min at 94ºC, annealing for 1 min at 50ºC, and extension for 2 min at 72ºC. The amplification was completed by a final extension step of 10 min at 72ºC, to allow complete extension of the PCR products.

Negative and positive controls were included in the same manner described for the RAPD reactions.

2.6 DNA fragment visualisation

Both RAPD and ISSR products were resolved on 2% agarose gels by electrophoresis, with the addition of 1 μL/250 mL ethidium bromide. Products were initially run at 100 V for 10 min to ensure even migration from the wells, and then run over 5 hours at 70 V, and finally visualised and photographed over UV light (GelDoc 1000 image system, BIORAD). Digital images were also captured and stored for later use in band scoring.

A 1Kb ladder (Life Technologies) was run alongside the PCR products to estimate fragment size and to facilitate the scoring of bands within and between populations. To further assist in band scoring and to ascertain inter-population band homology, additional gels were run containing 2 individuals from each of the six populations sampled.

A random sample of reactions was duplicated, and cross-checked with the original amplification products to ensure reaction repeatability. For comparison between species, it was important to maintain the same visualisation system, as the method in which fragments are visualised is as influential in detecting variation as the marker system employed (Hollingsworth *et al*. 1998a).

2.7 Data analysis

Each RAPD or ISSR product was assumed to represent a single locus and was scored as present (1) or absent (0) and entered into a binary data matrix. Bands with identical migration were considered as identical fragments, regardless of intensity. The long electrophoretic running times acted to minimise the scoring of non-homologous bands due to increased band separation. Only bands that

appeared in size ranges between, or closely adjacent to, visible monomorphic bands were scored. While this methodology substantially reduced the overall number of bands that could be scored, it importantly avoided scoring non-homologous, faint or artifactual bands. Monomorphic loci were not scored, as suggested by Nybom and Bartish (2000) in their analysis of RAPD studies, so as to make estimates of polymorphism more comparable across studies.

2.7.1 Statistical assessment of genetic diversity

Shannon's diversity estimates (Lewontin 1972) were calculated from band frequencies in the binary matrices in PopGene Ver. 1.31 (Yeh *et al*. 1999), and used to estimate the degree of genetic variation within each population. As previously mentioned, both RAPD and ISSR markers are dominant and therefore heterozygotes cannot be detected. The use of Shannon's index is recommended for dominant data since it is not sensitive to this bias (Meekins *et al*. 2001). The notation of Shannon's index as '*S*' and not '*H*' is used here in keeping with the suggestion of Allnutt *et al.* (1999) to avoid confusion with diversity measures such as heterozygosity, to which Shannon's index is not comparable. '*S*' was calculated using the following formula:

$$S = - \sum p_I \log_2 p_i$$

where p_i is the frequency of the presence or absence of each RAPD band. Total diversity across the populations was estimated using the mean value of *S* over all the populations sampled, and the pooled species-level value was also determined. An analysis of variance (ANOVA) was used, after arcsine transformation of the data, to examine differences in the levels of diversity across populations. Arcsine

transformation of estimates of genetic diversity for each locus was necessary as proportional data, with values encompassed by 0 and 1, are binomially rather than normally distributed (Zar 1996). This transformation means the resultant data will have an underlying distribution that is nearly normal. If significant differences were found among the populations sampled, post-hoc tests employing Least Significant Difference (LSD) were conducted in SPSS v. 10 to identify which populations accounted for the difference. The percentage of polymorphic loci (%P) was calculated for each population in PopGene, as was the mean value across all populations and a pooled species-level value.

2.7.2 Statistical assessment of genetic differentiation

A Euclidean distance matrix was calculated in Arlequin ver. 2.000 (Schneider *et al*. 2000), which measures the shortest distance between phenotypes produced from the binary matrix, and used as the input file for an Analysis of Molecular Variance (AMOVA) (Excoffier *et al.* 1992) to investigate the genetic structuring of the sampled populations. Variance components were estimated for i) within populations, ii) among populations within regions, and iii) among regions. Populations were permutated among regions until the structuring that best explained the observed data was identified. The best explanation of the observed data is the structure which maximises the inter-regional variance while minimising the inter-populational variance within regions. Additional hypotheses of structuring, based on ecological and biogeographical theory, were also examined and will be discussed separately. Probability values, that any random value is greater than the observed value, were calculated within Arlequin for each

of the AMOVA parameters using 10,000 random permutations. Significance is tested by permutating populations among regions and individuals among populations. All possible configurations of populations and regions should be investigated as more than one 'significant' association can be found. Only one structure, however, represents the most parsimonious explanation for the observed distribution of genetic variation across the populations sampled.

Pairwise Φ_{ST} values among all populations were calculated in Arlequin from the Euclidean distance matrix, and their significance tested through 10,000 random permutations of the data set. These values were then used to conduct a Principal Coordinates Analysis (PCA) in GenAlEx Ver. 5.04 (Peakall and Smouse 2001) to spatially examine the variation among populations and to highlight the resolving power of the ordination. Correlation between the two pairwise population matrices produced from RAPD and ISSR analyses was estimated using a Mantel matrix correspondence test, with 1000 random permutations to determine significance values. This was done to examine congruence between the analytical methods in determining population structure.

Genetic distances between samples and populations were also calculated using Nei's unbiased measure of genetic distance (Nei 1973) as implemented in PopGene. G_{ST} values derived from this distance measure were compared with Φ_{ST} estimates from the AMOVA analysis in Arlequin to examine congruence between statistical methods. Finally, unrooted UPGMA trees and phylograms were calculated in PopGene, from Nei's unbiased measure of genetic distance. These trees graphically display the relative divergence and relationships among populations sampled. These are also examined in conjunction with the results

from the PCA as, by their nature, UPGMA trees impose a dichotomous topology. PCA, however, plots the populations in multi-dimensional space and is therefore extremely useful in verifying the results gained from the topology of UPGMA trees.

2.8 Data management

All extractions were labeled with unique codes and are recorded in both electronic and hardcopy formats at the Molecular Biology Laboratory, James Cook University, Cairns. DNA extracts have been frozen and stored for later use. The binary matrices produced from both RAPD and ISSR analyses are also stored in both hardcopy and electronic formats at the above facility and are presented in the Appendices.

CHAPTER THREE

GENETIC STRUCTURING AND DIVERSITY IN *Araucaria bidwillii*

3.1 Introduction

3.1.1 Extant distribution

A. bidwillii has undergone extensive range reductions and fragmentation, and now occurs in only 2 disjunct regions in North and South Queensland, Australia. Extant populations occur from as far north as Mt Lewis in North Queensland (16°30' S) to as far south as the wider Brisbane area (26°15' S – 27°00' S), with a substantial break in distribution of approximately 2,000 kms or 9° in latitude (Figure 3.1). The northern populations at Mt Lewis and Cannabullen Falls (17° 40' S) are small and are suggested to be relictual, while the southern populations are generally more extensive, despite being more heavily exploited than the northern populations in the recent past. The sampled populations are discussed in more detail below.

3.2 Materials and methods

3.2.1 Sampling

Leaf samples were collected from a total of 131 individuals from six populations (Table 3.1) across the geographic range of the species (Figure 3.1). Collections were made at two different scales, reflecting the species extant, disjunct distribution in north and south Queensland respectively. Within south Queensland, where multiple populations occur, several populations were sampled. These include 'Jimna' from the coastal Conondale/Blackall Ranges, and four

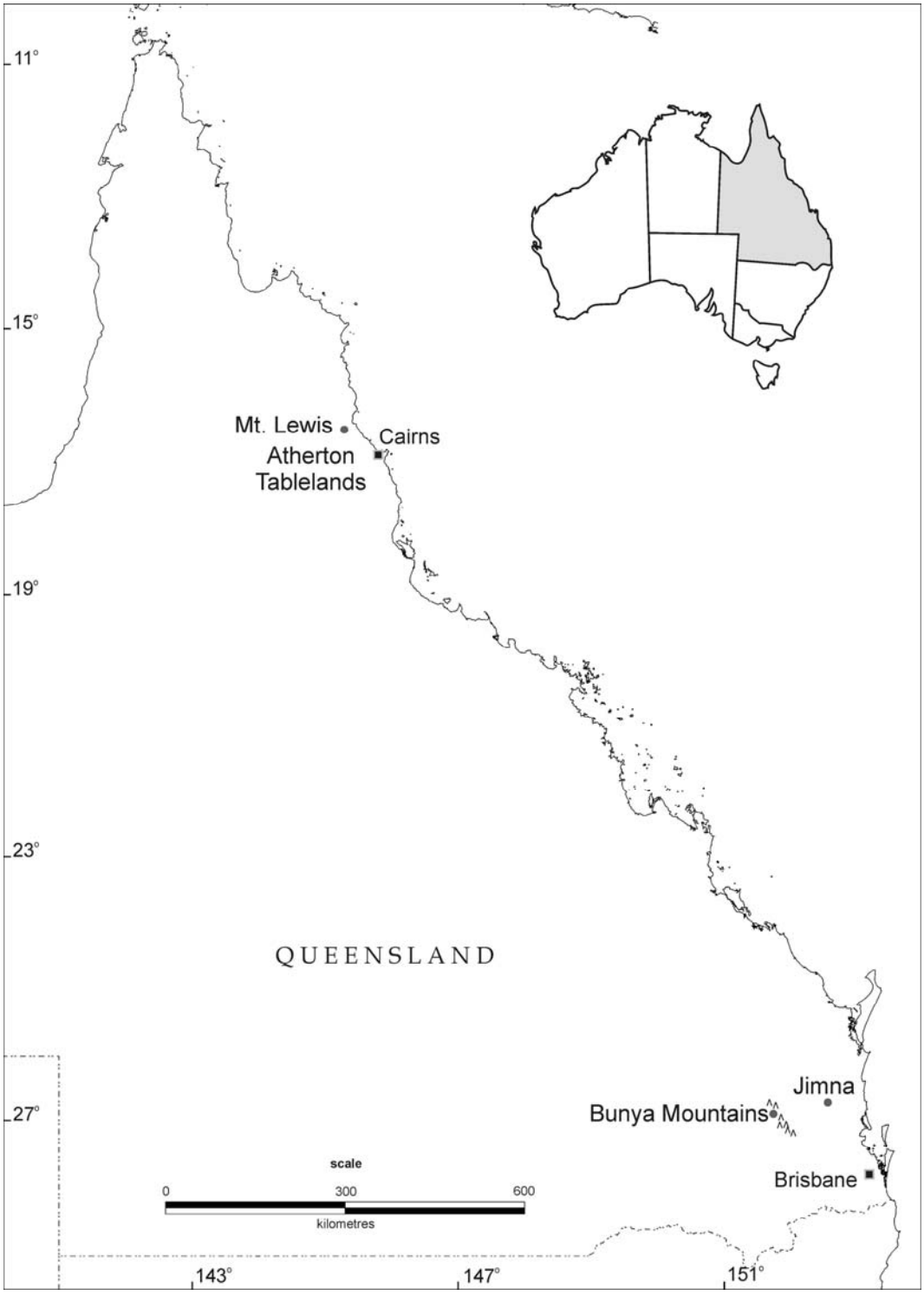

Figure 3.1. Extant distribution of *A. bidwillii*, which illustrates the broad disjunction in distribution and shows sampling localities covering the extant latitudinal distribution of the species in northern and southern Queensland respectively (figure prepared by A. Edwards).

Table 3.1. **Localities and descriptives of sampled populations of *A. bidwillii*.**

Population Name	Region	Latitude	Longitude	Altitude	Approx. population size	No. individuals sampled
Jimna	Jimna State Forest/ Conondale Range	26º 42’	152º 27’/ 38’	500	1000>x>100	21
Westcott	Bunya Mts	26º 51’	151º 34’	1000m	1000>x>100	21
Burton’s Well	Bunya Mts	26º 49’	151º 33’	800m	x<100	17
Dandabah	Bunya Mts	26º 52’	151º 35’	1000m	x~1000	26
Paradise	Bunya Mts	26º 52’	151º 35’	1000m	x~1000	22
Mt Lewis	North Qld	16º 30’	145º 22’	1000m	x<100	24

populations from the Bunya Mountains – ‘Dandabah’, ‘Paradise’, ‘Westcott’ and ‘Burton’s Well’. In north Queensland, there are only two small extant populations – north-west of Cairns at Mt Lewis (16º 30’ S) and south-west of Cairns at Cannabullen Falls (17º 40’ S). Due to physical and logistical problems with accessibility to the Cannabullen Falls population, only Mt Lewis was sampled from this region for this study.

Trees were selected arbitrarily from each population, but were separated as far as possible to minimise the chance of sampling closely related or genetically identical individuals. All samples included in this study were collected from mature (canopy forming) reproductive individuals so as to gain a purely historical signal from the data. Whilst the effects of recent anthropogenic fragmentation and exploitation are essential to quantify, it is only through a comparison with the historical genetic signal that any insight can be gained into the effects of more recent processes. For this reason, juveniles were collected for future analyses but were not included in the present study.

A minimum of 20 individuals per population was targeted, with only one population (Burton's Well) yielding less (17 individuals), due to a lower density of individuals in that population, and the drier environment that hinders the preservation of fallen green leaves that are required for appropriate DNA preservation. This environment also produced few specimens with epicormic buds, which were substantially relied upon in other populations for fresh leaf material when access to branches was unfeasible.

Approximately five leaves were collected from each tree, assigned a unique identifier, and placed into plastic sealable bags with a small amount of silica gel, to assist in the rapid drying and preservation of DNA, until they could be processed.

Vouchers for all specimens were retained and are stored at the Molecular Biology Laboratory, James Cook University, Cairns.

3.2.2 Site descriptions

3.2.2.1 Mt Lewis

This site represents the most northerly extant occurrence of the species and is incorporated within Mt Lewis State Forest. Mt Lewis is part of the Great Dividing Range and lies approximately 65 kms north-west of Cairns, within the Carbine Tableland, south of the Windsor Uplands (Figure 3.1). The predominant forest type has been classified as notophyll evergreen vine forest (Webb and Tracey 1981) of varying complexity, but in actuality a mosaic of vegetation types occurs in the area. The soils are composed of fine-grained Mareeba granites. Annual rainfall is in excess of 3,000 mm. Mt Lewis is home to many endemic

plant and animal species, and as such, appears to have historically been an important refugium for these lineages.

Mt Lewis itself is 1200m asl, however the Bunya population occurs lower down on its western slopes at approximately 900m. The forest type here may best be described as wet sclerophyll, occurring in a zone between moist rainforest and drier *Eucalyptus*/*Callitris* woodlands, and includes the representative wet sclerophyll taxon, *Eucalyptus grandis*. Other co-occurring coniferous taxa include *Podocarpus grayi* and *Agathis robusta*. In fact, this site is unique within Australia in that *Agathis* and *Araucaria bidwillii* co-occur. Other endemic coniferous taxa found and/or restricted on Mt Lewis (*Prumnopitys ladei* and *Podocarpus smithii*) are absent from this site.

The site contains relatively few mature individuals (< 100), but a range of stem size classes was noted and there was evidence of recent regeneration over multiple years. Most mature individuals are located adjacent to a permanent water source, Station Creek, and intrusion into the adjacent rainforest is rare. Fire is a common threat to the site and is promoted by the site's proximity to sclerophyll woodlands. Both seedling mortality due to fire, and seed predation by rodents, have personally been noted over repeated visits to the site. Intact seeds were not found, despite repeated visits to the site, which appears to substantiate early reports state that no viable seed could be found at the site (Smith and Butler 2002). However, the range of size classes and the presence of seedlings obviously indicate that not all seed is predated.

While the rainforests of Mt Lewis were substantially logged up until the 1980's, *A. bidwillii* was not targeted from this area (Hanrahan pers. comm.). The

entire area is now incorporated within the Wet Tropics World Heritage Management Area, and is therefore protected from any future logging.

3.2.2.2 Jimna

This population was named for this study from the central township of the area and the surrounding State Forest. The region is broadly referred to in this study as the coastal region, as it encompasses the coastal ranges of Conondale and Blackall. The site is approximately 100 kms north of Brisbane, 50 kms inland from the Sunshine Coast, and approximately 90 kms east of the Bunya Mts (Figure 3.1). The soils of this region are derived predominantly from basalt and annual mean rainfall for the region is approximately 1100 mm. The majority of the population's samples came from the "Araucaria Walk" within Jimna State Forest, along with other individuals from the adjacent Sunday Creek Road within the Conondale Range.

The State Forest encompasses 33,000 ha of plantation timber, surrounding pockets of native vegetation, of which 3,000 ha is occupied by the Bunya's native congener, *A. cunninghamii*. These pockets of native vegetation are in fact one of the few places where the two Australian species of *Araucaria* naturally co-occur (see section 3.2.2.3., where an altitudinal separation is usually present).

This region was extensively cleared from the 1870's, but is now protected within a network of National Parks and State Forests. Czechura (1991) maintains that "irreversible changes have already occurred in the Blackall-Conondale Ranges". Bunyas, as previously mentioned, were initially a target species of forestry operations, however, some sites within the region proved too difficult to

log, and as a result, occasional mature specimen trees survived. It is from this stock that the current population is derived. Regeneration at the site, and therefore population regrowth, has been said to be 'exponential' over the past 40 years (Smith and Butler 2002), which may suggest the damage, at least for this species, was not as 'irreversible' as first thought. While the site contains evidence of regeneration, the majority of individuals are mature, and are distributed more sparsely throughout the rainforest when compared to the Mt Lewis population. Of course, the possibility remains that irreversible genetic damage has occurred, the quantification of which is one of the aims of the present study.

The Jimna State Forest site may best be described as dry Araucarian vine thicket, while the Conondale/Blackall Range sites are more typical of the moist upland rainforests of the area. The area inhabited by *A. bidwillii* in south-east Queensland is of interest to plant biogeographers. The region between the coastal ranges and the Bunya Mountains is characterised by a distinct floristic community, broadly termed dry rainforest (Smith and Butler 2002). Many species associated with northern dry rainforests find their southern distribution limits within this zone. Conversely, many southern species have northern limits in the same region. This biogeographic overlap suggests that this broad region supported a band of rainforest refugia and may have also acted as a barrier to certain plant species' dispersal either north or south of their respective distributions (Smith and Butler 2002).

3.2.2.3 Bunya Mountains

The Bunya Mountains represent the most western section of the Great Dividing Range in south-east Queensland, and span approximately 20 kms. They rise 500 m above the surrounding plains, and the Mountains' highest point is 1135 m a.s.l. at Mt Kiangarow. The Mountains, which lie 150 kms inland from the coast and approximately 150 kms NW of Brisbane (Figure 3.1), are in fact the remnants of a broad shield volcano that erupted around 24 million years ago; the same volcanic complex that formed the Lamington Plateau and Border Ranges south of Brisbane (Willmott *et al*. 1995). All soils within the region are of basaltic origin. Average annual rainfall for the Mountains is 1050 mm, although rainfall is orographically controlled. For example, only 17 kms to the north-east at Kumbia at an elevation of 520 m, the values of average annual rainfall drop to 672 mm. Further evidence of this rainfall gradient may be gained by the increasingly drier forest types encountered north of Westcott (e.g. Burton's Well).

The populations sampled from the Bunya's most extensive extant stand, the Bunya Mountains, were sampled along an approximate north-south transect, following the Bunya Mountains road which traverses the Mountains. The actual localities of sampled populations are displayed, and are named according to the closest existing named feature of the National Park. Dandabah and Paradise occur within the core distribution of the species on the Mountains in what is more or less continuous moist Araucarian forest, although collection localities for the two populations were separated by at least 2 kms. Westcott and Burton's Well occur in the northern, drier regions of the National Park, with Westcott approximately 3 kms north of Paradise, and Burton's Well another 5 kms from the Westcott

population. The latter populations are clearly separated not only from each other, but also the Dandabah/Paradise core region of the Bunya Mountains.

The Park protects a mosaic of vegetation types, including the most westerly occurrence of rainforest in south-east Queensland, and the largest protected areas of dry vine thicket in Australia. The Park also contains patches of grassy balds, which have been compared to similar ecosystems in the Southern Appalachians (Fensham and Fairfax 1996). The maintenance, persistence and origin of these grasslands have attracted much attention, as they appear to be in decline due to changes in fire regimes since European Settlement (Fensham and Fairfax 1996).

While *A. cunninghamii* (Hoop Pine) occurs at several localities within the Bunya Mountains, it is usually associated with drier communities than *A. bidwillii*. The distributional separation of the two congeners, which is present within the core region – with *A. bidwillii* occurring higher up in moist rainforests, and *A. cunninghamii* occurring in drier rainforest on the lower slopes - does not occur in the northern, drier reaches of the Park. There the two species may be found side-by-side in what may be considered as an intermediate habitat for both species – their environmental overlap of distribution.

The National Park was declared in 1908 (the second to be declared in Queensland) and is one of the few in Queensland that was designated for the protection of a single species (see section 1.6.1.1). Thus, logging is no longer a threat to these populations. However, other threats common to all populations, including fire and pathogens, are of management concern. Of course,

(mis)management of populations in the absence of genetic information is also a potential threat.

3.3 Results

3.3.1 Molecular analyses

From the forty RAPD primers trialled, nine primers were chosen for the analysis of the entire sample set on the basis of their detection of polymorphism within and among populations (Table 3.2).

Table 3.2. **RAPD primer sequences used in the genetic analysis of *A. bidwillii*.**

Name	Sequence 5' – 3'	# loci scored
OPB 20	GGACCCTTAC	6
OPB18	CCACAGCAGT	6
OPB17	AGGGAACGAG	5
OPB15	GGAGGGTGTT	2
OPB10	CTGCTGGGAC	6
OPB9	TGGGGGACTC	6
OPB8	GTCCACACGG	6
OPB1	GTTTCGCTCC	8
OPB6	TGCTCTGCCC	5

From the forty-one ISSR primers screened, six primers that displayed appropriate levels of variation were selected for the analysis of the entire sample set (Table 3.3).

3.3.2 RAPD analysis

3.3.2.1 Reaction conditions

The addition of formamide (2%) did not improve band resolution in a series of RAPD PCR trials (cf. Tsumura *et al*. 1996) and as a result was omitted from all subsequent reactions.

Table 3.3. **ISSR primer sequences used in the genetic analysis of *A. bidwillii*.**

Name	Sequence 5' – 3'	# loci scored
17898B	CACACACACACAGT	12
808	AGAGAGAGAGAGAGAGC	11
810	GAGAGAGAGAGAGAGAT	11
807	AGAGAGAGAGAGAGAGT	8
811	GAGAGAGAGAGAGAGAC	5
818	CACACACACACACACAG	4
857	ACACACACACACACACYG	6

Repeatability and reproducibility of all reactions was ascertained through the aforementioned duplicate reactions.

3.3.2.2 Genetic diversity as detected by RAPD primers

The 9 RAPD primers scored from the 40 screened primers produced a total of 50 polymorphic loci. This represents a conservative proportion of the total number of loci, both variable and monomorphic, as the previously described scoring methodology was employed. All products scored were between 300 base pairs (bp) and 2,000 bp.

A minimum of three monomorphic markers was produced from each primer, but these were not included in the analysis. Two population-specific bands were recorded for the Mt Lewis population, both from primer OPB 15 (Table 3.2). Additionally, five loci present in all southern populations were absent from the Mt Lewis population. One of these loci, from OPB 10, was fixed in all populations with the exception of the Mt Lewis population.

The relative degree of diversity harboured within populations, as indicated by Shannon's index, varied from 0.43 for the Mt Lewis population to 0.53 for the Jimna population (Table 3.4), but these differences were not significant ($p = 0.37$, ANOVA). The mean population diversity was 0.48 and the pooled species-level estimate was 0.60. The percentage of polymorphic loci varied from 74% for Mt Lewis to 92% for Jimna (Table 3.4). The mean percentage of polymorphic loci across all populations was 84%. As only polymorphic bands were scored for this analysis, the pooled-species level of polymorphism was 100%.

3.3.2.3 Genetic differentiation as detected by RAPD primers

Genetic structuring was investigated by AMOVA. The hierarchical structure which best explains the distribution of RAPD phenotypes of *A. bidwillii* among the populations sampled comprised 5 regions. These were Mt Lewis, Jimna, Westcott, Burton's Well, and Dandabah plus Paradise. This structuring explained 21.19% of the variation as occurring among regions, 4.28% occurring between populations within a region (in this case Dandabah plus Paradise), with the remainder, 74.53% being contained within populations (Table 3.5).

Table 3.4. **Shannon's diversity index (*S*) and percent polymorphic RAPD loci (%P) for all populations of *A. bidwillii* sampled as well as mean values averaged over all populations and pooled species-level values. Standard errors are presented in parentheses.**

Population	*S* (± standard errors)	%P
Jimna/Conondale	0.5333 (0.2099)	92
Westcott	0.4751 (0.2401	86
Burton's Well	0.4628 (0.2677)	80
Mt Lewis	0.4261 (0.2894)	74
Dandabah	0.498 (0.216)	90
Paradise	0.464 (0.2499)	82
Mean, S_{pop}	0.4755	84
Species level	0.6043 (0.0898)	100

Table 3.5. **AMOVA analysis of RAPD variation for 6 *Araucaria bidwillii* populations. These values represent the most parsimonious explanation for the observed structuring in the RAPD data. Five regions are identified: Mt Lewis, Jimna, Burton's Well, Westcott, Paradise plus Dandabah.**

	d.f.*	Variance component	% of total variance	*p* value[+]
Among regions	4	1.93836	**21.19**	0.00000
Between populations within regions	1	0.39179	**4.28**	0.00000
Within populations	125	6.81783	**74.53**	0.00000

*Degrees of freedom

[+]Significance of the variance components

When the regions are combined so as to contrast Mt Lewis with the southern populations (Table 3.6), most of the variation (60.43%) is found within

populations but a significant ($p < 0.00001$) proportion is due to differences between northern and southern regions (30.80%) and among populations within the southern regions (8.77%).

Table 3.6. **AMOVA analysis of RAPD variation for 6 *Araucaria bidwillii* populations. Regions were defined as northern (Mt Lewis) and southern (the remainder) populations.**

	d.f.*	Variance component	% of total variance	*p* value[+]
Between regions	1	3.33174	**30.80**	0.00000
Among populations within regions	4	0.94813	**8.77**	0.00000
Within populations	125	6.53526	**60.43**	0.00000

*Degrees of freedom

[+]Significance of the variance components

When no regional hierarchy is invoked, 24.18% of the variation is partitioned among all sampled populations, with the remaining 75.82% being contained within populations ($p < 0.00001$; Table 3.7).

Table 3.7. **AMOVA analysis of RAPD variation for 6 *Araucaria bidwillii* populations. Regions were collapsed so as to identify global population structuring.**

	d.f.*	Variance component	% of total variance	*p* value[+]
Among populations	5	2.17548	**24.18**	0.00000
Within populations	125	6.81783	**75.82**	0.00000

*Degrees of freedom

[+]Significance of the variance components

To investigate the structuring of the southern populations, the Mt Lewis population was excluded from the analysis. Invoking no hierarchical structure gave a Φ_{ST} of 11.85%, with 88.15% of the variation residing within populations (Table 3.8; $p < 0.00001$).

Table 3.8. **AMOVA analysis of RAPD variation for 5 southern *Araucaria bidwillii* populations. Regions were collapsed so as to identify population structuring within the core distribution of *A. bidwillii*.**

	d.f.*	Variance component	% of total variance	*p* value+
Among populations	4	0.96499	**11.85**	0.00000
Within populations	102	7.17503	**88.15**	0.00000

*Degrees of freedom

+Significance of the variance components

All Φ_{ST} values between individual pairs of populations were significant (Table 3.9). Maximum values were recorded between Mt Lewis and all other populations sampled – from 39% to 44% divergence. The lowest values were recorded between Dandabah and Paradise with 5% divergence; other values ranged from 8% to 16% divergence (Table 3.9).

The unrooted UPGMA tree (Figure 3.2) graphically illustrates the relationships among populations and regions. The northern population is separated from all southern populations. A long branch leads to the Mt Lewis population, while the Jimna (coastal) population clusters within the Bunya Mts

populations. The population from Burton's Well is the most divergent of the southern populations, while Dandabah and Paradise are closest.

Table 3.9. **Matrix of pairwise differences between sampled populations of *A. bidwillii* (lower diagonal) with significance values (above diagonal), calculated from AMOVA derived Φ_{ST} values from analysis of the RAPD data set. The extreme divergence values for Mt Lewis are highlighted in bold.**

	Jimna	Westcott	Burtons Well	Mt Lewis	Dandabah	Paradise
Jimna	-	0.000	0.000	0.000	0.000	0.000
Westcott	0.08201	-	0.000	0.000	0.000	0.000
Burtons Well	0.07770	0.16918	-	0.000	0.000	0.000
Mt Lewis	**0.38840**	**0.43379**	**0.34846**	-	0.000	0.000
Dandabah	0.10947	0.12827	0.12133	**0.41227**	-	0.000
Paradise	0.11061	0.12320	0.15651	**0.43828**	0.04657	-

The UPGMA tree, displayed as a phylogram, shows the divergence of the northern population from the southern populations (Figure 3.3). Burton's Well separates from the other southern populations, with Jimna and Westcott, and Dandabah and Paradise, once again clustering into two groups. There is also a slightly longer branch length leading to the Jimna and Westcott populations, relative to that leading to the Dandabah plus Paradise group.

The same pattern was obtained using principal coordinates analysis of pairwise Φ_{ST} values (Figure 3.4). There is a clear separation of Mt Lewis and Burton's Well from all other southern populations. The first three axes of this ordination explain 99.4% of the variation present (87%, 7% and 5%, respectively).

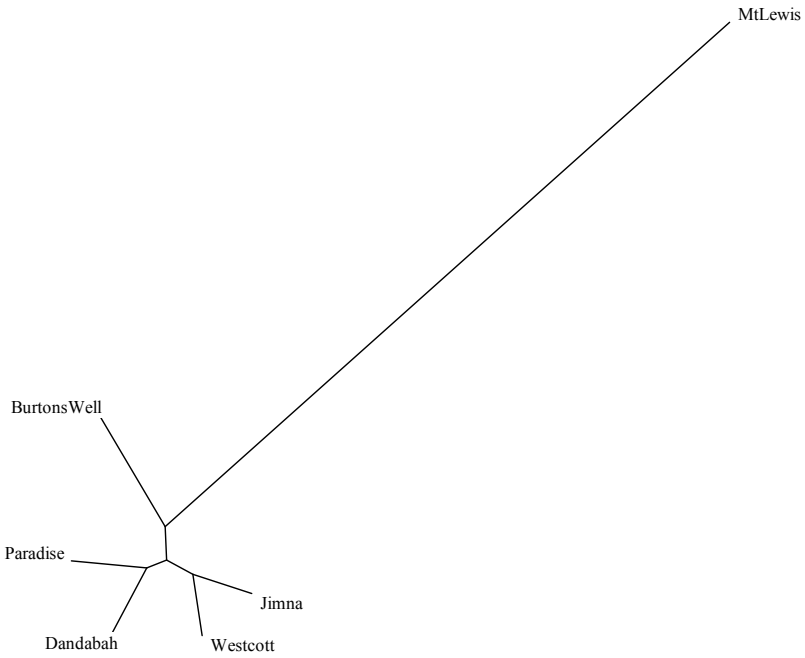

Figure 3.2. UPGMA tree, unrooted, displaying the relative divergence among sampled populations of *A. bidwillii* as determined from the RAPD data set.

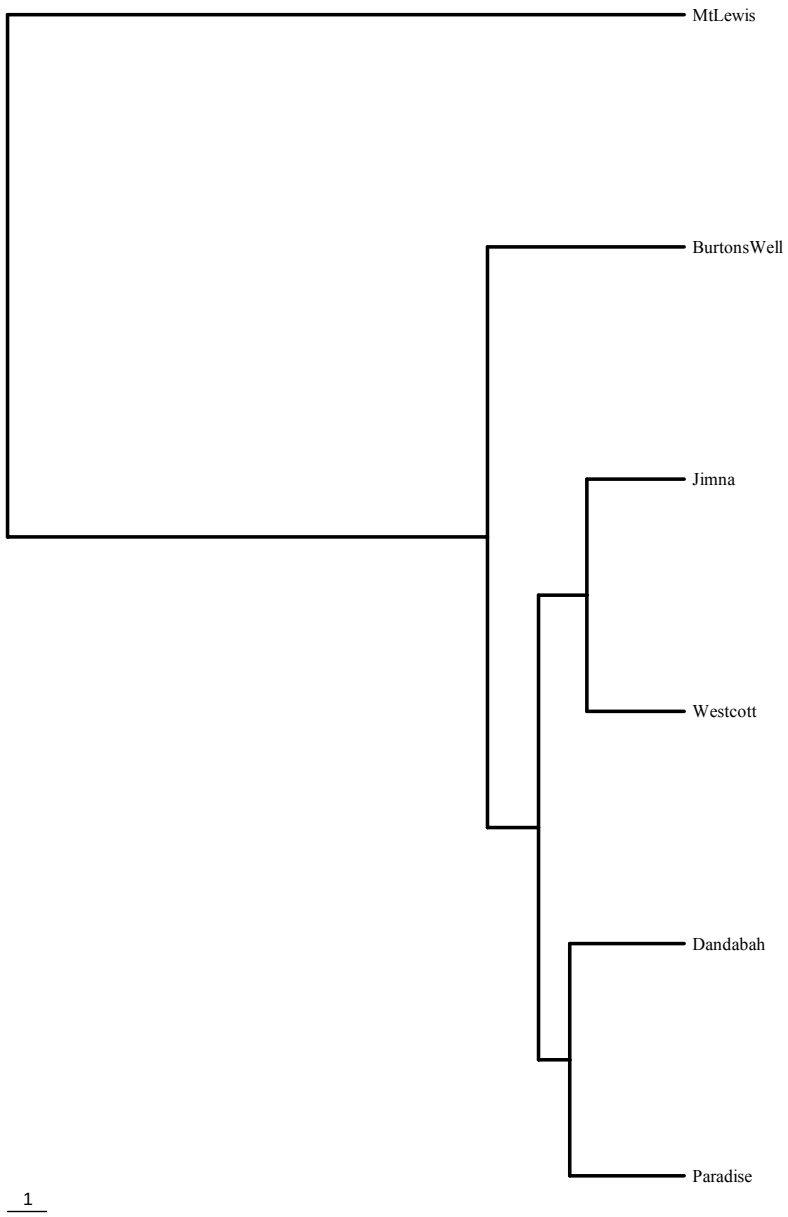

Figure 3.3. UPGMA tree derived from the RAPD data set, as displayed as a phylogram displaying proportional branch lengths, and highlighting the relative divergence among sampled populations of *A. bidwillii*.

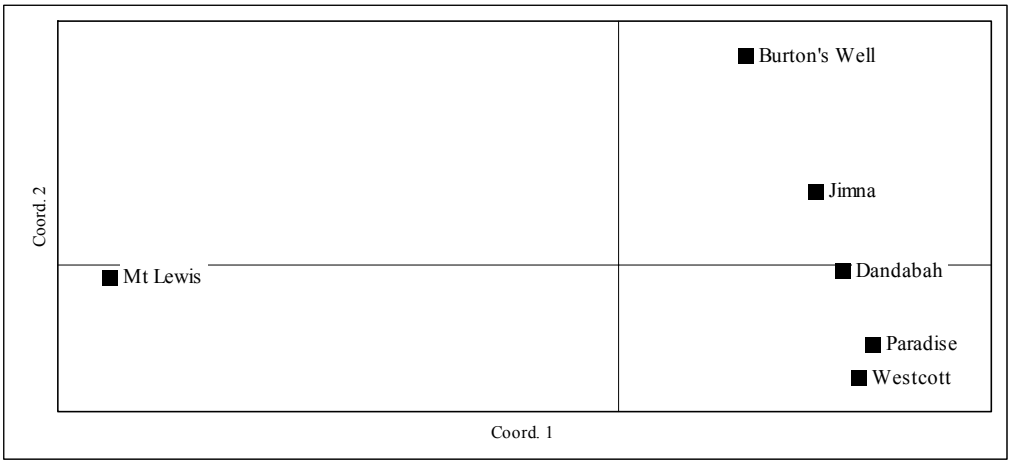

Figure 3.4. PCA ordination, derived from RAPD data, of pairwise Φ_{ST} values among populations of *A. bidwillii* sampled for this study. Axis one contains 87% of the variation present, clearly separating Mt Lewis from all other sampled populations. Axis two accounts for 7% of the variation, and separates the southern populations sampled.

For all analyses, Φ_{ST} (derived from a Euclidean distance metric) and G_{ST} estimates (derived from Nei's unbiased measure of diversity) are in broad agreement. For example, the global Φ_{ST} value for all populations (24.2%) agrees with the estimate from G_{ST} (21.7%). The structure which best explained the distribution of RAPD variation among the populations sampled gave a Φ_{ST} of 21.19%, while that estimated using G_{ST} gave a value of 19.8%. Similarly, when the Mt Lewis population is excluded, the value of Φ_{ST} was 11.9%, compared with a G_{ST} value of 12.5%.

3.3.3 ISSR analysis

3.3.3.1 Reaction conditions

The addition of formamide to the ISSR reactions did not improve band resolution in trials (cf. Tsumura *et al*. 1996) and was therefore omitted from subsequent reactions.

Although AT repeats have been suggested to be the most frequently encountered microsatellite repeat motif in plants (Lagercrantz *et al*. 1993), AT based ISSR primers failed to generate PCR products in this species. Other ISSR primers which failed to produce PCR products in *A. bidwillii* include 5' labelled primers (regardless of the microsatellite motif), as well as tri-nucleotide repeats (regardless of the anchoring sequence or position). However, di-nucleotide repeats are known to be more common than tri-nucleotide repeats in plant genomes (Smith and Devey 1994). Of the di-nucleotide repeats reported previously in another conifer, *Pinus radiata* (Smith and Devey 1994), GA repeats and CA repeats were the most common, with GA repeats being more common than CA repeats. These results concur in that four of the seven ISSR primers employed in this study were based on GA repeat motifs, while three were from CA repeat primers (Table 3.3).

Of the two ISSR primers designed in this study specifically for *Araucaria*, which were derived from previous reported microsatellite motifs in the Araucariaceae (Scott *et al*. 2003), one failed to produce polymorphic products (GAGAGAGAGAGAGAGAAC) and the other (TTCTTCTTCTTC TTCTTCTG) produced a number of bands that could not be unequivocally scored across populations or even individuals within a population.

All reactions were reproducible and no implicit differences were noted in the repeatability of the two molecular marker systems.

3.3.3.2 Genetic diversity as detected by ISSRs

The 7 ISSR primers chosen from the 41 screened primers (Table 3.3) produced a total of 57 polymorphic loci. As with the RAPD analysis, this also represents a conservative proportion of the total number of loci, both variable and monomorphic, as the previously described conservative scoring methodology was employed. All products scored were between 300 bp and 2,000 bp in length.

From one to five monomorphic markers were produced from each ISSR primer, but these were not included in the analysis. No population-specific bands were recorded, although Burton's Well and Mt Lewis both lacked a marker from primer 810 (Table 3.3) that was present in all other populations. Additionally, three loci present in all southern populations were absent from the Mt Lewis population – these were from primers 808, 811, and 857 (Table 3.3).

The relative degree of diversity harboured within populations, as indicated by Shannon's index (S), varied from 0.46 for the Mt Lewis population to 0.55 for the population at Dandabah (Table 3.10), but there was no significant difference in the level of diversity between populations ($p = 0.22$, ANOVA). The mean population diversity was 0.50 and the pooled species-level estimate was 0.57 (Table 3.10). Percent polymorphic loci (%P) varied from 83% for Mt Lewis to 98% for Dandabah (Table 3.10). The mean percentage of polymorphic loci across all populations was 92%. As only polymorphic bands were scored for both analyses the pooled-species level of polymorphism was 100%.

Table 3.10. **Shannon's diversity index (*S*) and percent polymorphic ISSR loci (%P) for all populations of *A. bidwillii* sampled as well as mean values averaged over all populations and pooled species-level values. Standard errors are presented in parentheses.**

Population	*S* **ISSRs**	%P
Jimna/Conondale	0.4737 (0.2252)	88
Westcott	0.4920 (0.2012)	93
Burton's Well	0.5118 (0.2020)	95
Mt Lewis	0.4644 (0.2500)	83
Dandabah	0.5500 (0.1521)	98
Paradise	0.5251 (0.1915)	95
Mean, S_{pop}	0.5028	92
Species level	0.5665 (0.1281)	100

3.3.3.3 Genetic differentiation as detected by ISSRs

Genetic structuring was investigated in the ISSR data set by AMOVA. The hierarchical structure which best explains the distribution of ISSR phenotypes identified the same 5 regions as the RAPD analysis: Mt Lewis, Jimna, Burton's Well, Westcott, and Dandabah plus Paradise (Table 3.11). This structuring partitioned 6.47% of the ISSR variation between regions, 3.59% between population within a region, with the remainder (89.94%) being distributed within populations ($p < 0.00001$; Table 3.11).

Once again regions were combined so as to contrast northern and southern populations (Table 3.12). However, more variation was found among populations

within a region (7.21%) than existed between regions (5.7%), and these values were not significant ($p = 0.17$; cf. RAPD analysis, $p = 0.000001$).

Table 3.11. **AMOVA analysis of ISSR variation for 6 *Araucaria bidwillii* populations. These values represent the most parsimonious explanation for the observed structuring in the RAPD data. Five regions are identified: Mt Lewis, Jimna, Burton's Well, Westcott, Paradise plus Dandabah.**

	d.f.*	Variance component	% of total variance	*p* value+
Among regions	4	0.64046	**6.47**	0.00000
Between populations within regions	1	0.35492	**3.59**	0.00000
Within populations	123	8.90125	**89.94**	0.00000

*Degrees of freedom

+Significance of the variance components

Table 3.12. **AMOVA analysis of ISSR variation for 6 *Araucaria bidwillii* populations. Regions were defined as northern (Mt Lewis) and southern (the remainder) populations.**

	d.f.*	Variance component	% of total variance	*p* value+
Between regions	1	0.58273	**5.70**	0.00000
Among populations within regions	4	0.73666	**7.21**	0.00000
Within populations	123	8.90125	**87.09**	0.00000

*Degrees of freedom

+Significance of the variance components

Collapsing all regional hierarchy gave a significant Φ_{ST} value of 9.57% (cf. 24.2% from RAPDs), with 90.43% of the variation contained within populations ($p < 0.00001$; Table 3.13).

Table 3.13. **AMOVA analysis of ISSR variation for 6 *Araucaria bidwillii* populations. Regions were collapsed so as to identify global population structuring.**

	d.f.*	Variance component	% of total variance	*p* value+
Among populations	5	0.94235	**9.57**	0.00000
Within populations	123	8.90125	**90.43**	0.00000

*Degrees of freedom

+Significance of the variance components

Mt Lewis was excluded from the ISSR analysis to further investigate structuring among the southern populations. Invoking no regional hierarchy, Φ_{ST} estimates indicated that 7.38% of the ISSR variation (cf. 11.85% from RAPD) was shared between populations (Table 3.14; $p < 0.00001$). The remainder of the variation (92.62%) was contained within populations (Table 3.14).

All Φ_{ST} values between individual pairs of populations as calculated from the ISSR data were significant (Table 3.15). Maximum values were once again recorded between Mt Lewis and all other populations sampled (10% – 16% divergence; cf. 39% to 44% from RAPDs; Table 3.15). The lowest values were recorded between Dandabah and Paradise (4%, cf. 5% from RAPDs), while all other values between populations were between 5% and 11% (Table 3.15, cf. 8% to 16% from RAPDs).

Table 3.14. **AMOVA analysis of ISSR variation for 5 southern *Araucaria bidwillii* populations. Regions were collapsed so as to identify population structuring within the core distribution of *A. bidwillii*.**

	d.f.*	Variance component	% of total variance	*p* value[+]
Among populations	4	0.72657	**7.38**	0.00000
Within populations	101	9.11392	**92.62**	0.00000

*Degrees of freedom

[+]Significance of the variance components

Table 3.15. **Matrix of pairwise differences between populations (lower diagonal) with significance values (above diagonal), calculated from AMOVA derived Φ_{ST} values from analysis of the ISSR data set.**

	Jimna	Westcott	Burtons Well	Mt Lewis	Dandabah	Paradise
Jimna	-	0.000	0.000	0.000	0.000	0.000
Westcott	0.08057	-	0.001	0.000	0.000	0.000
Burtons Well	0.10788	0.05801	-	0.000	0.000	0.000
Mt Lewis	0.13081	0.15535	0.09901	-	0.000	0.000
Dandabah	0.04778	0.07313	0.08463	0.12881	-	0.002
Paradise	0.09579	0.08577	0.08550	0.15822	0.03722	-

The unrooted UPGMA tree (Figure 3.5) was again used to graphically illustrate the relationships among populations and regions, as determined from the ISSR data. The northern population is once again separated from all southern

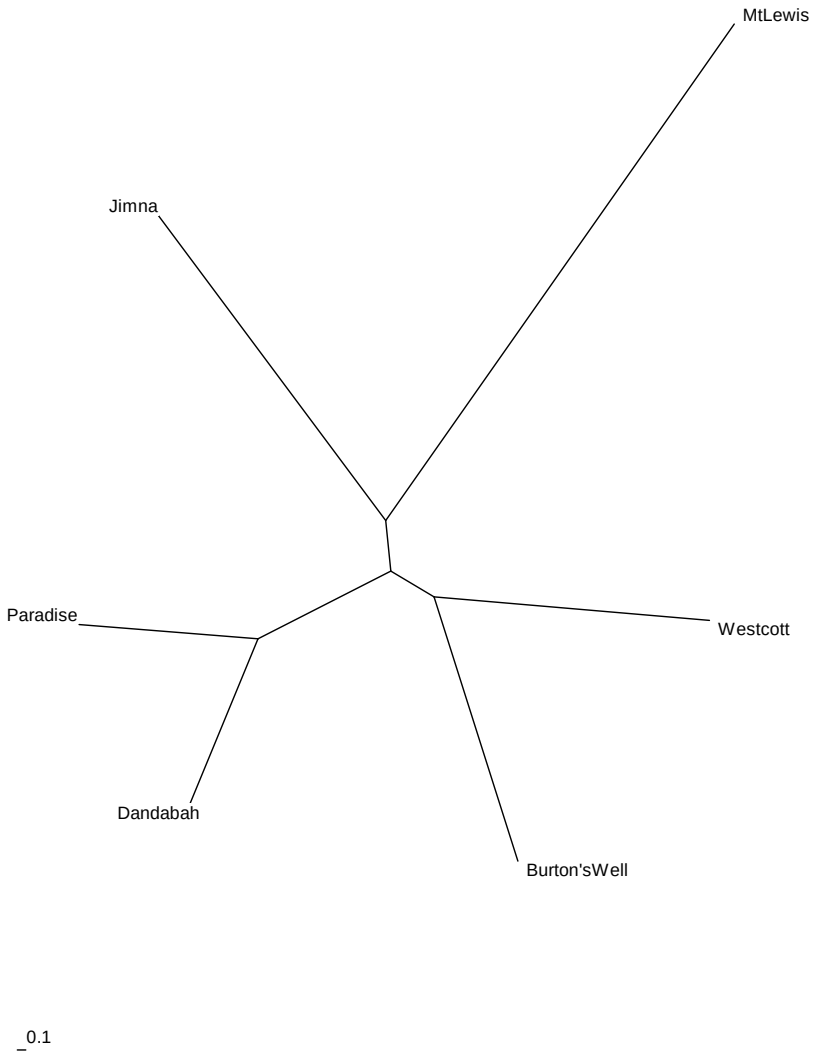

Figure 3.5. UPGMA tree, unrooted, displaying the relative divergence among sampled populations of *A. bidwillii* as determined from the ISSR data set.

populations, however the separation, as determined by shorter branch lengths, was not as great as that for the RAPD analysis. The populations of Westcott and Burton's Well, along with Paradise and Dandabah, cluster together, away from relatively long branches leading to the populations of Mt Lewis and Jimna. It

should be noted that the UPGMA diagrams derived from the different data sets (Figures 3.3 and 3.5) are at differing scales and thus the branch lengths in the ISSR UPGMA tree are much shorter than those in the tree derived from the RAPD data set.

The UPGMA tree, when displayed as a phylogram as determined from the ISSR data, again shows the divergence of the northern population from the southern populations (Figure 3.6). However, in line with the unrooted representation, the divergence is not as great as for the RAPD data. Mt Lewis is again the most divergent of the populations sampled. In contrast to the tree determined from the RAPD data, the Jimna population displays some divergence from the Bunya Mts populations. The populations from the Bunya Mts form a separate group, with Westcott and Burton's Well, and Dandabah and Paradise clustering respectively. Once again, the branch lengths in the ISSR phylogram are substantially shorter than those from the RAPD analysis.

The PCA produced from analysis of pairwise ISSR Φ_{ST} values (Figure 3.7) supports the ISSR UPGMA tree topology. There is less separation of populations, relative to that obtained from the RAPD data set. Nevertheless, Mt Lewis is clearly separated from all other populations. Although to a lesser extent than the Mt Lewis population, Burton's Well is separated from the other southern populations. This separation mirrors that obtained from a PCA analysis of the RAPD data, in that Mt Lewis and Burton's Well display increased divergence

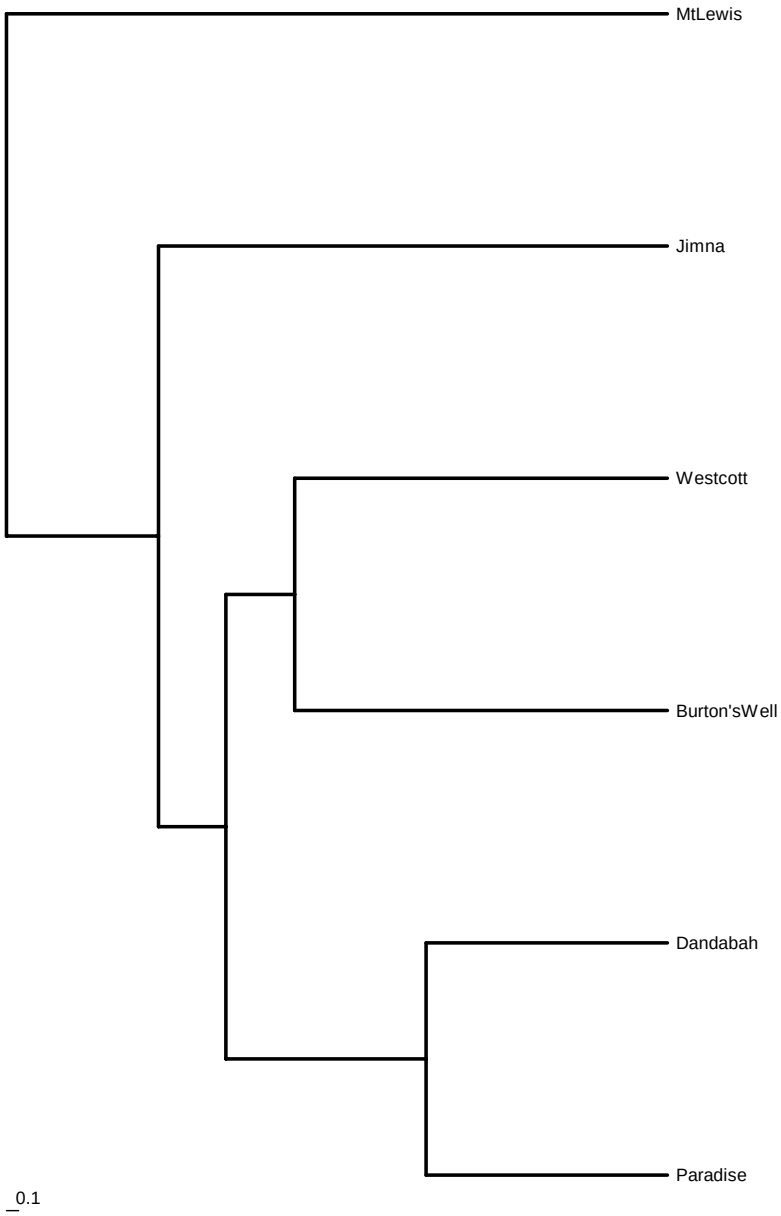

Figure 3.6. UPGMA tree, shown as a phylogram with branch lengths proportional to change. This displays the relative divergence among sampled populations of *A. bidwillii* as determined from the ISSR data set.

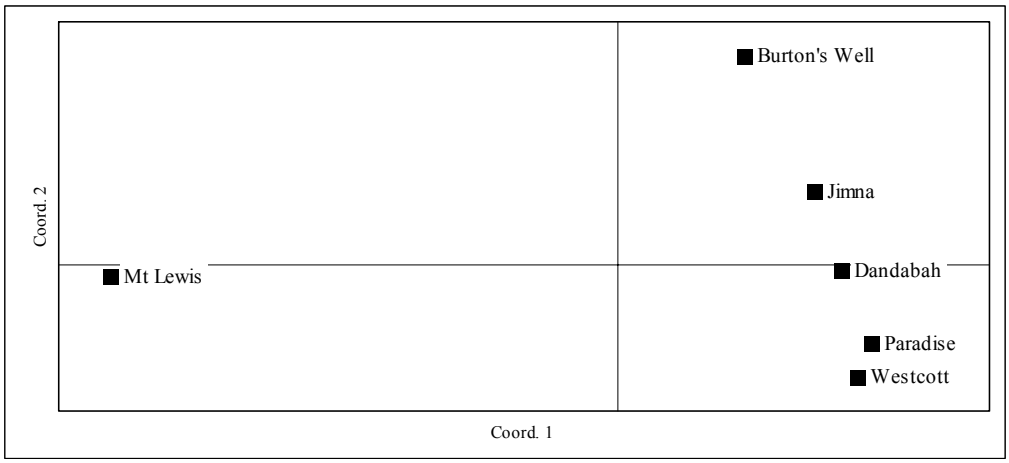

Figure 3.7. PCA ordination of pairwise Φ_{ST} values among populations of *A. bidwillii* derived from the ISSR data set. Axis one contains 61% of the variation present, clearly separating Mt Lewis from all other sampled populations. Axis two accounts for 24% of the variation, and separates the southern populations sampled.

relative to the other sampled populations (Figure 3.4). The first three axes of this ordination explain 60.9%, 85.3% and 100%, respectively.

3.3.3.4 Congruence test between data sets and population parameters

The Mantel Test conducted between pairwise matrices derived from the different dominant molecular markers indicated a high degree of congruence ($r = 0.8$; $p < 0.05$).

As for the RAPD analyses, Φ_{ST} and G_{ST} estimates were in broad agreement for the ISSR analyses. For example, the global Φ_{ST} value from the ISSR data set for all populations (7.38%) generally agrees with the estimate from G_{ST} (9.59%). Given this broad congruence both across markers and data sets, and

the robustness of AMOVA analyses for examining dominant molecular data sets, only values derived from the AMOVA analyses will be further discussed.

3.4 Discussion

Two results from these analyses are striking: firstly, the high genetic variation recorded for a 'relictual' southern conifer, and secondly, the extreme population differentiation, predominantly between northern and southern populations. This is in contrast to the majority of studies that have found genetic diversity within populations to be negatively correlated with the degree of population differentiation (Nybom and Bartish 2000).

3.4.1 Genetic diversity in Bunyas

Genetic diversity in Bunyas, as revealed by a mean Shannon's index of 0.48, is in line with that reported in RAPD studies for other Southern conifers, which have also revealed high levels of genetic variation - *Fitzroya cupressoides*, $S = 0.54$ (Allnutt *et al*. 1999); *Araucaria araucana*, $S = 0.65$ (Bekessy *et al*. 2002). These results are in contrast to a recent report that genetic diversity within some members of the Australian Araucariaceae is low (Peakall *et al.* 2003). However, low genetic variation does not characterise the family as a whole, as Bekessy *et al.* (2002) also reported substantial genetic variation in their study of the South American *Araucaria araucana*.

Despite evidence of range reductions in the past, there is no evidence for loss of genetic variation in any of the populations examined, using either RAPDs or ISSRs as molecular markers. Such loss, for example through population

bottlenecks brought about by inbreeding, or founder events, would be reflected in lower diversity values in affected populations. The mean values of '*S*' obtained for Bunyas from the RAPD and ISSR analyses, 0.48 and 0.5 respectively, are only slightly lower than values reported for other southern conifers using the same methodologies - *Araucaria araucana* 0.65 (Bekessy *et al*. 2002), *Fitzroya cupressoides* 0.54 (Allnutt *et al*. 1999), *Podocarpus salignus* 0.69 (Allnutt *et al*. 2001), *Pilgerodendron uviferum* 0.57 (Allnutt *et al*. 2003).

Using either molecular methodology, there is no significant difference in the variation found among populations, regardless of size or degree of isolation of the population (ANOVA, $p > 0.05$). This suggests that extant population size in Bunyas cannot predict the level of diversity it contains – smaller populations (<100) may harbour equal amounts of genetic diversity as larger populations (>1000). It should be noted that in both analyses Mt Lewis recorded the lowest values of genetic diversity and may therefore be displaying the initial impacts of genetic erosion. Quantifying the genetic diversity in that population's progeny could substantiate this theory in the future.

The percentage of polymorphic RAPD markers was also high regardless of the population size or isolation. The Mt Lewis population recorded the lowest polymorphism (74%) and the Jimna population the highest (92%) with a mean value across populations of 84%; a result which is in agreement with estimates of genetic diversity derived from Shannon's Diversity Index (Table 3.4).

Polymorphism in the ISSR data set was higher, ranging from 83% for Mt Lewis to 98% for Dandabah, with a mean value of 92%. Further comparisons across studies are not made here, due to the inherent subjectivity of primer choice

to maximise polymorphic loci and the inclusion, in some studies, of monomorphic loci in their calculations of total polymorphism (see Chapter 2). The slight discrepancy between data sets in their estimation of polymorphism will be examined in more detail in Chapter Five.

Small endemic populations may lose genetic variation by genetic drift (Frankham 1996). For conifers, the lagging effects of extremely long generation times and the indirect methodology (i.e. genetic methods) used may mean that the genetic effects of relatively recent size reductions are not yet detectable, particularly in currently small populations of *A. bidwillii*. Populations that are marginal, small or geographically isolated (eg. Mt Lewis, Burtons Well, Jimna) are expected to be more susceptible to the effects of genetic drift due to lower effective population size and/or increased selection pressures. In any case the loss of genetic variation and its restoration are slow processes, made slower in this instance by extremely long generation times inherent in the Araucariaceae.

Long generation times appear to act to distribute genetic variation temporally within Bunya populations, through the maintenance of a range of age classes in any given population. The spatial distribution of genetic variation (i.e. genetic structuring) will be maintained through a balance of population divergence and gene flow, and is examined below.

These results suggest that effective population size in Bunyas may be independent of actual census size. More likely is the scenario that current population sizes are of insufficient age to allow a genetic equilibrium to be reached and therefore any reduction in genetic variability due to the effects of small contemporary population size remains undetectable at this stage. However,

historically, population size fluctuations do not yet appear to have affected the genetic diversity of any of these populations. The high diversity detected has most likely been maintained by distributing the variation temporally across a range of age classes in this long-lived species. In doing this, smaller populations may be able to maintain similar levels of genetic variation to larger populations. As only mature individuals were sampled for this study, it should also be noted that these results are providing an historical signal, and thus the commonality of genetic variation, within southern populations at least, should not be surprising under an historical scenario of population connectivity.

3.4.2 Regional differentiation in Bunyas

It is important, but rarely conducted in published population genetic studies, to test all permutations of hierarchical structure, as significant differentiation of regions/ populations may be found in a number of differing structures. That which minimises among population within region variation and maximises regional differentiation can be considered as the best hypothesis for the variation observed.

Significant genetic structuring is predicted among historically isolated plant populations, which may have acted as refugia, due to previously low levels of gene flow in the past. From these results it is evident that the hypothesis supporting three separate Pleistocene refugia for the species (Bunya Mts, Jimna, Mt Lewis) as proposed by Webb and Tracey (1981) is not supported by either the RAPD ($p = 0.13$) or ISSR ($p = 0.06$) data (data not shown). Thus, while these three regions may have acted as Pleistocene refugial localities for some rainforest

species, as predicted by phylogenetic diversity of rainforest endemics (Webb and Tracey 1981), there is no evidence from the RAPD or ISSR data that these localities acted as refugial areas for Bunyas. This result is interesting given the present-day association of this conifer with rainforest environments and the expectation that rainforest contractions and expansions would have affected all species inhabiting that ecosystem in the same manner. The broader ecological tolerance of this species, relative to angiosperm rainforest endemics, may partially explain this lack of association with rainforest refugia.

The hierarchical structure that bests explains the partitioning of variation for both the RAPD and ISSR data sets (Tables 3.5 and 3.11) provides evidence for five distinct regions: Mt Lewis/ Jimna/ Burton's Well/ Westcott/ Dandabah plus Paradise. These results not only mirror the extant distribution of the populations sampled but also allow insight into smaller scale population processes. For example, the divergence of both Jimna and Mt Lewis is not surprising given their extant geographic isolation. However, the significant structuring within the Bunya Mts is not predicted by population genetic theory which would predict that these populations form a homogenous group, based on the longevity of the species and wind-dispersed pollen (Nybom 2004). The fine-scale structuring will be further discussed below by examining the population pairwise values of differentiation.

The difference in resolution gained from the two molecular markers was quite substantial. Regional differentiation accounted for 21.19% of variation in the RAPD data set, but only 8.18% in the ISSR data set. This discrepancy will be discussed in Chapter 5.

Regardless of the molecular marker employed, pairwise differences between populations indicate that all sampled populations are significantly differentiated from each other (Tables 3.9 and 3.15), thereby emphasising the high degree of population structuring in Bunyas. Significant differentiation occurs between populations separated by as much as 2000 kms (Mt Lewis – Bunya Mts) or as little as 2 kms (within Bunya Mts) and is independent of the degree of isolation of the populations. As previously mentioned, the distribution of *A. bidwillii* within the Bunya Mts is more or less continuous while Jimna and Mt Lewis may be considered as isolates.

Once again, the resolution of the markers in detecting population differentiation differed, however this difference was predominantly confined to the values between Mt Lewis and all southern populations. RAPDs detected substantially more differentiation between Mt Lewis and the southern population (35% - 44%) than did the ISSRs (10% - 16%). Pairwise values among the southern populations from the different markers were congruent, and ranged from 4% to 16% for the RAPDs and 4% to 11 % for the ISSRs.

While distance alone can explain the significant differentiation of the Mt Lewis population, this cannot be generalised for the southern populations. Remarkably, the RAPD analysis suggests that the coastal population (Jimna) is more genetically alike to 'core' Bunya Mts populations than some of these populations are to each other (Figure 3.4; Table 3.9). The ISSR analysis also showed some populations within the Bunya Mts (e.g. Burton's Well vs Dandabah or Paradise, Westcott vs Paradise) displaying more divergence than was found between the coastal population and populations (Westcott, Dandabah) from the

Bunya Mts (Table 3.15). The occurrence of indigenous 'festivals', concurrent with the Bunyas' masting events (see Introduction) raises the possibility of dispersal by human activity, at least within southern Queensland to the coastal Ranges. Whether the population associations detected represent historical habitat connectivity or comparatively recent dispersal by humans cannot be ascertained from the present data. However, it seems likely from historical records that a more or less continuous araucarian forest connected the coast to the Bunya Mts, over which a mixing of genotypes may be expected. Loss of intervening habitat and selective logging over the past few centuries may simply have left *in situ* genotypes that are more closely related than present geographic 'isolation' of these populations would suggest. In either case, due to distance alone, the potential for pollen-mediated gene flow would be expected to be greater among southern populations than between northern and southern populations, which may explain this association. Thus the present 'isolation' of the coastal and northern populations appears to be the product of different temporal events, recent versus historical.

The different origins of population isolation are also interesting when considering the potential impacts on genetic variation. As previously shown, there was no significant difference in the amount of variation detected between the sampled populations, which adds further support to the theory of the temporal maintenance of genetic diversity in small populations of long-lived southern conifers. These results suggest that Bunyas can buffer the deleterious effects of small population size for substantial periods, as long as a range of age classes is maintained.

The differentiation of the Mt Lewis population may be either due to drift, caused by small population size and low levels of historical gene flow, selection in absence of gene flow without significant loss of diversity, or simply reflect a different historical origin. The presence of slight phenotypic divergence in leaf morphology (Pye pers. obs.) provides further support for this theory. In any case, the Mt Lewis genotypes may represent a significant entity given models of global climate change that predict a warmer climate than at any time during the recent geological past (Huntley 1998), as this population represents an evolutionary lineage already exposed to higher temperatures. Research is underway to assess current management strategies and taxonomic status of this distinct population.

The Mt Lewis population is currently protected within a Forestry Reserve within the Wet Tropics World Heritage Management Area. Current threats to the population include: stochastic genetic effects associated with small population size, fire (which may ironically also promote colonisation), increased seed predation due to small population size and higher abundance of potential predators in a tropical environment (which lowers the effectiveness of the Bunya's strategy of masting strategy), cyclones, pathogens (a significant threat identified for *Wollemia nobilis*, Araucariaceae (Bullock *et al*. 2000)) and other stochastic environmental threats common to all small populations.

Given the strong divergence detected in the RAPD data between northern and southern populations, additional threats may be posed by outbreeding depression and/or loss of historical patterns of differentiation (Young *et al*. 1996). This could be brought about by pollen flow from the large number of Bunyas planted on the adjacent Atherton Tablelands over the last few centuries, which,

based on leaf morphology, appear to be derived from the southern populations, as they possess pungent leaf tips; a characteristic confined to the southern populations of Bunyas. Genetic analysis of seedlings, by means of the aforementioned Mt Lewis population-specific markers, is currently being undertaken to assess this potential threat to the genetic integrity of the population. If the seedlings lack the markers associated with the Mt Lewis adult population, we can infer that inter-population pollination may be occurring, which may lead to the risks associated with outbreeding depression together with the loss of historical differentiation detected in this study (Young *et al*. 1996). Albeit to a lesser extent, this risk is also present for the southern populations as all populations displayed significant genetic structuring. Therefore, movement of germplasm should be avoided, even within the Bunya Mts.

3.4.3 Differentiation among the southern populations

A surprising result from this study was the significant structuring found between all populations, regardless of size or degree of isolation. As previously mentioned, this pattern was detected over all spatial scales, from as few as 2 kms to as much as 2,000 kms. Wind-pollinated conifers are expected to show low differentiation, especially over short distances, as enhanced gene flow acts to negate differentiation. However, significant genetic structuring was found between what are effectively continuous populations in the Bunya Mountains (e.g. Dandabah and Paradise). The reasons for this are unclear but may be related to restricted dispersal through limitations in either pollen and/or seed dispersal. While seed predation and caching occur (Pye pers. obs.), seeds may only be

dispersed over short distances which may partially explain this structure. Wind-dispersed pollen should encounter no barrier to gene flow in continuous or adjacent populations. However, recent studies, reviewed in Koenig and Ashley (2003), suggest that short distance pollen dispersal in wind pollinated trees might be relatively common. Furthermore, the magnitude and direction of prevailing air currents together with the physical characteristics of the pollen grain could act to modify generalisations of widespread gene flow via wind-dispersed pollen..

Studies of pollen dispersal in the genus are few, but a recent study of pollination in *Araucaria angustifolia* showed that the large non-saccate pollen of that species does not travel great distances (Sousa and Hattemer 2003). The pollen grains of *A. angustifolia*, and presumably the genus, are significantly larger than other anemophilous species (~ 61.5 μm) and lack the floatation devices of other conifers with similar sized pollen grains. The possibility remains that the density of forest on the Bunya Mountains acts to physically impede pollen dispersal, thereby producing a neighbourhood effect. The emergent habit is obviously advantageous in facilitating pollen distribution, however in a dominant stand only particularly old specimens may emerge above the 'emergents'. While today the canopy is fairly heterogeneous this may not have always been the case. Given the logging history of the site, there is also the possibility that each population was preferentially pollinated by a few emergent 'donor' individuals, which would act to increase local divergence. As only mature individuals were sampled this signal is historical and will necessarily reflect the pre-European structure of the forests. More research is required specifically for the

characteristics of pollen and its dispersal in *A. bidwillii*, and the implications of these on extant genetic structuring after an intensive logging regime.

The discrepancy noted in the resolution of the two molecular markers is examined in further detail in Chapter 5. The implications of these results for the conservation of the species is further discussed in Chapter 7.

CHAPTER FOUR

GENETIC STRUCTURING AND DIVERSITY IN *Araucaria cunninghamii*

4.1 Introduction

4.1.1 Extant distribution

The latitudinal distribution of *A. cunninghamii* is the largest of all extant Araucarian species, covering a latitudinal range from 1º S (PNG/ Irian Jaya) to 31º S (northern NSW, Australia). However this distribution is by no means even, in terms of abundance, or continuous, in terms of occurrence. The areas of maximum abundance centre on southeastern Queensland, within 300 kms of Brisbane, and the Bulolu / Wau region of PNG (Dieters *et al*. 2002). The distribution of the species within Queensland is disjunct (Figure 4.1), but does not mirror the broad north/south disjunction of *A. bidwillii*. There are more intervening populations of *A. cunninghamii* throughout central Queensland (Figure 4.1), and it is a frequently encountered dominant species of offshore islands. However, the majority of populations now appear to be isolated and/or associated with refugia for other species.

The disjunct distribution is the product of historical range contractions that presumably occurred under the same similar environmental fluctuations that led to the extant distribution of *A. bidwillii*. Of course, contemporary exploitation has further fragmented these populations (see section 1.6.2.2.). However the broader ecological tolerance and, potentially, differences in the species' biology of *A. cunninghamii* has resulted in more extant populations than its congener.

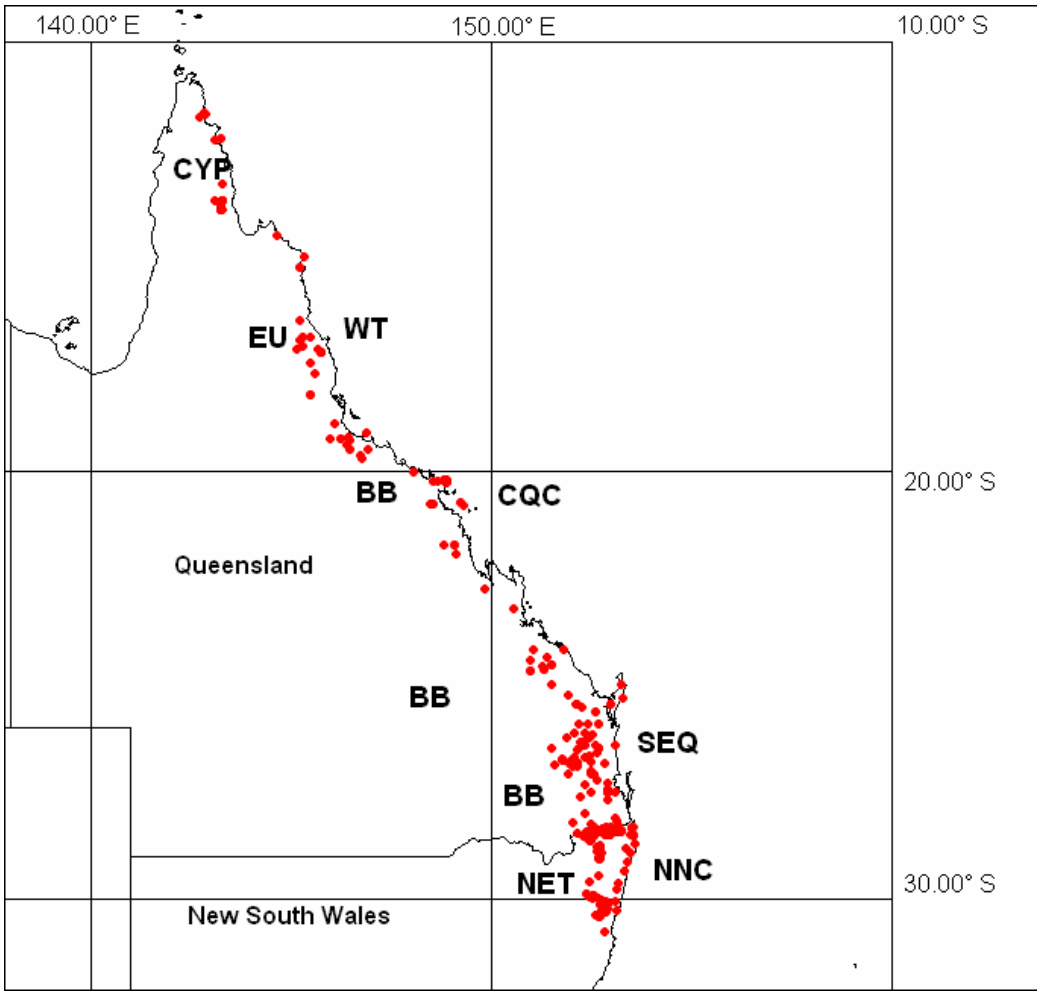

Figure 4.1. The extant distribution of *A. cunninghamii* in Australia (map provided by T. Jovanavich). CYP, WT, EU, CQC, BB, SEQ, NNC and NET stand for the Bioregions in which the species occurs- Cape York Peninsula, Wet Tropics, Einasleigh Uplands, Central Queensland Coast, Brigalow Belt, South East Queensland, New South Wales North Coast and New England Tableland respectively.

4.2 Materials and methods

4.2.1 Sampling

Leaf samples were collected from a total of 147 individuals from eight populations (Table 4.1) across a comparable geographic range to that sampled for the study of *A. bidwillii* (Figure 4.2). Collections were made at two different

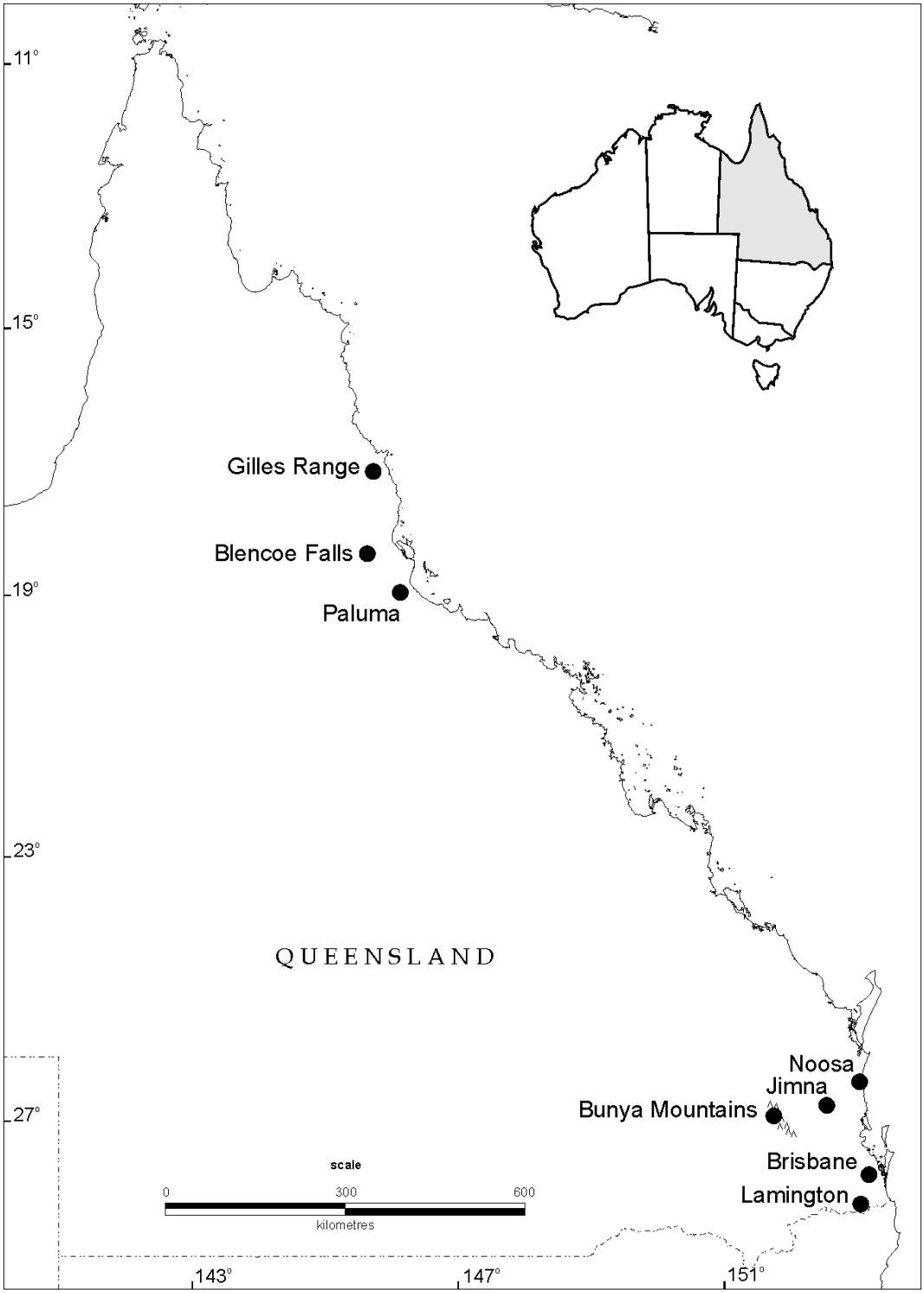

Figure 4.2. Localities of populations of *A. cunninghamii* sampled for the study. Populations were sampled over a comparable range to *A. bidwillii*.

scales, reflecting the sampling strategy employed for *A. bidwillii*. While the distribution of *A. cunninghamii* is more continuous, populations were sampled from north and south Queensland respectively, to facilitate direct comparisons with its congener. For ease of discussion, I will refer to broad areas of Hoop pine distribution in Queensland as follows: Cape York – any populations north of the World Heritage Wet tropics Management Area; North Queensland – any populations bordered by Cooktown in the north to Townsville in the south; Central Queensland – any populations north of Bundaberg and south of Townsville; Southern Queensland – any population between the New South Wales/ Queensland border and Bundaberg (Figure 4.1).

To provide direct and meaningful comparisons with its congener, only extant populations of *A. cunninghamii* found within and/or adjacent to the current distribution of *A. bidwillii* were sampled for this study. For North Queensland, populations were sampled at Huntsbrook Creek on the Gillies Range, Blencoe Falls and Little Crystal Creek, Paluma (Figure 4.2). An additional population from the Hann Tableland was also targeted, however problems with accessibility hindered the collection of plant material from this site.

Within south Queensland, populations were sampled to mirror the sampling of *A. bidwillii* in the preceding chapter, and to extend the resolution gained in that study by sampling additional populations within the region (Figure 4.2). Accordingly, populations from Festoon Falls in the Bunya Mountains (Bunya Mts) and Jimna State Forest (Jimna) were sampled where the two species co-occur. Other populations sampled include Noosa National Park (Noosa),

Binna Burra section of Lamington National Park (Lamington), and Jolly's Lookout in Brisbane Forest Park (Brisbane).

Trees were selected randomly from each population, but were separated as far as possible to minimise the chance of sampling closely related or genetically identical individuals. All samples included in this study were once again collected from mature (canopy forming) reproductive individuals, firstly to gain a historical perspective on genetic structuring, but also to avoid sampling the products of closely related reproductive events. A minimum of 20 individuals per population was targeted, however for several populations this was not feasible, either due to accessibility or simply due to the number of individuals at the site (Table 4.1). Approximately 20 cm of branch, containing several of the short, imbricate leaves, was collected from each tree, assigned a unique identifier, and placed into plastic sealable bags with a small amount of silica gel, to assist in the rapid drying and preservation of DNA, until they could be processed.

Table 4.1. **Localities and descriptives of sampled populations of *A. cunninghamii*.**

Population Name	Region	Latitude	Longitude	Approx. population size	No. individuals sampled
Lamington	South Qld	28º 10'	153º 08'	1000<x>100	12
Blencoe	North Qld	18º 15'	145º 45'	1000<x>100	20
Brisbane	South Qld	27º 20'	152º 46'	x < 100	10
Bunya Mts	South Qld	26º 52'	151º 35'	x >1000	20
Gillies	North Qld	17º 11'	145º 11'	x < 100	18
Jimna	South Qld	26º 42'	152º 27'	x>1000	23
Noosa	South Qld	26º 25'	153º 07'	1000 < x > 100	22
Paluma	North Qld	18º 58'	146º 15'	x < 100	22

Vouchers for all specimens were retained and are stored at the Molecular Biology Laboratory, James Cook University, Cairns.

4.2.2 Site descriptions

4.2.2.1 Paluma

This population lies along Little Crystal Creek, at approximately 400 m in altitude. The site is predominantly comprised of sclerophyll vegetation on granite-derived soils, however Hoop pines line the creek and rainforest vegetation is also present within these small patches. This area marks the boundary between open forest that occurs lower down, and the rainforest habitat which occurs higher on the Paluma Range (1000 m a.s.l.).

4.2.2.2 Gillies

The Gillies population occurs adjacent to the Gillies Highway on Huntsbrook Creek at approximately 670 m a.s.l. This small population (less than 20 individuals) occurs in a small patch of rainforest, surrounded by fire-promoting sclerophyll vegetation, that appears to be protected from fire due to the permanent watercourse and the presence of less flammable rainforest vegetation. The substrate is derived from granites, although supplementation by basalts that flow from a lake upstream may also occur (Whitehead pers. comm.).

4.2.2.3 Blencoe

This population is also isolated and although situated in drier savanna-type woodlands, Hoop pines line the sheltered gorge along granite outcrops within and above Blencoe Falls. The site is 95 kms inland from Cardwell.

4.2.2.4 Jimna

This population was sampled from the Araucaria Walk in Jimna State Forest, where both *A. bidwillii* and *A. cunninghamii* co-occur. The site is described in Chapter 3.

4.2.2.5 Bunya Mountains

This population was sampled from Festoon Falls at Dandabah within the Bunya Mountains. The site is described in Chapter 3.

As previously mentioned *A. cunninghamii* occurs at several localities within the Bunya Mountains, where it is usually associated with drier communities than the Bunya. At Festoon Falls, a few individuals of both species co-occur, however, there is a distributional gradient, with *A. bidwillii* occurring higher up in moist rainforests relative to *A. cunninghamii* which is more dominant in drier rainforest on lower slopes.

4.2.2.6 Noosa

The Noosa population was sampled from Noosa National Park, along the Palm Grove circuit, where Hoop pines emerge above a rainforest canopy. The site is approximately 10 m a.s.l. and occurs on sandy soils. This population of *A. cunninghamii* is relatively small when compared to other southern Queensland populations.

4.2.2.7 Brisbane

This population was collected from Jolly's Lookout in Brisbane Forest Park, in what was formerly an extensive Hoop pine forest. This site has seen dramatic reductions in abundance of *A. cunninghamii* due to an intensive logging regime beginning in the early 1930s. The surrounding vegetation is predominantly rainforest, and occurs on basaltic soils.

4.2.2.8 Lamington

The Lamington population was collected from the Binna Burra section of the Lamington National Park, along the 'Rainforest circuit'. The soils of the area are derived from basalt and the region supports a diverse rainforest community, including the most northerly occurrence of *Nothofagus* in Australia. The site is approximately 800 m a.s.l., and unlike the northern populations, *A. cunninghamii* is generally found on ridgetops throughout the area.

4.3 Results

4.3.1 RAPD analysis

4.3.1.1 Genetic diversity in A. cunninghamii as detected by RAPD primers

The 7 RAPD primers scored from the twelve screened primers that were screened produced a total of 75 polymorphic loci (Table 4.2). This represents a conservative proportion of the total number of loci, both variable and monomorphic, as the previously described scoring methodology was employed. All products scored were between 300 bp and 2,000 bp

Table 4.2. **RAPD primer sequences used in the genetic analysis of *A. cunninghamii*.**

Name	Sequence 5' – 3'	# loci scored
OPB 20	GGACCCTTAC	4
OPB17	AGGGAACGAG	4
OPB12	CCTTGACGCA	11
OPB10	CTGCTGGGAC	16
OPB9	TGGGGGACTC	10
OPB8	GTCCACACGG	13
OPB3	CATCCCCCTG	17

A minimum of two monomorphic markers was produced from each primer, but these were not included in the analysis. No fixed population–specific bands were recorded. One locus, OPB 17-1, was present only in the Gillies population but was not fixed. Another locus, OPB10-1, separated northern from southern populations, as this locus, although not fixed, was present in all northern populations and uniformly absent from southern populations. Locus OPB8-12 was fixed for all southern populations, and while present at lower frequencies in the Blencoe and Paluma populations, it was absent in all individuals sampled from the Gillies Range. Finally, locus 20-3 was invariably absent in all northern populations (fixed absence), occurring only in southern populations.

Shannon's diversity index (S) varied from 0.28 for the Gillies range population, to 0.46 for the population at Paluma (Table 4.3). An analysis of variance, (ANOVA) revealed a significant difference in the levels of diversity

Table 4.3. **Shannon's diversity index (*S*) and percent polymorphic RAPD loci (%P) for all populations of *A. cunninghamii* sampled as well as mean values averaged over all populations and pooled species-level values. Standard errors are presented in parentheses.**

Population	*S* **RAPDs** (± standard errors)	%P
Lamington	0.4134 (0.2812)	71
Blencoe	0.3883 (0.2835)	71
Brisbane	0.4043 (0.2810)	72
Bunya Mts	0.4081 (0.2694)	75
Gillies	0.2822 (0.2971)	53
Jimna	0.4516 (0.2328)	85
Noosa	0.4568 (0.2409)	84
Paluma	0.4582 (0.2488)	84
Mean, S_{pop}	0.4079	74
Species level	0.5266 (0.1565)	100

detected among populations (F = 2.27; $p = 0.03$). Subsequent post-hoc tests identified the diversity within Gillies population as being significantly different from all other populations sampled. All other populations contained similar levels of diversity, however the variation detected in the Gillies population was significantly lower when compared to all other populations ($p \leq 0.05$; Table 4.4). The mean population diversity was 0.41 and the pooled species-level estimate was 0.53 (Table 4.3). The percentage of polymorphic loci varied from 53% for the Gillies population to 85% for Jimna (Table 4.3). The mean percentage of polymorphic loci across all populations was 74%. Once again, as only polymorphic bands were scored for this analysis the pooled-species level of polymorphism was 100%.

Table 4.4. Significance values from post-hoc tests from ANOVA employing the test of Least Significant Differences (LSD), which identify the Gillies population as the sole divergent population ($p \leq 0.05$) according to extant levels of genetic diversity as assessed by RAPDs of the sampled populations of *A. cunninghamii*.

	Lamington	Blencoe	Brisbane	Bunya Mts	**Gillies**	Jimna	Noosa
Lamington	-	-	-	-	-	-	-
Blencoe	0.165	-	-	-	-	-	-
Brisbane	0.174	0.976	-		-	-	-
Bunya Mts	0.379	0.611	0.631	-	-	-	-
Gillies	**0.001**	**0.052**	**0.048**	**0.014**	-	-	-
Jimna	0.973	0.155	0.164	0.361	**0.001**	-	-
Noosa	0.528	0.448	0.466	0.803	**0.007**	0.506	-
Paluma	0.292	0.737	0.760	0.862	**0.023**	0.277	0.672

4.3.1.2 Genetic differentiation in A. cunninghamii as detected by RAPD primers

Genetic structuring in the RAPD data set of *A. cunninghamii* was investigated by AMOVA. The hierarchical structure that best explained the distribution of RAPD phenotypes among the sampled populations of *A. cunninghamii* comprised 6 regions. These were Gillies, Blencoe, Paluma, Lamington, Bunya Mts plus Brisbane, and Noosa plus Jimna. This structuring explained 19.11% of the variation as occurring among regions, 2.27% occurring between populations within regions, with the remainder, 78.62% being contained within populations ($p < 0.00001$; Table 4.5). As this structure has minimised the variation among populations within each region and maximised the variation among regions, this structure best explains the distribution of RAPD phenotypes of *A. cunninghamii* across the sampled range of the species.

Table 4.5. **AMOVA analysis of RAPD variation for 8 *Araucaria cunninghamii* populations. These values represent the most parsimonious explanation for the observed structuring in the RAPD data. Six regions are identified: Gillies, Blencoe, Paluma, Lamington, Jimna plus Noosa, Brisbane plus Bunya Mts.**

	d.f.*	Variance component	% of total variance	*p* value[+]
Among regions	5	2.10193	**19.11**	0.00000
Between populations within regions	2	0.24960	**2.27**	0.00000
Within populations	137	8.64525	**78.62**	0.00000

*Degrees of freedom

[+]Significance of the variance components

When no regional hierarchy is invoked, 20.25% of the variation is partitioned among all sampled populations, with the remaining 79.75% being contained within populations ($p < 0.00001$; Table 4.6).

Table 4.6. **AMOVA analysis of RAPD variation for 8 *Araucaria cunninghamii* populations. Regions were collapsed so as to identify global population structuring.**

	d.f.*	Variance component	% of total variance	*p* value[+]
Among populations	7	2.19531	**20.25**	0.00000
Within populations	137	8.64525	**79.75**	0.00000

*Degrees of freedom

[+]Significance of the variance components

To investigate the structuring of the southern populations, all northern populations were excluded from the analysis. Invoking no hierarchical structure gave a Φ_{ST} of 5.67%, with 94.33% of the variation residing within populations ($p < 0.00001$; Table 4.7).

Table 4.7. **AMOVA analysis of RAPD variation for 5 southern *Araucaria cunninghamii* populations. Regions were collapsed so as to identify population structuring within the most abundant extant distribution of *A. cunninghamii*.**

	d.f.*	Variance component	% of total variance	*p* value[+]
Among populations	4	0.54047	**5.67**	0.00000
Within populations	80	8.98982	**94.33**	0.00000

*Degrees of freedom

[+]Significance of the variance components

Similarly, to investigate the structuring of northern populations alone, all southern populations were excluded from the analysis. Invoking no hierarchical structure gave a Φ_{ST} of 23.42%, with 76.58% of the variation residing within populations ($p < 0.00001$; Table 4.8). This represents an approximate four-fold

Table 4.8. **AMOVA analysis of RAPD variation for 3 *Araucaria cunninghamii* populations in northern Queensland. Regions were collapsed so as to identify population structuring within this region.**

	d.f.*	Variance component	% of total variance	*p* value[+]
Among populations	2	2.49655	**23.42**	0.00000
Within populations	57	8.16165	**76.58**	0.00000

*Degrees of freedom

[+]Significance of the variance components

increase in the divergence among northern populations relative to that among the southern populations.

All but three Φ_{ST} values between individual pairs of populations were significant (Table 4.9). Non-significant values were found between Brisbane and the Bunya Mountains, Jimna and the Bunya Mountains, and between the Bunya Mountains and Noosa. The maximum value of 42% divergence was recorded between the Gillies range population and the Lamington population (Table 4.9). Surprisingly, the Gillies Range population showed less divergence from the population at Paluma (28%) than it did from the geographically closer Blencoe Falls population (30%; Table 4.9). The divergence between the populations at Paluma and Blencoe was 14% (Table 4.9), which is above that recorded between all southern populations (Table 4.9).

The lowest significant value of differentiation was recorded between the Bunya Mountains and Noosa populations (4%). The highest values of divergence among the southern populations occurred between Noosa and Lamington (12%). In addition there was less significant divergence between the Paluma and Blencoe Falls populations versus any southern population (18 - 26%) than there was between each of these two northern populations and the population slightly to the north of them at the Gillies Range (28% and 30% respectively).

The unrooted UPGMA tree (Figure 4.3) derived from the RAPD data graphically illustrates the relationships among populations and regions and confirms the groupings identified in the AMOVA analyses. The northern populations are clearly separated from all southern populations. However, that separation appears to be as great among the northern populations as it is between

Table 4.9. Matrix of pairwise differences between sampled populations of *A. cunninghamii* (lower diagonal) with significance values (above diagonal) calculated from AMOVA derived Φ_{ST} values from analysis of the RAPD data set. Non-significant associations are highlighted in bold.

	Lamington	Blencoe	Brisbane	Bunya Mts	Gillies	Jimna	Noosa	Paluma
Lamington	-	0.000	0.002	0.002	0.000	0.000	0.000	0.000
Blencoe	0.27475	-	0.000	0.000	0.000	0.000	0.000	0.000
Brisbane	0.09941	0.21699	-	0.817	0.000	0.000	0.001	0.000
Bunya Mts	0.08053	0.18213	**-0.02424**	-	0.000	0.951	0.159	0.000
Gillies	0.41462	0.29814	0.34262	0.37068	-	0.000	0.000	0.000
Jimna	0.10700	0.24341	0.06325	**-0.02445**	0.31793	-	0.000	0.000
Noosa	0.11575	0.25739	0.09473	**0.03670**	0.33047	0.04447	-	0.000
Paluma	0.26412	0.14038	0.20849	0.17526	0.27624	0.20937	0.21369	-

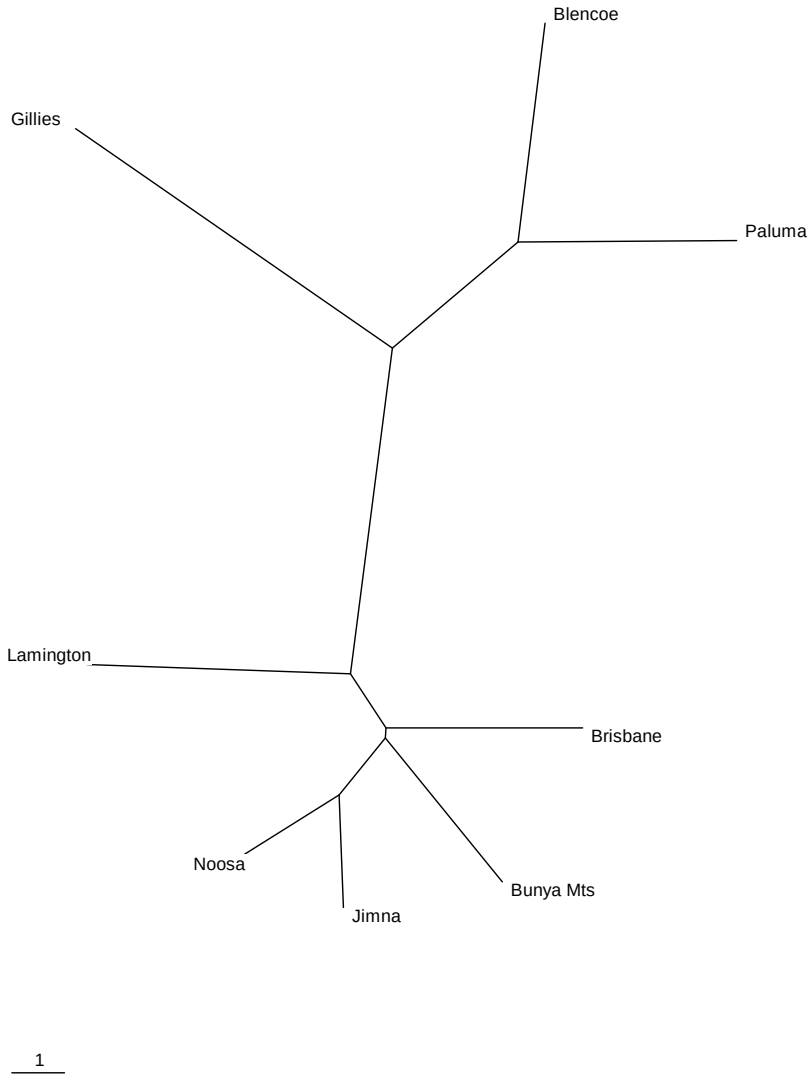

Figure 4.3. UPGMA tree, unrooted, displaying the relative divergence among sampled populations of *A. cunninghamii* as determined from the RAPD data set.

the northern and southern populations. The branch length leading to the Gillies population appears to be as long as that separating the northern and southern populations. Its affinity to the northern populations is evident from the much shorter branch length leading to the grouping of Paluma and Blencoe populations.

Three southern groups are identified, in agreement with the AMOVA analysis that best described the distribution of RAPD phenotypes. These groups separate the Lamington population from the other southern populations by a relatively short branch length. The population from Brisbane groups with the Bunya Mountains' population, and that group appears to be equidistant from both the Lamington population and the grouping of the Jimna and Noosa populations.

The UPGMA tree, when displayed as a phylogram, again graphically displays the divergence of the northern populations from the southern populations (Figure 4.4). The divergence recorded between Blencoe and Paluma, as determined by proportional branch lengths, is as great as that differentiating Brisbane from Jimna or Noosa. The divergence of the Border Ranges population at Lamington from the other southern populations is greater than that between the aforementioned northern populations but less than that between the Gillies population and other northern populations. The greatest divergence among all populations sampled occurs between the northern and southern populations, which cluster into two discrete groups.

This pattern was confirmed using principal coordinates analysis of pairwise Φ_{ST} values (Figure 4.5). There is a clear separation of all northern populations from a closely clustered southern group. The Gillies population is the most divergent among all sampled populations, followed by the clustering of Blencoe and Paluma from all remaining populations. There appears to be some evidence for the separation of the Lamington population from the other southern sampled populations. The first three axes of this ordination explain 96% of the variation present (the first three axes explaining 67%, 22% and 7% respectively).

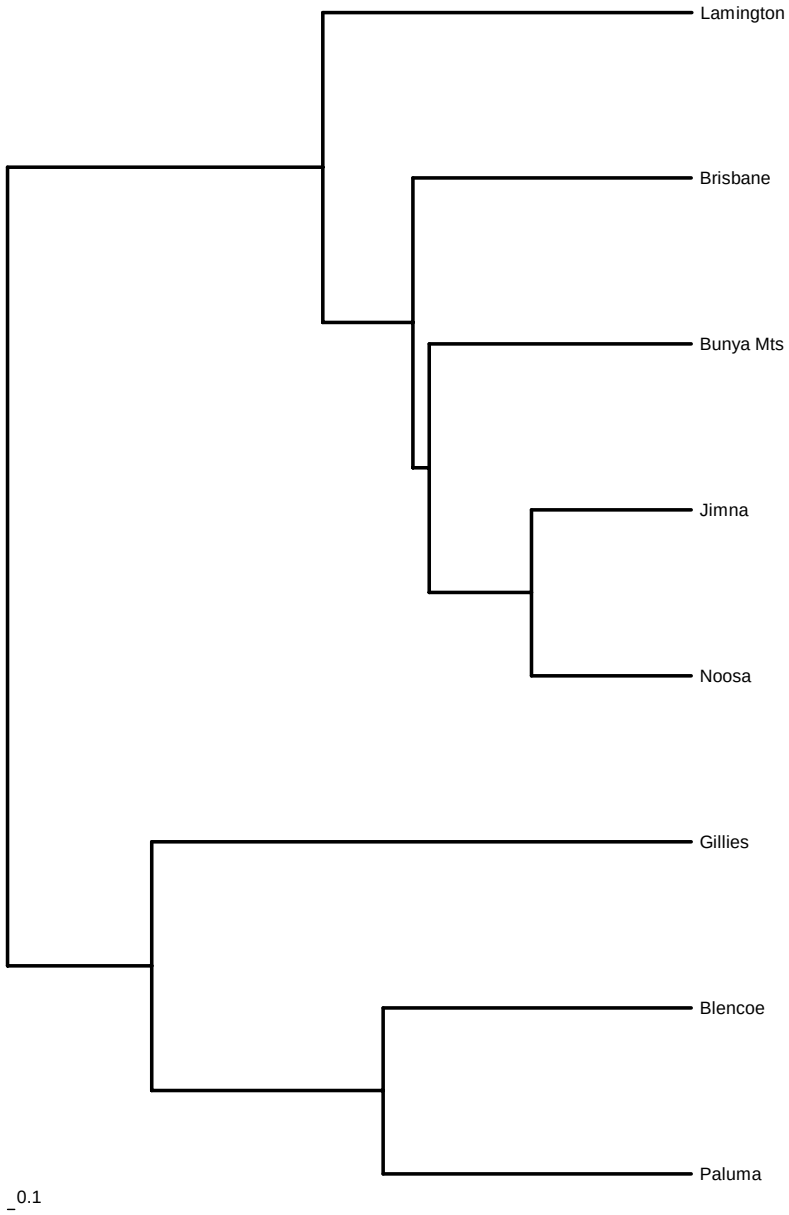

Figure 4.4. UPGMA tree derived from the RAPD data set, as displayed as a phylogram displaying proportional branch lengths, and highlighting the relative divergence among sampled populations of *A. cunninghamii.*

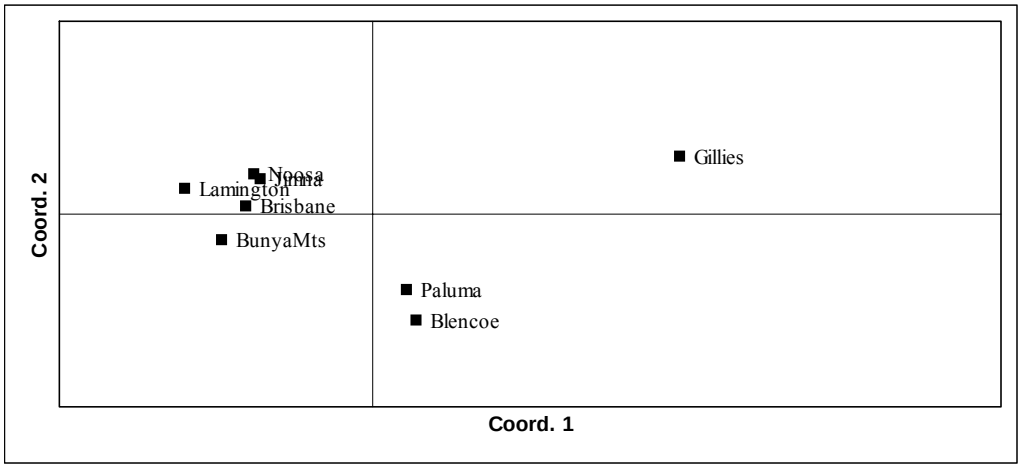

Figure 4.5. PCA ordination of pairwise Φ_{ST} values derived from RAPD data among populations of *A. cunninghamii* sampled for this study. Axis one contains 67% of the variation present, clearly separating the Gillies population from all other sampled populations. Axis two accounts for22% of the variation, and separates the southern populations from the northern populations.

For all analyses, Φ_{ST} and G_{ST} estimates are again in broad agreement. For example, the global Φ_{ST} value for all populations (20.1%) agrees with the estimate from G_{ST} (21.1%). The structure which best explained the distribution of variation among all populations sampled gave a Φ_{ST} of 19%, while that estimated using G_{ST} gave a value of 18%. Given this congruence only the results from AMOVA are discussed below, in keeping with the previous analysis.

4.3.2 ISSR reactions

4.3.2.1 Genetic diversity in A. cunninghamii as detected by ISSRs

The 6 ISSR primers chosen from the twelve screened primers (Table 4.10) produced a total of 59 polymorphic loci. As with the RAPD analysis, this also

***Table 4.10*. ISSR primer sequences used in the genetic analysis of *A. cunninghamii*.**

Name	Sequence 5' – 3'	# loci scored
807	AGAGAGAGAGAGAGAGT	11
808	AGAGAGAGAGAGAGAGC	7
809	AGAGAGAGAGAGAGAGG	9
810	GAGAGAGAGAGAGAGAT	9
811	GAGAGAGAGAGAGAGAC	11
857	ACACACACACACACACYG	12

represents a conservative proportion of the total number of loci, both variable and monomorphic, as the conservative scoring methodology described in Chapter 3 was employed. All products scored were between 300 bp and 2,000 bp in length.

From one to five monomorphic markers were produced from each ISSR primer, but these were not included in the analysis. No population–specific bands were recorded although a number of loci reached fixation in several populations. Locus 857-4 and locus 857-6 were both uniformly absent from all northern populations (fixed absence; Table 4.10). Similarly two loci, 807-11 and 809-2 displayed fixed presence in the northern populations. Locus 811-4 was present in all individuals sampled from the Paluma and Blencoe populations. Finally, locus 809-4 displayed fixation in all populations except the Gillies population where it was absent in approximately one quarter of the sampled population.

Shannon's index (S) indicated that the genetic diversity harboured in the sampled populations of *A. cunninghamii* varied from 0.29 for the Gillies population to 0.45 for the Jimna population (Table 4.11; cf. 0.28 to 0.46 from RAPDs). An ANOVA was conducted which indicated a significant difference

***Table 4.11*. Shannon's diversity index (*S*) and percent polymorphic ISSR loci (%P) for all sampled *A. cunninghamii* populations and mean values averaged over all populations and pooled species-level values. Standard errors are presented in parentheses.**

Population	***S* ISSRs**	%P
Lamington	0.4475 (0.2281)	86
Blencoe	0.3816 (0.2764)	71
Brisbane	0.3851 (0.2540)	78
Bunya Mts	0.4079 (0.2314)	88
Gillies	0.2937 (0.2705)	63
Jimna	0.4511 (0.2028)	93
Noosa	0.4190 (0.2345)	88
Paluma	0.3987 (0.2568)	78
Mean, S_{pop}	0.3947	81
Species level	0.4971 (0.1618)	100

among the populations in the diversity they contained ($F = 3.29$; $p < 0.005$). Subsequent post-hoc multiple comparison tests, employing the test of LSD, identified the Gillies population as the sole divergent population ($p < 0.05$; Table 4.12). The Gillies population displayed significantly lower levels of genetic diversity when compared to all other populations. All other populations contained a similar amount of diversity. The mean population diversity was 0.40 and the pooled species-level estimate was 0.50 (Table 4.11; cf. 0.41 and 0.53 respectively for RAPDs). The percentage of polymorphic loci varied from 63% for the Gillies population to 93% for Jimna (Table 4.11). The mean percentage of polymorphic loci across all populations was 81% (cf. 74% from RAPDs). As only

Table 4.12. Significance values from post-hoc tests from ANOVA employing the test of Least Significant Differences (LSD), which identify the Gillies population ($p < 0.05$) as the sole divergent population according to extant levels of genetic diversity as assessed by ISSRs for *A. cunninghamii*.

	Lamington	Blencoe	Brisbane	Bunya Mts	**Gillies**	Jimna	Noosa
Lamington	-	-	-	-	-	-	-
Blencoe	0.567	-	-	-	-	-	-
Brisbane	0.847	0.705	-		-	-	-
Bunya Mts	0.884	0.670	0.963	-	-	-	-
Gillies	**0.003**	**0.017**	**0.006**	**0.005**	-	-	-
Jimna	0.425	0.171	0.321	0.345	**0.000**	-	-
Noosa	0.347	0.131	0.257	0.278	**0.000**	0.888	-
Paluma	0.314	0.114	0.230	0.249	**0.000**	0.834	0.946

polymorphic bands were scored for both analyses the pooled-species level of polymorphism was 100%.

4.3.2.2 Genetic differentiation in A. cunninghamii as detected by ISSRs

Genetic structuring was investigated in the ISSR data set by AMOVA. The most parsimonious explanation of the partitioning of variance identified 6 regions: Gillies, Blencoe, Paluma, Lamington, Brisbane plus Bunya Mts, and Jimna plus Noosa. This structure best explained the distribution of ISSR phenotypes in the sampled landscape. All AMOVA values were significant ($p < 0.005$) with regional variation accounting for 13.76% of the total variation, 5.24% was attributed to the variation between populations within regions, with the remainder (81%) being retained within populations (Table 4.13; cf. 19%, 2%, 79% respectively from RAPDs).

Table 4.13. **AMOVA analysis of ISSR variation for 8 *Araucaria cunninghamii* populations. These values represent the most parsimonious explanation for the observed structuring in the ISSR data. Six regions are identified: Gillies, Blencoe, Paluma, Lamington, Jimna plus Noosa, Brisbane plus Bunya Mts.**

	d.f.*	Variance component	% of total variance	*p* value+
Among regions	5	1.40474	**13.76**	0.00000
Between populations within regions	2	0.53514	**5.24**	0.00000
Within populations	130	8.26759	**81.00**	0.00000

*Degrees of freedom

+Significance of the variance components

Collapsing all regional hierarchy gave a significant Φ_{ST} value of 18.12%, with 81.88% of the variation contained within populations ($p < 0.00001$; Table 4.14; cf. $\Phi_{ST} = 20\%$ from RAPDs).

Table 4.14. **AMOVA analysis of RAPD variation for 8 *Araucaria cunninghamii* populations. Regions were collapsed so as to identify global population structuring among the sampled populations.**

	d.f.*	Variance component	% of total variance	*p* value+
Among populations	7	1.82910	**18.12**	0.00000
Within populations	130	8.26759	**81.88**	0.00000

*Degrees of freedom

+Significance of the variance components

The structuring of both northern and southern populations was further investigated by alternately excluding each group from the analysis. Examining

the southern populations separately gave a Φ_{ST} value of 10.90% ($p < 0.000001$; Table 4.15; cf. 6% from RAPDs), with the remainder, 89.10%, contained within populations.

Table 4.15. **AMOVA analysis of RAPD variation for 5 southern *Araucaria cunninghamii* populations. Regions were collapsed so as to identify population structuring within the most abundant extant distribution of *A. cunninghamii*.**

	d.f.*	Variance component	% of total variance	*p* value+
Among populations	4	1.09629	**10.90**	0.00000
Within populations	76	8.96171	**89.10**	0.00000

*Degrees of freedom

+Significance of the variance components

The same analysis examining only northern populations gave a Φ_{ST} value of 20.01% ($p < 0.000001$, Table 4.16; cf. 23% from RAPDs), with 79.99% of the variation contained within populations. This represents a two-fold increase in divergence among northern populations relative to the southern populations.

Table 4.16. **AMOVA analysis of ISSR variation for 3 *Araucaria cunninghamii* populations in northern Queensland. Regions were collapsed so as to identify population structuring among the northern populations sampled.**

	d.f.*	Variance component	% of total variance	*p* value+
Among populations	2	1.82367	**20.01**	0.00000
Within populations	54	7.29069	**79.99**	0.00000

*Degrees of freedom

+Significance of the variance components

All pairwise population Φ_{ST} values were significant except between the Brisbane and Bunya Mountains populations (Table 4.17). A maximum value of divergence (34%) was recorded between the Gillies population and both the Brisbane and Lamington populations (Table 4.17). The smallest significant amount of divergence, 7%, was detected between the Jimna population and the Bunya Mountains. The Gillies population is as divergent from the Paluma population as it is from the Blencoe Falls population (23%; Table 4.17).

The unrooted UPGMA tree (Figure 4.6) was used to graphically illustrate the relationships among populations and regions, as determined from the ISSR data. The northern populations are separated from all southern populations, however the separation is not great. The divergence of the Gillies population is evident as a long branch, arising equidistant from the northern and southern populations, which leads away from the much shorter branches separating all other populations. The divergence of the Lamington population is also clear, being separated from the two southern groupings of Jimna and Noosa, and Brisbane and the Bunya Mountains. These results confirm the significant regional structuring of *A. cunninghamii* as determined by AMOVA.

The ISSR UPGMA tree, when displayed as a phylogram, again shows the divergence of the northern populations from the southern populations (Figure 4.7). However, in line with the unrooted representation, the divergence is not substantial, especially among the southern populations, which display much shorter branch lengths. The Gillies population, however, is once again clearly separated from all other populations. The remaining northern populations cluster away from the southern populations. Within the southern populations there is

Table 4.17. Matrix of pairwise differences between sampled populations of *A. cunninghamii* (lower diagonal) with significance values (above diagonal) calculated from AMOVA derived Φ_{ST} values from analysis of the ISSR data set. Non-significant values are highlighted in bold.

	Lamington	Blencoe	Brisbane	Bunya Mts	Gillies	Jimna	Noosa	Paluma
Lamington	-	0.000	0.002	0.000	0.000	0.000	0.000	0.000
Blencoe	0.19928	-	0.000	0.000	0.000	0.000	0.000	0.000
Brisbane	0.09683	0.15865	-	0.25988	0.000	0.000	0.001	0.000
Bunya Mts	0.08655	0.14861	**0.01259**	-	0.000	0.000	0.000	0.000
Gillies	0.34262	0.23441	0.34157	0.29591	-	0.000	0.000	0.000
Jimna	0.08645	0.19087	0.11527	0.07178	0.28692	-	0.000	0.000
Noosa	0.14661	0.23795	0.22952	0.16498	0.32788	0.07661	-	0.000
Paluma	0.22626	0.14622	0.16420	0.15922	0.22996	0.11492	0.14751	-

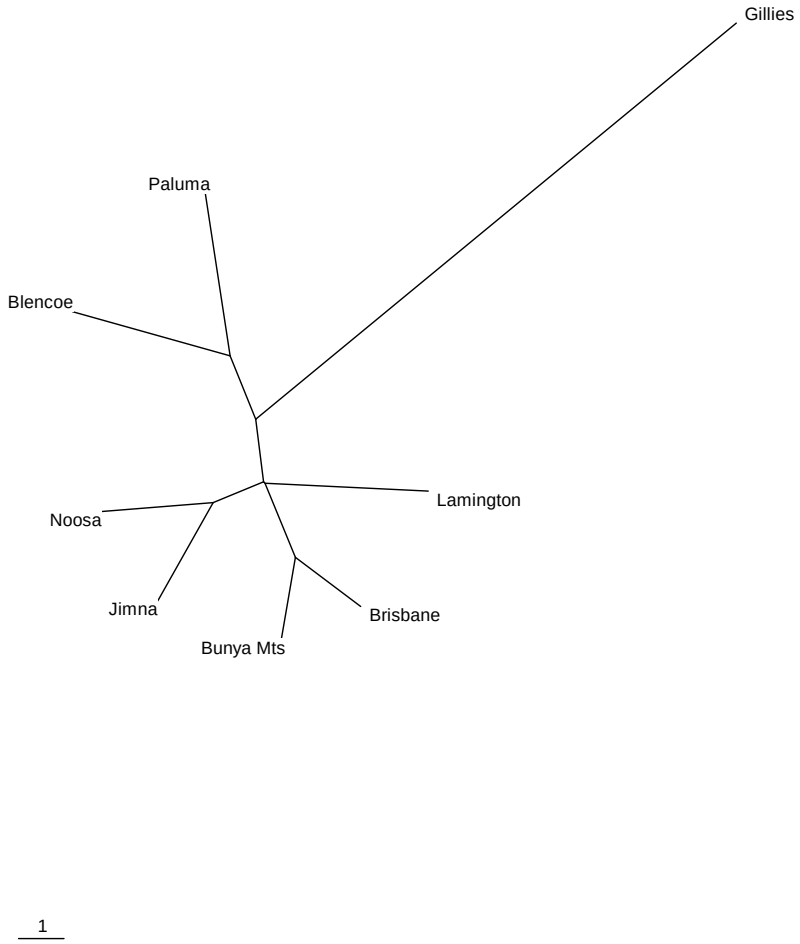

Figure 4.6. UPGMA tree, unrooted, displaying the relative divergence among sampled populations of *A. cunninghamii* as determined from the ISSR data set.

evidence to support the AMOVA results, which suggested the divergence of the Lamington population from the groupings of Jimna and Noosa, and the Bunya Mountains and Brisbane (Figure 4.7).

The PCA produced from analysis of pairwise ISSR Φ_{ST} values (Figure 4.8) supports the ISSR UPGMA tree topology. The separation of populations is over a relatively small spatial scale. However, the separation of the Gillies population from all other sampled populations is substantial and supports the topology of the UPGMA trees (Figure 4.8). The two other northern populations cluster together along the first axis, which separates northern from southern populations, and accounts for 60% of the observed variation. The separation of the southern populations along the second axis is increased relative to the RAPD results. The first three axes of this ordination explain 60%, 22% and 12% respectively (94% cumulatively).

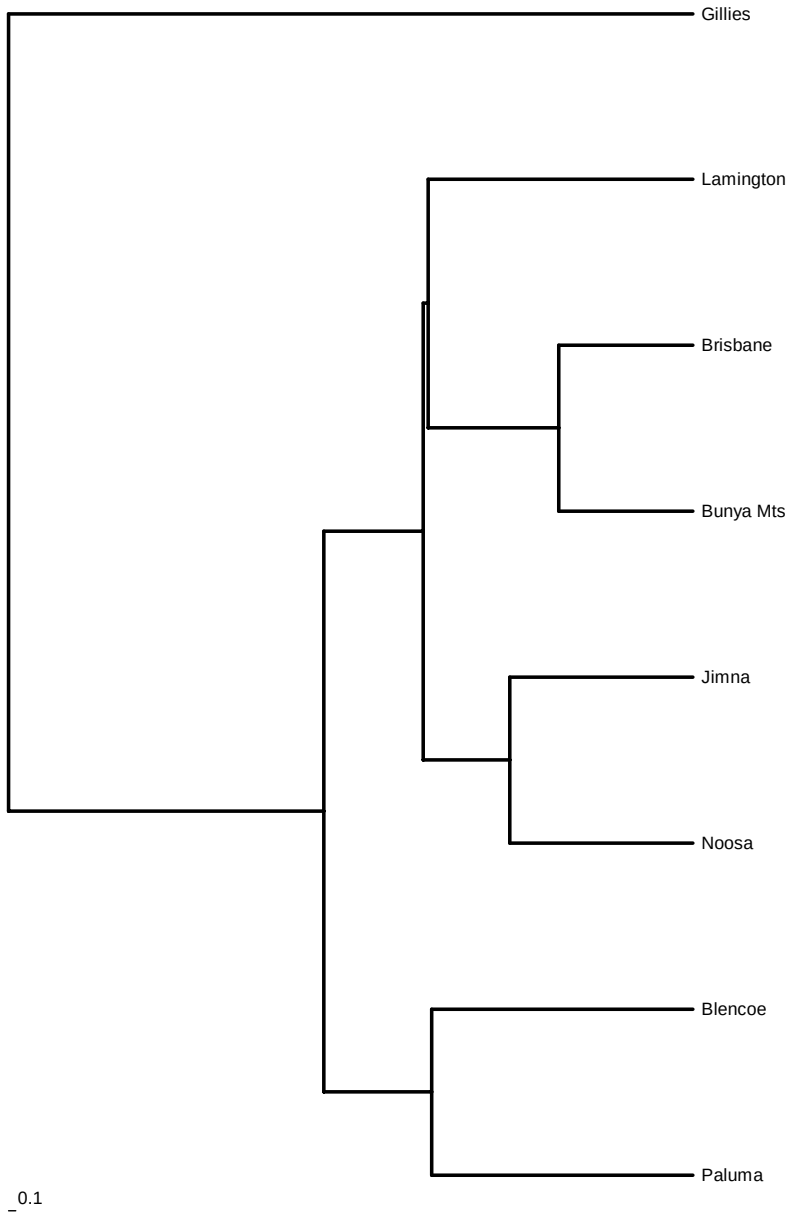

Figure 4.7. UPGMA tree derived from the ISSR data set, as displayed as a phylogram displaying proportional branch lengths, and highlighting the relative divergence among sampled populations of *A. cunninghamii.*

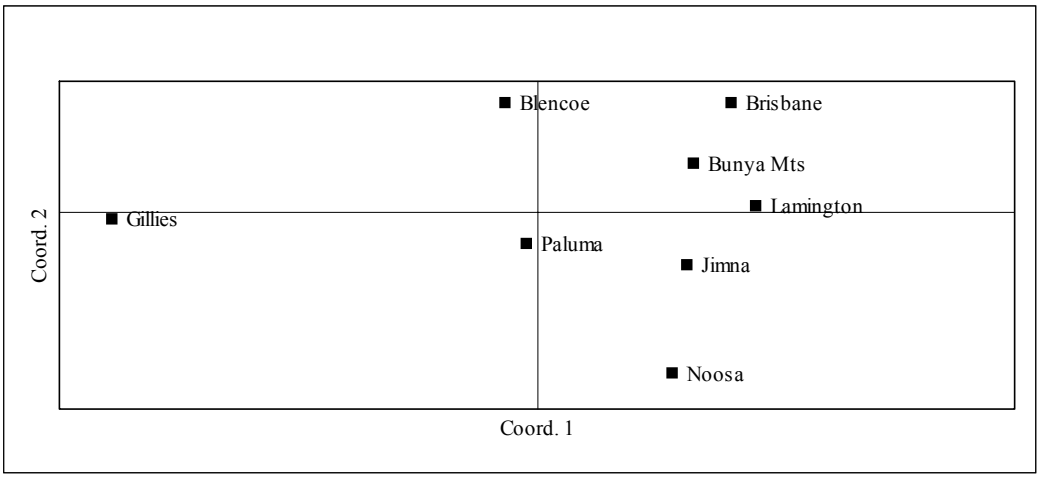

Figure 4.8. PCA ordination of pairwise Φ_{ST} values derived from ISSR data among populations of *A. cunninghamii* sampled for this study. Axis one contains 60% of the variation present, clearly separating the Gillies population from all other sampled populations. Axis two accounts for22% of the variation, and separates the southern populations from the northern populations.

4.4 Discussion

Substantial levels of genetic variation and population divergence were detected amongst the populations of *A. cunninghamii* sampled for this study. The genetic structuring of both species of Australian *Araucaria* revealed in these studies contrast with the majority of plant population genetic studies which suggest that genetic diversity within plant populations is negatively correlated with the degree of population divergence (Nybom and Bartish 2000).

4.4.1 Genetic diversity in A. cunninghamii

Genetic erosion has been cited as a likely outcome of small population size in species that formerly had more extensive distributions (Pluess and Stöcklin 2004). However despite conforming to this model, genetic diversity in *A. cunninghamii* is moderate and only slightly lower than those reported in RAPD studies for other Southern conifers with similar life-history traits - *Fitzroya cupressoides*, S = 0.54 (Allnutt *et al*. 1999); *Araucaria araucana*, S = 0.65 (Bekessy *et al*. 2002). The estimates of genetic diversity from both RAPD and ISSR analyses in *A. cunninghamii* are in very close agreement (S [ISSR] = 0.40, S [RAPD] = 0.41).

The ANOVA detected significantly lower levels of diversity in the Gillies Range population. All other populations contained similar levels of diversity, which suggests that the Gillies population may represent an anomaly among extant Hoop pine populations, and indeed among Australian *Araucaria* populations. Other populations show similar degrees of geographical isolation (e.g. Blencoe, Bunya Mountains, Noosa) or similar extant populations sizes (e.g. Brisbane, Paluma) to the Gillies population, yet appear to have maintained similar levels of genetic diversity to comparatively more extensive and connected populations. Low genetic diversity may be brought about by a number of evolutionary processes associated with small population size. However, small population size does not seem to be the most parsimonious explanation for the distribution of genetic diversity, since all northern populations are effectively small and isolated, were not heavily logged and therefore should have encountered the same historical, evolutionary pressures. It is possible that the significantly lower diversity in the Gillies population is a result of a genetic

bottleneck caused by a founder effect with subsequent high levels of inbreeding and/or restricted gene flow. As the use of dominant markers precludes the quantification of heterozygosity, the hypothesis of inbreeding requires further investigation with suitable co-dominant molecular markers, such as microsatellites, as they become available. If this low diversity is a result of a founder effect then this population should display a close genetic association with its source population, however the above results suggest it does not. This will be investigated below.

In any case, the results from Forestry trials indicate that this small, isolated, genetically depauperate population represents a substantial resource for the future of Australia's forestry industry (Dieters *et al*. 2002). Inter-provenance trials, which have hybridised the Gillies population with individuals from Jimna, display increased vigour – earlier and more sustained growth (Dieters *et al*. 2002). These results, together with the detection of significantly lower levels of genetic diversity, indicate that this population requires a different model of management and conservation than is applicable to other populations of *A. cunninghamii* sampled in this study. The importance of the conservation of this small population is emphasised when the number of individuals (~20) responsible for the potential increase, through inter-provenance hybridisation (Dieters *et al*. 2002), in a $ 23 billion economy is considered. These individuals are in an extremely fire-prone area, which is burned almost annually, due to its proximity to a highway and the surrounding sclerophyll vegetation. Thus the potential for complete loss of this unique genetic resource is high, without appropriate conservation measures being undertaken. The most cost-effective option would

be to establish an ex-situ plantation, derived from cuttings from the remaining individuals, thereby ensuring the future of this potentially massive economic resource. This plantation should of course be situated as far away as possible from all extant populations to minimise the potential for genetic pollution of divergent provenances as determined in this study.

There appears to be no evidence of widespread genetic depletion in *A. cunninghamii*. Supporting this is the fact that the percentage of polymorphic RAPD markers was also high regardless of the population size or isolation, with the exception of the Gillies population. Polymorphism was greater in the ISSR data set than in the RAPD data set, which agrees with the few comparative studies conducted (Nybom 2004), and also concurs with the results from the previous chapter. Across molecular markers, there was no trend of increasing polymorphism with increasing population size, as would be expected from theoretical population genetic models (Hamrick and Godt 1996). For example, the small, isolated population at Noosa displays similar (ISSRs) or higher (RAPDs) values of polymorphism than the much more extensive population in the Bunya Mountains (Tables 4.3 and 4.11). For both data sets, Jimna recorded the highest levels of polymorphism, which probably reflects the historical abundance of the species in this area as opposed to its currently restricted distribution in the area due to an intensive logging regime. Comparisons across studies are not made here, due to the inherent subjectivity of primer choice to maximise polymorphic loci and the inclusion, in some studies, of monomorphic loci (see Chapter 2).

Small endemic populations are expected to be susceptible to the loss of genetic variation by genetic drift (Frankham 1996). However, the results

presented here, together with those from the preceding chapter indicate that these conifers may maintain substantial amounts of genetic diversity in very small populations. For conifers, the lagging effects of extremely long generation times may mean that the genetic effects of relatively recent size reductions (i.e. anthropogenic exploitation over the past two centuries) are not yet detectable. Populations that are marginal, small and/or geographically isolated (in this case all sampled populations of *A. cunninghamii*) are expected to be more susceptible to the effects of genetic drift due to lower effective population size and/or increased selection pressures. However, historical gene flow may have been sufficient to negate these forces, at least among the southern populations which have been recorded to be more continuous, prior to European exploitation, than the northern populations (Petrie 1904).

These results may also suggest that effective population size in *A. cunninghamii* may be independent of actual census size, as was suggested in the Bunya study. However again, more likely is the scenario that current population sizes are of insufficient age to allow a genetic equilibrium to be reached and therefore any reduction in genetic variability due to the effects of small contemporary population size remains undetectable at this stage. This is especially the case for those populations where recent exploitation has severely reduced the size of the population (e.g. Brisbane). We can say that historical population size fluctuations, associated with climatic variability (Kershaw and Wagstaff 2001) do not appear to have affected the genetic diversity of any of these populations, with the potential exception of the Gillies population. Within-population diversity has most likely been maintained by distributing the variation

temporally across a range of age classes in this long-lived species. In doing so, small populations may be able to maintain similar levels of genetic variation to larger populations for substantial periods of time.

The long generation times, inherent in the Araucariaceae, appear to act to distribute genetic variation temporally within *A. cunninghamii* via the maintenance of a range of age classes in most populations. The spatial distribution of this genetic variation will obviously be governed by a balance of population divergence and gene flow and is examined below.

4.4.2 Regional differentiation in A. cunninghamii

Both data sets identified a high degree of differentiation among the populations of *A. cunninghamii* sampled. The hierarchical structure that best explains the partitioning of variation for both the RAPD and ISSR data (Tables 4.5 and 4.13) provides evidence for six distinct regions: Gillies, Blencoe, Paluma, Lamington, Brisbane plus Bunya Mts, and Noosa plus Jimna. These congruent results, which are also supported by the UPGMA and PCA figures, mirror the extant geographic locations of these populations but also reflects the historical association of these populations. For example, the grouping of Brisbane plus Bunya Mts, and Noosa plus Jimna, is also reflected in non-significant pairwise population divergence values from the RAPD data set for both groups, and the ISSR data set for the former group only. Historical records suggest that these populations, prior to European settlement, formed continuous tracts of Araucarian forest, and as such their extant isolation is the product of contemporary exploitation which is not expected to be reflected in the historical genetic signal. The effects of this recent

exploitation may be investigated through the assessment of the progeny of these populations, and is discussed further in Chapter 5.

The substantial differentiation of the Gillies population in both data sets, as well as the aforementioned significant reduction in diversity in this population, appears to represent an anomaly among the populations sampled. These results suggest that the isolation of this population may have preceded that of any other population sampled, and most likely reflects the initial effects of small population size on genetic integrity, as predicted by genetic theory. Given enough time, all populations may be similarly affected, as is the case in *Wollemia nobilis* (Peakall *et al*. 2003). The other northern populations show a greater affinity with the southern populations than they do to this population, which suggests that low diversity along with genetic drift and potential inbreeding has lead to its extreme divergence. Given the congruence in life-history traits, habitat and environmental pressures that the northern populations have encountered, this result is surprising and requires further research with markers suitable for assessing levels of homozygosity. It appears highly likely that this population is suffering genetic erosion, along with subsequent genetic drift, due to the effects of small population size, most likely manifested through an older isolation event than all other populations sampled. The broad regional differences detected likely resulted from historical processes that occurred from the Late Pliocene onwards, and have been enforced by comparatively recent (Pleistocene/Holocene) selective pressures and barriers to gene flow. This will be further discussed in the chapter 6.

Regardless of the molecular marker employed, pairwise differences between populations indicate that most sampled populations are significantly

differentiated from each other (Tables 4.9 and 4.17), thereby emphasising the high degree of population structuring in *A. cunninghamii*. Significant differentiation occurs between populations separated by as much as 2000 kms (e.g. Blencoe to Lamington) or as little as 50 kms (Lamington to Brisbane) and is independent of the degree of the current isolation of the populations.

Once again, the resolution of the markers in detecting population differentiation differed, however this difference was predominantly confined to the values between northern and all southern populations. In other words, this discrepancy occurred mainly over large geographical ranges. RAPDs detected substantially more differentiation between northern and the southern population (18% - 42%) than did the ISSRs (12% - 34%). Pairwise values among the southern populations from the different markers were broadly congruent, and ranged from 4% to 12% for RAPDs (Table 4.9) and 7% to 17 % for ISSRs (Table 4.17).

From historical records (Petrie 1904), it seems that a more or less continuous araucarian forest connected the coast to the Bunya Mts, over which a mixing of genotypes may be expected. Loss of intervening habitat and intensive logging over the past few centuries may simply have left in situ genotypes that are more closely related than present geographic 'isolation' of these populations would suggest. This pattern is more exaggerated for *A. cunninghamii* than *A. bidwillii* as the former species was far more extensively exploited in the early settlement of southeastern Queensland (Petrie 1904).

In either case, due to distance alone, the potential for pollen-mediated gene flow would be expected to be greater among historical southern populations than

between northern and southern populations. Thus the present 'isolation' of populations appears to be the product of different temporal events, recent versus historical, and this is reflected in the higher values of divergence amongst the northern populations relative to the southern populations. The different origins of population isolation are also interesting when considering the potential impacts on genetic variation. As previously shown, there was no significant difference in the variation detected between these populations (with the exception of the Gillies population), which adds further support to the theory of the temporal maintenance of genetic diversity in small populations of long-lived southern conifers. These results suggest that *A. cunninghamii* may be able to buffer the deleterious effects of small population size for substantial periods, as long as a range of age classes is maintained. Furthermore, the extant isolation of the northern population is reflected in values of population divergence, which suggests these populations have been isolated for some time (cf. southern populations).

It should be noted that the inclusion of central Queensland populations and those from off-shore islands in future research may be important in increasing our understanding of the patterns of intra-regional differentiation among *A. cunninghamii* populations.

4.4.3 Differentiation among the southern populations of A. cunninghamii

When AMOVA analyses of the were restricted to southern populations only, the value of population differentiation (Φ_{ST}) was 6% for RAPDs (Table 4.7), which is substantially lower than values from RAPD studies of other southern conifers with

similar pollination and dispersal mechanisms (e.g. *Araucaria araucana*, Φ_{ST} = 12%, Bekessy *et al*. 2002).

A surprising result from this study was the significant structuring found between most populations, regardless of size or degree of isolation. As previously mentioned, this pattern was detected over most spatial scales, from as few as 50 kms to as much as 2,000 kms. Wind-pollinated conifers are expected to show low differentiation, especially over shorter distances, as enhanced gene flow acts to negate differentiation. The possibility remains that seed dispersal is restricted and is the main factor in determining genetic differentiation of these populations, through fewer opportunities for inter-population dispersal. However, as previously mentioned, recent studies (reviewed in Koenig and Ashley (2003)) suggest that further research is required on the dispersal limitations of wind-pollinated trees.

Studies of pollen dispersal in the genus are few, but a recent study of pollination in *Araucaria angustifolia* showed that the large non-saccate pollen of this species does not travel great distances (Sousa and Hattemer 2003). The pollen grains of this species, and presumably the genus, are significantly larger than other anemophilous species (~ 61.5 μm). The emergent habit is obviously advantageous in facilitating pollen distribution, however in a dominant stand only particularly old specimens may emerge above the 'emergents'. Given the logging history of the sites, there is also the possibility that each population was preferentially pollinated by a few emergent 'donor' individuals, which would act to increase local divergence. As only mature individuals were sampled for this analysis this signal is historical and will necessarily reflect the pre-European structure of the forests. More research is required specifically for the

characteristics of pollen and its dispersal in *A. cunninghamii*, and the implications of these on extant genetic structuring after an intensive logging regime.

CHAPTER FIVE

COMPARISON OF NUCLEAR DNA DOMINANT MARKERS FOR THE DETECTION OF GENETIC DIVERSITY AND DIVERGENCE IN *ARAUCARIA*.

This chapter contrasts the results gained from RAPDs and ISSRs in the preceding chapters to assess the utility of these markers in assessing the genetic diversity and divergence in *Araucaria* in Australia. In doing so, an understanding of the underlying population genetics of species of Australian *Araucaria* can be greatly enhanced, and the utility of the molecular markers themselves can be directly contrasted. Furthermore, these results can now be placed within a global framework of comparable studies which have utilised the same markers (i.e. RAPDs) and have analysed the results in a comparable fashion. It is only through a meaningful comparison across a range of taxa with similar life-histories that our understanding of the population dynamics of southern conifer can be evaluated and increased. From this, the broad aims of the thesis can then be addressed in terms of a global response to similar environmental pressures.

5.1 Contrasting molecular markers

Although both molecular markers used in this study are now widely employed in population genetics there are few studies that have directly compared the genetic signal detected using ISSRs and RAPDs in native plant populations. While RAPDs have been the most popular molecular tool for the investigation of plant

population genetics (Nybom and Bartish 2000), ISSRs have only more recently been employed for this purpose. ISSRs are now beginning to be used in the analysis of genetic structuring in natural populations (Hollingsworth *et al*. 1998b), however they have been most widely employed in the assessment of the relationships between cultivars, and crop species (e.g. Nagaoka and Ogihara 1997; Lai *et al*. 2001; Fernández *et al*. 2002; Galván *et al*. 2003; Vijayan 2004) as well as the assessment of taxonomic relationships among species (e.g. Mattioni *et al*. 1999). There are some studies that have employed both markers, however these have been largely confined to the assessment of crop species, which may be expected to have been dramatically modified, both morphologically and genetically, over the past centuries. While studies of native plant populations using ISSRs are now becoming more frequent (e.g. Wolfe *et al*. 1998; Baumel *et al*. 2001), there are few comparative studies using ISSRs and RAPDs independently for the investigation of native plant populations. This is reflected in a recent summary analysis of nuclear DNA markers in plants (Nybom 2004) where there were too few ISSR studies to allow a full statistical evaluation of correlations of genetic diversity and divergence with life-history traits. However emerging trends were noted and are discussed further below and are placed within the context of the results presented in this thesis.

This chapter examines both the congruence and the lack thereof between these markers in the genetic assessment of long-lived native *Araucaria* over a wide geographical area in eastern Australia. Direct comparisons with other studies must be conducted cautiously as the history and selection of crop cultivars may have produced differential selection pressures, resulting in different patterns

of diversity and divergence, to that which has occurred in native plant populations.

There is also a tendency in previous studies to combine the data sets derived from these two molecular markers (e.g. Hollingsworth *et al*. 1998b; Clausing *et al*. 2000; Hess *et al*. 2000). While theoretically there is good evidence to allow these data sets to be combined, including their dominant nature, and random and abundant distribution of these markers throughout the genome, few studies assess the goodness of fit or correlation between the data sets before combining them (e.g. Clausing *et al*. 2000; Hess *et al*. 2000). Furthermore, by combining data sets prior to separate analyses, any discrepancy or differential patterns in either marker may be lost. Given the high correlation of the data sets in this study, and their broad congruence in detecting genetic diversity (see below), data sets were not combined, which provides an opportunity for the comparison of two independent assessments of genetic diversity. In treating the data sets independently some important trends have been revealed in this study, which mirror the few comparative studies that have been conducted to date using these two molecular markers independently.

5.2 Estimation of genetic diversity in *Araucaria* using RAPDs and ISSRs.

The sampling structure employed in this study allows direct comparisons to be made between the two species regarding genetic structuring and its effects on genetic variation. Within this comparable range, both species are expected to have experienced similar historical pressures and are expected to display similar levels of variation and divergence, as a consequence of their shared life histories.

Both ISSRs and RAPDs proved to be useful and efficient indicators of genetic diversity in both *A. bidwillii* and *A. cunninghamii*. This is revealed in a direct comparison of data sets for each species, which displayed high levels of correlation (*A. cunninghamii*, $r = 0.9$, $p < 0.05$; *A. bidwillii*, $r = 0.8$; $p < 0.05$) and produced very similar estimates of diversity (*A. cunninghamii*, S [RAPD] = 0.41, S [ISSR] = 0.40; *A. bidwillii*, S [RAPD] = 0.48, S [ISSR] = 0.50). These results concur with the few comparative studies to date using these markers, in that both markers detected similar levels of variation (e.g. Mattioni *et al*. 2000; Qian *et al*. 2001; Nybom 2004). This congruence is also predicted by molecular theory which suggests these markers are 'neutral' and randomly sample a large portion of the genome (Williams *et al*. 1990; Zietkiewicz *et al*. 1994).

The percentage of polymorphic markers differed slightly between ISSRs and RAPDs for both species. In both species, increased polymorphism was detected from ISSRs relative to RAPDs. For *A. bidwillii* this ranged from 84% using RAPDs to 92% using ISSRs. The same trend was evident in the analysis of *A. cunninghamii*, with 74% polymorphism detected using RAPDs, and 81% using ISSRs. This trend has also been noted in previous studies (Nagaoka and Ogihara 1997; Lai *et al*. 2001; Qian *et al*. 2001; Fernández *et al*. 2002; Galván *et al*. 2003; Vijayan 2004), and is congruent with the present results.

Previous studies have explained this discrepancy as being due to functional constraints associated with RAPD loci (Esselman *et al*. 1999) and/or a faster evolutionary rate in ISSRs, including a higher abundance and higher polymorphism of microsatellites in the plant genome (Jain *et al*. 1999; Qian *et al*. 1999; Lai *et al*. 2001). Further research is required to elucidate this slight

discrepancy in the degree of polymorphism each marker detects, especially given the above tendency for data sets to be combined prior to testing for combinability.

5.3 Estimation of genetic divergence in *Araucaria* using RAPDs and ISSRs.

The major discrepancy between the molecular markers, as revealed in the analyses of *Araucaria*, lies in the degree of divergence each marker attributes to that occurring over wide geographic distances. The patterns detected by both markers, as assessed by AMOVA, were completely congruent and identified 5 regions for *A. bidwillii*, and 6 regions for *A. cunninghamii* across the populations sampled. The discrepancy between the markers lies in the degree of divergence they attribute to regional differentiation relative to that occurring within populations or among populations within a region. This trend is clearly evident in a comparison of the unrooted UPGMA trees derived from both species (Figure 5.1).

For example, the AMOVA of *A. bidwillii* using RAPDs attributed 21% to that occurring among regions, while AMOVA using ISSRs attributed only 7% to regional differentiation. ISSRs tended to distribute more variation within populations (90%) in *A. bidwillii*, relative to RAPDs (75%). This trend was also mirrored in the analysis of *A. cunninghamii*, however the discrepancy was of a lesser degree. AMOVA analyses of RAPD data attributed 19.11% of the variance to that among regions, 5.24% to between populations and 78.62% to within population differentiation. In comparison, ISSRs attributed 13.76% to regional differentiation, 5.24% to between population differentiation and 81% to within population divergence.

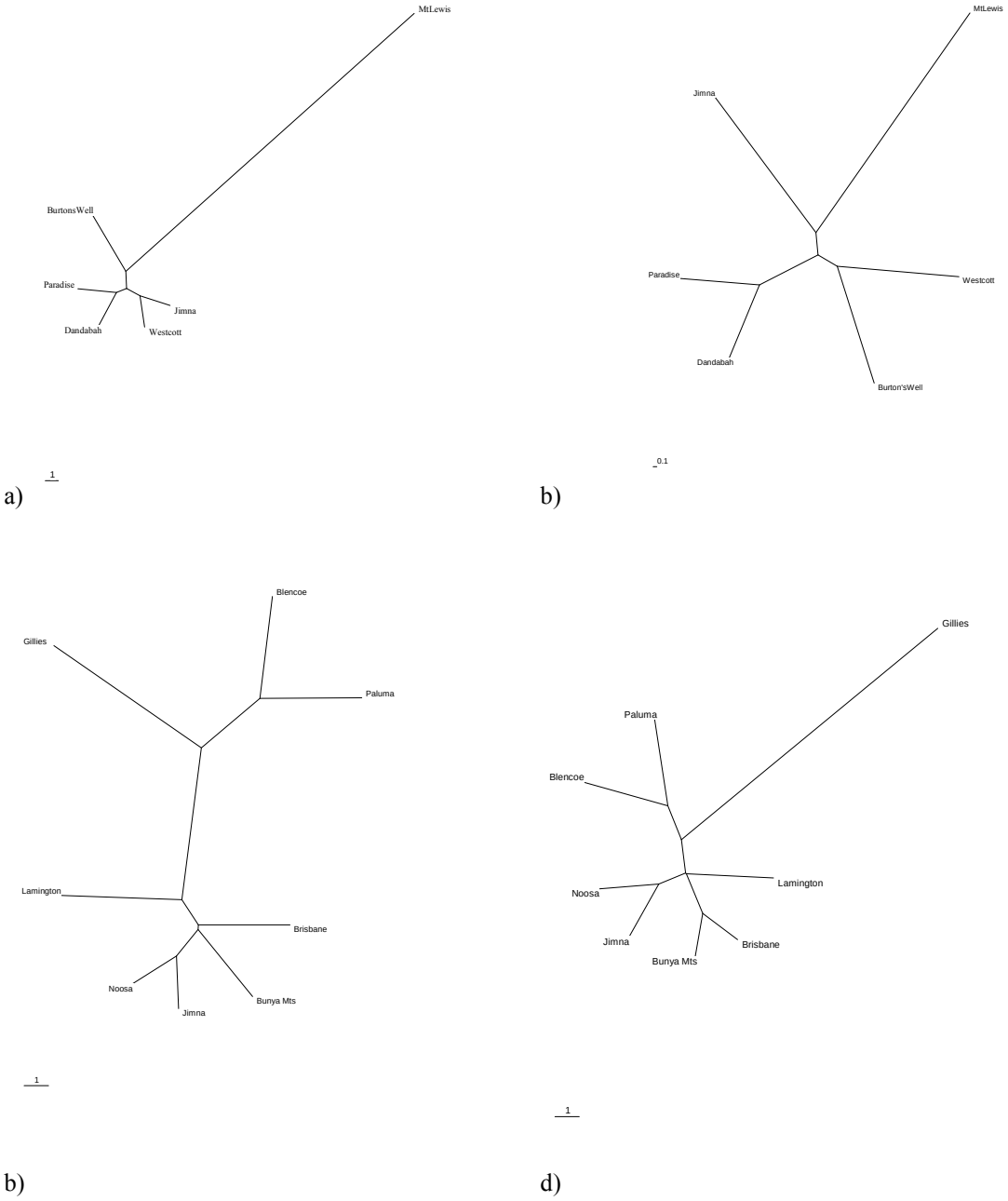

Figure 5.1. Comparison of molecular marker resolution in species of Australian Araucaria as displayed by unrooted UPGMA trees. a) derived from RAPD analysis of *A. bidwillii*. b) derived from ISSR analysis of *A. bidwillii*. c) derived from RAPD analysis of *A. cunninghamii* d) derived from ISSR analysis of *A. cunninghamii*. Branch lengths and scale bars illustrate the discrepancy of these markers when assessing genetic diversity in *Araucaria* over large geographical areas.

Values of pairwise population differentiation were for *A. bidwillii* were similar between markers for the southern populations but differed substantially in the degree of differentiation detected between Mt Lewis and the southern populations. This is clearly illustrated when comparing the branch lengths in the UPGMA figures (Figure 5.1). Similarly, AMOVAs testing the grouping of northern versus southern populations were significant for the RAPD data but not significant for the ISSR data (see chapter 3).

Unfortunately the trend to combine data sets from ISSRs and RAPDs in natural plant populations precludes additional interpretations of this trend until further studies are conducted. However from this and previous studies, it appears likely that the higher polymorphism associated with ISSRs means that their utility may be confined to closely related populations when assessing geographic and genetic structuring over large geographical distances. Nybom (2004) noted that ISSRs tend to over-emphasise differences between closely related populations, and attribute less of the variation to difference over large geographical distances (compared to RAPDs, AFLPs and microsatellites). It was also noted that ISSRs tended to attribute more of the variation to within populations relative to RAPDs and AFLPs (Nybom 2004). These trends were confirmed in the present study of *Araucaria* populations. The reasons for this remain unclear, but could be potentially related to differences in the rate of accumulation of mutations in ISSR microsatellite priming sites relative to that at RAPD priming sites, especially under a scenario of long-term historical isolation. In comparison to RAPDs, homology was difficult to ascertain in several of the banding profiles obtained from ISSRs for *Araucaria* due to small variations in fragment size. These bands

were not scored and may also attribute to the lack of resolution at larger scales, as many of these loci appeared to be population-specific.

The larger discrepancy between the markers in the AMOVA of *A. bidwillii* relative to those detected in *A. cunninghamii* may be a result of their historical and extant distribution. The potential taxonomic distinction of northern and southern populations of *A. bidwillii*, as noted in chapter 3, may have exacerbated this discrepancy. Northern and southern populations of *A. bidwillii* are widely separated and have presumably been isolated for some time. On the other hand, populations of *A. cunninghamii* are more continuous which may act to lessen overall population divergence via a stepping stone effect of gene flow. This theory could be further examined through additional sampling of the numerous intervening populations of *A. cunninghamii* in central Queensland which were not the focus of the present study.

Accordingly, the utility of ISSRs in the assessment of genetic structuring of plant populations over large distances may be compromised. However once again reflecting the paucity of comparative studies of natural plant populations using ISSRs, only one other study has reported the tendency of ISSRs to underestimate divergence over large geographical areas (i.e. Qian *et al*.2001). Further comparative studies of natural plant populations utilising these molecular markers over a range of geographic distances will be required to elucidate this trend. These results highlight the importance of the use of multiple molecular markers in the assessment of genetic diversity and divergence in plant populations.

5.4 Comparisons with previous molecular studies of Southern Conifers

In a summary article of genetic structuring in plants, G_{ST} (analogous to Φ_{ST}) was found to be significantly correlated with breeding system, life cycle, stage of succession, and floral morphology (Loveless and Hamrick 1984). Outbreeding, wind pollination, late successional status and long life cycles are all expected to encourage gene flow and inhibit population differentiation (Hamrick and Godt 1996, Nybom and Bartish 2000), thereby maintaining the majority of diversity within populations. Gymnosperms, albeit a paraphyletic concept, are further expected to display low Φ_{ST} / G_{ST} values based on averages from previous studies (G_{ST} = 7%; Nybom and Bartish 2000).

The mean values of 'S' obtained for *A. cunninghamii* from the RAPD and ISSR analyses, 0.41 and 0.40 respectively, are only slightly lower than values reported for other southern conifers using dominant molecular markers (Table 5.1). Similarly, the mean values of 'S' for *A. bidwillii* generally concur with the values recorded for southern conifers (Table 5.1).

Table 5.1. Mean values of genetic diversity recorded for southern conifers from the analysis of RAPD data, as determined by Shannon's diversity index (S),

Species	Mean value of RAPD diversity (S)	Author
Araucaria bidwillii	**0.48**	**This study**
Araucaria cunninghamii	**0.41**	**This study**
Araucaria araucana	0.65	Bekessy *et al*. 2002
Fitzroya cupressoides	0.54	Allnutt *et al*. 1999
Pilgerodendron uviferum	0.57	Allnutt *et al*. 2003
Podocarpus salignus	0.69	Allnutt *et al*. 2001

The ranges of values of genetic diversity detected in this study also fall within those previously reported for southern conifers (Figure 5.2). Thus there appears to be no evidence of widespread genetic depletion in the sampled populations of *A. cunninghamii* and *A. bidwillii*, despite potential fluctuations in their distribution and abundance (Kershaw and Wagstaff 2001).

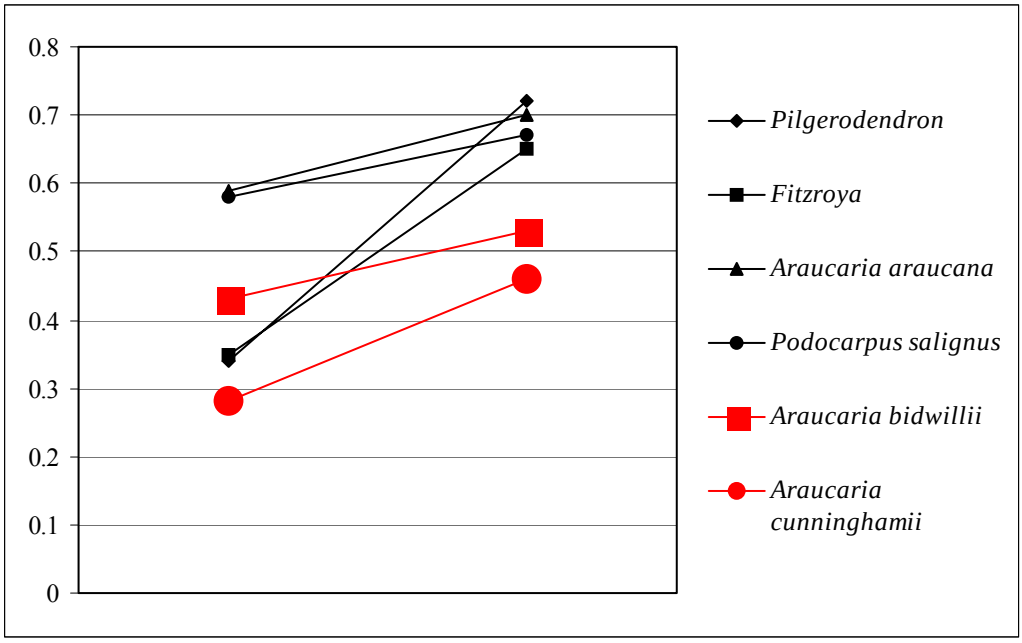

Figure 5.2. Ranges of values of 'S' recorded for southern conifers from RAPD analyses. Values recorded for *Araucaria araucana* range from 0.59 to 0.7 (Bekessy *et al.* 2002); *Podocarpus salignus* ranges from 0.58 to 0.67 (Allnutt *et al.* 2001); *Fitzroya cupressoides* ranges from 0.35 to 0.65 (Allnutt *et al.* 1999); *Pilgerodendron uviferum* ranges from 0.34 to 0.72 (Allnutt *et al.* 2003). The values recorded in this study for *A. bidwillii* and *A. cunninghamii* are highlighted in red.

The high degree of significant population differentiation in *A. bidwillii* is surprising for a wind-pollinated, long-lived conifer as all of these traits (breeding, system, longevity and taxonomic status – gymnosperm vs angiosperm) are expected to minimise differentiation among populations (Nybom and Bartish 2000).

When AMOVA analyses of the RAPD data were restricted to southern populations of *A. bidwillii*, the value of population differentiation (Φ_{ST}) was 12%, which is in close agreement with range-wide values from RAPD studies of other southern conifers with similar pollination and dispersal mechanisms (*Araucaria araucana*, Φ_{ST} = 12%, Bekessy *et al*. 2002; *Fitzroya cupressoides*, Φ_{ST} = 14%, Allnutt *et al*. 1999; *Pilgerodendron uviferum*, Φ_{ST} = 18%, Allnutt *et al*. 2003). The ISSR value was slightly lower at 7.4%. Comparing these results in a global context, the RAPD AMOVAs, together with the UPGMA and PCA figures derived from the RAPD data, suggest that the Mt Lewis population of *A. bidwillii* is a distinct evolutionary lineage.

The global estimates of population divergence for *A. cunninghamii* (20.25%, RAPDs; 18.12% ISSRs) are also substantially higher than the average value of 7% estimated across a range of gymnosperms (Hamrick and Godt 1996), which would suggest a lack of historical connectivity between most of the sampled populations. These results also highlight the need for caution when applying genetic theories across broad taxonomic groups with a range of differing life-histories, such as the paraphyletic concept of 'gymnosperms'.

Studies of conifers from the Northern Hemisphere constitute the majority of population statistics relating to generalised "gymnosperm" values, so further research is also required on the enigmatic group of conifers now confined to southern latitudes, to ascertain why they are so inherently different with regard to population dynamics and/or their response to past environmental pressures.

5.5 Project aims revisited

Having contrasted these results with the current available genetic data for southern conifers, the broad aims of the project can now be specifically addressed. To reiterate the following questions were addressed in this thesis:

1. *Given the dynamic history of Araucaria in Australia what levels of diversity do extant populations of A. bidwillii and A. cunninghamii maintain? In other words, has the evolutionary potential of these species, or particular populations, been compromised through historical range reductions and fragmentation?*

It appears from these results that both species have been able to maintain substantial amounts of genetic diversity despite a long history of range contractions and fluctuations in their abundance. There is no evidence for any widespread genetic erosion, however the significantly lower genetic diversity recorded in the Gillies population suggests that all populations may not respond in the same way to similar environmental pressures. Despite the slight reduction in diversity in *A. cunninghamii*, and the agreement in values across molecular markers and its congener *A. bidwillii*, there appears to be no evidence of a widespread dramatic reduction in genetic diversity among members of the Australian Araucariaceae, as suggested by Peakall *et al*. (2003). As previously mentioned, the study by Peakall *et al*. (2003) used different molecular methodologies and sampling strategies in their comparisons of diversity among *Araucaria cunninghamii*, *Agathis robusta* and *Wollemia nobilis*, which may partially explain the conflicting results. Given the utility of ISSRs and RAPDs in

detecting diversity and divergence in this study among individuals, populations and the species of Australian *Araucaria*, these markers may offer a more sensitive technique for the detection of diversity in other related taxa. These include the highly restricted *Wollemia nobilis* where the detection of any substantial diversity has proved elusive (Peakall *et al*. 2003), as well as the taxonomy of *Agathis,* which has thus far been unresolvable (Setoguchi *et al*. 1998).

It should be noted, however, that given the historical reduction in range and abundance of this genus that reduced genetic diversity, comparative to what was originally present, is likely to have occurred across most members of the genus.

2. *Can the level of genetic diversity within a population be predicted from extant population size?*

Once again, with the exception of the Gillies population there appears to be no evidence for decreased genetic diversity in smaller populations relative to larger ones. Contemporary exploitation of these species has probably left *in situ* a sub-sample of the genetic diversity that was previously present and may partially explain the high levels of diversity recorded for what are now small and isolated populations (e.g. the Brisbane population of *A. cunninghamii*). Thus genetic diversity cannot be predicted from extant population size in these species. Future management of these populations should not use population size as an indicator of the conservation 'worth' of a given population.

3. *Do these populations form a cohesive unit (metapopulation) or does significant genetic structuring exist?*

Surprisingly, a high amount of genetic structuring was detected in both species, which appears initially to contradict genetic theories regarding the cohesiveness of conifer populations (Hamrick and Godt 1996). However, as will be discussed in the following chapter, it is highly likely these species have been subject to a substantially different history of fragmentation than the conifers of the Northern Hemisphere. A longer history of fragmentation events has most likely lead to increased divergence among populations of these southern conifers. These concepts are explored further in the following chapter.

CHAPTER SIX

RECONCILING HISTORICAL MICROFOSSIL AND GENETIC SIGNALS IN *ARAUCARIA*.

An understanding of the distribution and degree of extant genetic variation in these species is imperative if we are to disentangle the generalised pattern provided by the micro-fossil record. Given the data provided in the preceding chapters, interpretation of these patterns can now be more directly assessed. Furthermore, as the study of the genetic variation in southern conifers is in its infancy, and has been identified as an IUCN priority, comparative data is essential in deriving meaningful interpretations of the population dynamics of southern conifers, not only on this continent but also across the Southern Hemisphere.

6.1. The fossil record and its relationship to the genetic assessment of extant species of *Araucaria*

The fossil record of *Araucaria* on the Australian continent is excellent and has offered us a unique opportunity to investigate historical fluctuations in vegetation types and their localities on the continent over the past 100 million years (Kershaw and Wagstaff 2001). These studies have also facilitated our understanding of the impacts of historical climatic fluctuations on the extant distribution and abundance of species. However, while nearly all Araucarians are closely associated with rainforest types, there is evidence to suggest that as many as four times the number of extant *Araucaria* spp. existed on the Gondwanan continent at the beginning of the Cretaceous (Hill and Brodbribb 1999; Kershaw

and Wagstaff 2001). There remains the possibility that some of these species may have had differing ecological requirements to their extant congeners. Thus, extrapolating patterns of distribution and/or abundance should be conducted cautiously, especially when extrapolating patterns to those of extant taxa. This is not to diminish the usefulness of the genus in reconstructing paleoenvironment conditions, as these records are extremely useful in determining broad regional vegetation responses to increasing aridity (MacPhail 1997; Kershaw and Wagstaff 2001). For example, by the Late Pliocene to Pleistocene all records of Araucariaceae had contracted to the northeastern part of the continent, within their present-day distribution, which mirrors the contraction of rainforest to the eastern coast under conditions of increasing aridity (Kershaw and Wagstaff 2001).

Detailed information on changes in vegetation communities in more recent geological times, i.e. throughout the Quaternary, has been gained through the analysis of pollen cores throughout Queensland (e.g. Kershaw 1983, Kershaw 1994, Kershaw *et al*. 1994; Longmore 1997; Moss and Kershaw 2000). These records not only provide evidence of community types in existence at particular geological times but also the relative abundance of species and/or genera in the region and their relationship to climatic change. As stated previously, the pollen of *Araucaria* is morphologically similar and thus precludes the identification of species. However, both extant Australian *Araucaria* species are associated with rainforest communities within the study areas and by incorporating their genetic signal, as determined in the previous chapters, we can gain further insight into the impacts of climatic fluctuations on this group of species. Furthermore, the Quaternary microfossil records, extracted from within, or closely adjacent to, the

present distribution of *Araucaria* are far more likely to be reflective of extant species and the impacts on their populations than records predating that period.

6.2 North Queensland Araucarian microfossil record

There is much evidence to suggest that *Araucaria* was formerly a dominant component of the vegetation of northeastern Australia (Kershaw 1994). However, these records, when confined to the Quaternary period, also indicate substantial changes in both the distribution and abundance of the genus within the region over the past 1.4 million years (Moss and Kershaw 2000).

Of the previous palynological studies in northeastern Queensland, that derived from Lynch's Crater has provided an important insight into Araucarian population dynamics and changes in vegetation patterns during the Pleistocene and Holocene (Kershaw 1994). The overall pattern indicates that Araucarian forests in the area were replaced by fire-promoting *Eucalyptus* forests between 38,000 and 26,000 BP (Kershaw 1994). While suggested to be previously widespread, araucarian vine forest did not re-establish subsequent to the Late Pleistocene eucalypt expansion (Hopkins *et al*. 1993) and Moss and Kershaw (2000) suggest that it was during this period that *Araucaria* became 'relictual' in northeastern Australia. This period also marked the extinction of other coniferous species in the area; genera of which are now absent from the Australian mainland (e.g. *Dacrydium*, *Dacrycarpus*). Kershaw (1994) argued that changing landscape usage, associated with Indigenous burning regimes, most likely brought about the demise of these forests. In contrast, other cores within the region show relatively poor representation of Araucarian pollen, such as Strenekoff's Crater (Kershaw

1994), which suggests some patchiness in the abundance of the genus in the region, even within similar geological time frames. Thus it is plausible to suggest that these records are highly restricted spatially and may mask the underlying importance of *Araucaria* in the region. A contemporary pollen profile conducted by Moss and Kershaw (2000) supports this, as they found the aerial pollen dispersal of *Araucaria* to be restricted and limited to deposition close to source populations. No *Araucaria* pollen was found in a survey of modern deposition sites within the Wet Tropics (Moss and Kershaw 2000).

Further evidence for the historical importance of this genus in northeastern Australia may be gained from an examination of off-shore marine cores, such as ODP site 820 (Kershaw *et al*. 1993). Rather than being 'closed' systems of deposition, the palynology of marine sediments is thought to be representative of regional, rather than local, vegetation patterns (Kershaw *et al*. 1993). This difference arises due to deposition occurring from a much wider catchment area, with varying proximity to the source populations as a result of changing sea levels. These sediments are also thought to be deposited by fluvial processes, rather than direct deposition from adjacent source trees, which again increases their regional representation (Longmore 1997). As stated by Moss and Kershaw (2000), pollen from this site is thought to be derived from water transport from the Barron, Mulgrave and Russell rivers, which would substantially increase the pollen spectra, but may preferentially reflect streamside vegetation as a result. Evidence from this core suggests an increase in *Araucaria* pollen from the Late Miocene to Pliocene, with subsequent domination of the spectra until the Late Pleistocene when the signal almost disappears from the record (Kershaw *et al*.

1993). Kershaw (1994), and later Moss and Kershaw (2000), estimated this initial decline at somewhere around 140 ka, with a subsequent decline mirroring that recorded from Lynch's Crater (~ 40 ka). Other pollen profiles (e.g. Butchers Creek; Kershaw 1994) confirm this trend of decreasing *Araucaria* representation, which is accompanied in most cores by an increase in Poaceae and Myrtaceae pollen.

Kershaw (1994) explains the dominance of *Araucaria* in the offshore core as being representative of an Araucarian forest that may have dominated the coastal plain until rising sea levels caused its demise. He supports this claim through the extant presence of Araucarian forests that occur on offshore islands throughout Queensland. He goes on to suggest that rainforest refugia may have been surrounded by araucarian forest, rather than the sclerophyll woodlands of today. Having initially postulated the decline at Lynch's Crater as being due to anthropogenic causes, the much earlier decline in the ODP820 core required an alternative explanation, as humans were not thought to inhabit the area until around 100,000 yrs later (Moss and Kershaw 2000). Dry conditions, during the peak of the penultimate glacial, were suggested to have induced increased burning and promoted sclerophyll vegetation, leading to the initial decline of *Araucaria*. The more recent decline (45,000 ka) is suggested to be anthropogenically induced as this period was not associated with a period of climatic change (Moss and Kershaw 2000). Kershaw and Wagstaff (2001) later refine their explanation and suggest these declines were interwoven with ENSO (El Niño Southern Oscillation) events. These events would have created drier conditions, thereby promoting fire, regardless of mean precipitation values. The first decline is

associated with an ENSO event during a dry glacial period, while the second decline occurred also during a period of high ENSO activity but also coincides with the arrival of humans to the continent (Kershaw and Wagstaff 2001).

Webb and Tracey (1981) had previously proposed locations of glacial refugia based on topographic and edaphic features of the landscape, which identified cloudy mountain tops and wet lowland communities as likely localities. However, Hopkins *et al.* (1993) provided evidence to suggest that even the largest extant areas of rainforest were at some time penetrated by sclerophyll vegetation and fire. Furthermore, they suggest that there were few, if any, large Late Pleistocene rainforest refugia. Thus, rather than forming large discrete patches, the rainforest refugial areas were most likely highly fragmented and confined to fire-protected areas. Hopkins *et al*. (1993) also suggest that the timing of these events, and thus differential signals across the pollen cores, may reflect the differential ability for both sclerophyll vegetation and associated fire to penetrate larger versus small areas of rainforest.

6.3 South Queensland Araucarian microfossil record

An excellent Quaternary pollen profile also exists for southern Queensland, and is derived from perched lake sediments from Fraser Island (Longmore 1997). This area now supports one of the remaining rainforest communities in southern Queensland, and contains two extant coniferous genera, both from the Araucariaceae: *Agathis robusta*, and *Araucaria cunninghamii*. However the extant representation of these two genera on the island has been severely reduced

due to their heavy exploitation until the declaration of World Heritage for the area.

This core shows fluctuations in the abundance of *Araucaria* pollen from 600 ka to 120 ka. A decrease is then noted from around 21 ka to 12 ka, when *Araucaria* is replaced once again by *Eucalyptus* and may represent the effects of the Last Glacial Maximum (Longmore 1997). During the Holocene, *Araucaria* pollen levels never recover to those recorded prior to 21 ka. These patterns mirror those described above for northern Queensland from both the ODP820 core (Moss and Kershaw) as well those described from other sites in the Atherton Tablelands (Kershaw 1994). These simultaneous declines are surprising given the higher extant abundance of *Araucaria* in southern Queensland, and the evidence provided in this thesis which suggests a more recent decline of the genus in southern Queensland.

6.4 Genetic structuring in *Araucaria* in relation to the microfossil record

6.4.1 Divergence among northern and southern populations of Araucaria

The genetic differentiation recorded between northern and southern populations of *Araucaria* in this study reflects the broad-scale reduction in distribution, and associated population divergence, seen across the Araucariaceae (Kershaw and Wagstaff 2001) and mirrors the large-scale climatic cycles from the Triassic to the Late Quaternary. By the Late Miocene, pollen profiles suggest the presence of *Araucaria* in northern Queensland, with a contraction of all fossil representation to eastern Australia by the Late Pliocene (Kershaw and Wagstaff 2001). Thus, it is plausible to suggest that the extreme differentiation recorded between northern

and southern populations of both *A. bidwillii* and *A. cunninghamii* in this study reflect this ancient contraction of the genus to the northern and southern areas of Queensland. This large disjunction would have been maintained and reinforced during the subsequent Quaternary oscillations, which are examined below.

6.4.2 Divergence among northern populations of Araucaria.

The hypotheses of rainforest distribution and survival throughout the Quaternary have largely been based on the above pollen profiles and the work of Webb and Tracey (1981). As previously mentioned, rainforests were initially thought to have contracted to large areas during unfavourable climatic periods (Webb and Tracey 1981), however the evidence provided by the charcoal analysis of Hopkins *et al*. (1993) suggests this view may be too simplistic. Kershaw (1994) speculated that isolated rainforest refugia may have been surrounded by Araucarian forest in a similar manner to which sclerophyll vegetation does today. However, the genetic analysis of *Araucaria*, presented in Chapters 2 and 3, initially appears to contradict this theory.

Both high diversity and significant genetic structuring characterises the *Araucaria* populations sampled in this study. Populations derived from single refugial localities should display minimal population divergence and maximum genetic variation, due to historically high levels of gene flow. The populations sampled for this study obviously do not conform to this model. Hopkins *et al*. (1993) proposed a theory of discrete rainforest refugia dissected by sclerophyll vegetation, which is more in line with the genetic structuring and diversity detected in the genetic analyses.

The genetic analysis of *A. bidwillii* precluded any detailed analysis of structuring in northern Queensland as only one of the two extant populations was sampled, due to difficulties in accessing the second site. However, the Mt Lewis population did not contain significantly different amounts of genetic variation to southern populations, despite its obvious isolation and small population size. Given its substantial genetic diversity, the possibility remained that this species was responsible for the micro-fossil pattern of continued reduction in abundance throughout the Quaternary, through a severe reduction in population size.

In contrast, the occurrence of more populations of *A. cunninghamii* within the Wet Tropics allowed for a more detailed examination of these results. Significant and extreme population structuring was detected among the northern populations of *A. cunninghamii*. This divergence was four times as great for the RAPD markers, and twice as great for the ISSR markers, than was recorded among the southern populations (see below). This suggests an earlier and/or more sustained period of isolation for the northern populations than for the southern populations. Obviously, the high degree of genetic structuring present, together with the maintenance of high genetic diversity within these populations, conflicts with simplistic hypotheses of a single rainforest refugium for the Wet Tropics, south of the Black Mountain Barrier (Webb and Tracey 1981; Nix 1991). More likely is the 'mosaic' scenario suggested by Hopkins *et al*. (1993).

To reiterate, Hopkins *et al*. (1993) suggested that all rainforest refugia were frequently penetrated by sclerophyll vegetation and fire. This would substantially increase the number of 'refugia' but decrease the connectivity of these isolates. The genetic structuring evident in *A. cunninghamii* concurs with

this pattern in that it suggests the maintenance of several distinct isolates, that given their extant ecological preferences, were most likely indicative of regions of rainforest refugia. Whether these were located within the present sites in which *A. cunninghamii* is restricted cannot be ascertained from the present data.

Furthermore, the dominance and abundance of the genus in the historical micro-fossil record at first appears to conflict with this theory. However, of all faunal and floral taxa within the wet Tropics, conifers were arguably the most affected by the Pleistocene climatic oscillations. It was during this period that two coniferous genera became extinct on the Australian mainland, and it remains highly likely that localised extinctions of *Araucaria* populations occurred. For example, as previously discussed, there is strong evidence to suggest that *Araucaria* was a dominant component of the vegetation surrounding Lynch's Crater. However, its current absence from the area, coupled with the extreme genetic divergence among northern populations, suggests that this population, along with *Dacrydium* and *Dacrycarpus* recorded from the area, became extinct. Thus the extant populations of *Araucaria* in the Wet Tropics may not have impacted dramatically on the micro-fossil record. There is no evidence in this study for a continuous araucarian forest on the Atherton Tablelands historically, as the divergence among northern populations exceeds that recorded among southern populations which were more continuous in the past.

The significantly lower genetic diversity recorded from the Gillies Range is interesting when viewed in this context. As suggested by Hopkins *et al*. (1993), the penetration of smaller rainforest areas may have occurred faster and had more dramatic impacts than the penetration of larger refugial areas. Thus it is possible,

given its present day isolation and the surrounding dominant sclerophyll vegetation, that the Gillies population has encountered these effects for a longer period and with a higher frequency and associated impacts than other northern populations which are more closely associated with rainforest environments (e.g. the Paluma population). Even given the extant isolation of *Araucaria* populations, this population is extremely small and contained within an extremely fire-prone area, where the occurrence of rainforest may have been historically limited.

6.4.3 Divergence among southern populations of Araucaria

As detailed above the divergence among southern populations was approximately one quarter to one half of that recorded for the northern populations of *A. cunninghamii*. Values of divergence among the southern populations as detected by pairwise population values ranged from 7% to 12% for *A. bidwillii* and 6% to 11% for *A. cunninghamii*; values which are broadly congruent, but substantially lower than those recorded among northern populations for *A. cunninghamii*. This suggests a much more recent episode of isolation for the southern populations than for the northern populations. This is surprising given the congruence of declines in the Quaternary micro-fossil records of *Araucaria* between northern and southern Queensland (Longmore 1997; Moss and Kershaw 2000), and the extant higher abundance of *Araucaria* within the southern region relative to northern Queensland. However, as suggested for *Araucaria* pollen representation at Lynch's Crater, the possibility remains that this represents a highly localised record which does not reflect the broad regional distribution of the genus. Historical records covering the past two centuries suggest that *Araucaria* was a

dominant component of the vegetation of southern Queensland, prior to its heavy exploitation (Petrie 1904; Baker and Smith 1910). This is reflected in the substantially lower levels of differentiation recorded among southern populations of *Araucaria* relative to their northern counterparts.

A caveat from both these results is that the extant distribution of the genus is unlikely to reflect the full potential climatic range of these species (see Kershaw 1997). Thus localised extinctions could have left simply in place the current pattern of genetic diversity and divergence that has been detected in this study. With ancient taxa, such as *Araucaria*, stochastic events and limited dispersal opportunities may mean that the current ranges do not reflect the full potential climatic range of these species. Further research, concentrating on both central Queensland populations and offshore island populations of *A. cunninghamii* may further assist in elucidating the historical demise of *Araucaria* in both northern and southern Queensland. A further caveat is that while the microfossil record only covers the Quaternary, the patterns of genetic differentiation observed in this study could just as well result from historical events dating much further in time.

CHAPTER SEVEN

IMPLICATIONS FOR THE ONGOING CONSERVATION AND MANAGEMENT OF *ARAUCARIA* POPULATIONS IN AUSTRALIA: EVIDENCE FROM GENETIC DATA.

7.1 Application of genetics to conservation

The application of genetic information to conservation strategies has been somewhat controversial, however the incorporation of genetic information is now recognised as integral to any informed conservation effort. In fact intraspecific variation has generally been accepted as a focus for conservation (Newton *et al*. 1999), as the preservation of genetic material is thought to maintain the evolutionary potential of species and populations. Part of the debate surrounding the use of genetics for the identification of conservation units has focussed on the use of neutral genetic markers (e.g. Bekessy *et al*. 2003). However this debate is primarily centred on whether historical or adaptive variation should be preserved. If the goal of conservation is to be focussed on long-term processes then historical information is essential to informed management. The preservation of adaptive variation, which may not always be detected by genetic investigations, focusses more on short-term demographic processes in populations. It may be argued that adaptive variation, having arisen from historical genetic variability, may be replaced if lost, whereas historical variability may not. Furthermore, single and or multiple genes may control some adaptive traits, and are thus not expected to be detected in the analysis of populations using neutral molecular markers which simultaneously assess large amounts of loci. Problems also arise when the

logistics of demographic studies of long-lived species, such as *Araucaria*, are considered. The goal of this study was to assess and identify populations that show significant historical divergence, which may inform future conservation and management strategies, some of which are currently based on adaptive traits specific to increased yields for forestry (Dieters *et al*. 2002). Information on the amount and distribution of genetic diversity have facilitated the development of *in situ* conservation strategies for forest tree species, and complemented studies of quantitative traits where available (Moran *et al*. 2000). These studies have also identified populations that require *ex situ* conservation because of both low genetic variability and/or the assessed risk of population extinction (Moran *et al*. 2000).

7.2 The identification of Evolutionary Significant Units

The field of conservation genetics was borne out of a perceived need to identify conservation units below the species level, where presumably the impacts of genetic variation are manifested and expressed. Several criteria have been proposed for this assessment, including Evolutionary Significant Units (ESUs). However, while there is general consensus on what constitutes an ESU, a consensus on how to identify one has proved elusive (Fraser and Bernatchez 2001). Furthermore, many of these criteria used for ESU identification have been developed based on zoological models, and are simply not suited to the study of plant populations. For example, Moritz (1994) proposed that ESUs be identified on the basis of reciprocally monophyletic mitochondrial DNA (mtDNA) lineages. While this approach may be feasible for zoological studies, the slower

evolutionary rate of mtDNA in plants, which is estimated to be 40 – 100 times slower than in animals (Palmer 1992), limits the utility and application of this criterion in plants. Few, if any, plant ESUs could be identified using this criterion. Fraser and Bernatchez (2001) propose that the designation of ESUs should be conducted on a case-by-case basis and be flexible, but recognise that a strictly objective decision using these criteria may be difficult to reach, and that a universal definition of what constitutes an ESU may not be possible. Crandall *et al*. (2000) agree with this proposal and go further to suggest the complete abandonment of the terminology, due to its dichotomous (conserve or not) nature.

However, there is general agreement that the central goal to conservation should be the preservation of both evolutionary processes and ecological viability of populations through the maintenance of both genetic diversity and divergence (Crandall *et al*. 2000). From this there is growing consensus that both adaptive divergence and historical isolation should be jointly considered.

The results presented in Chapters 3 and 4 provide much insight into the historical population dynamics of *Araucaria* in Australia and have advanced our understanding of the genetic integrity of these populations. Here, a conservative approach is taken to the recommendation of conservation and management priorities, which incorporates information on genetic diversity and divergence as well as current threats to natural populations of *Araucaria* in Australia.

7.3 Conservation recommendations for *A. bidwillii*

As mentioned above, both genetic and ecological studies complement each other with regard to the conservation of species (Sherwin and Moritz 2000), and as such

ecological knowledge is required specifically for those populations of *A. bidwillii* that display the largest divergence (Mt Lewis, Burton's Well [RAPDs], and Jimna [ISSRs]). The first two populations appear in ecotonal environments suggesting, in line with ecological theory, that selection may be stronger in these environments than in their 'core' habitat. This divergence may also be a consequence of lower densities in these populations, as Gram and Sork (1999) found that populations with low densities may harbour a different suite of genotypes to denser populations. This is thought to be due to adaptive genetic variation and thus, Gram and Sork (1999) suggest a strategic conservation plan should aim to preserve a range of populations with differing densities in order to maintain adaptive genetic variation. It should be noted that while ISSRs and RAPDs are 'neutral' genetic markers and are not expected to specifically detect adaptive variation (see Bekessy *et al*. 2003), it is expected that at least some of the large number of loci sampled by neutral markers will be under some degree of selection (Penner 1996; Esselman *et al*. 1999). Therefore, the possibility remains that at least some of the observed structure may be adaptive (Esselman *et al*. 1999). This may also in part explain the discrepancies between the two markers in detecting genetic structuring over large geographic distances (see chapter 5).

Further research on the adaptive significance of these results will require a different application of these markers, such as Quantative Trait Loci (QTLs). The difficulty in developing QTLs may now be overcome given this initial molecular investigation in *A. bidwillii* and the fact that some of the population–specific bands detected may now be sequenced (Sequence characterised amplified regions – SCARS) and investigated for correlation with adaptive traits. Such efforts have

proved to be useful for the identification of *Araucaria angustifolia* seeds from differing localities in Brazil (Hampp *et al*. 2000) through the production of diagnostic primers. Six markers were identified in this study that show great promise for their future development as diagnostic population markers. Three of these were derived from RAPD markers (2 from primer OPB 15, and 1 from primer OPB10) and three were from the ISSR markers (808, 811, 857). All six markers discriminate Mt Lewis from the southern populations and will prove to be extremely useful for the ongoing assessment of pollen introgression in this population, especially once diagnostic primers are developed. Juveniles have been collected from this site and further research will investigate the potential pollen introgression from the numerous trees of *A. bidwillii* which have been planted on the Atherton Tablelands, and which appear to display morphological affinities to the southern populations (Pye pers. obs.).

It is apparent from this study that both the northern and southern populations of *A. bidwillii* are on independent evolutionary trajectories, which suggests that their management, and subsequent conservation, should be considered independently.

The Mt Lewis population is already subject to a different climatic regime to the southern populations; a regime which may be more similar to that expected under a global climate change scenario. Given the high degree of genetic divergence observed in the RAPD data set, the presence of population-specific markers from both ISSRs and RAPDs, together with phenotypic divergence, the conservation of this population may be imperative for the future of the species – acting as a genetic reservoir that may be already adapted to increased

temperatures. However, as suggested by Petit *et al*. (1998), conservation attempts should encompass both genetic diversity and divergence, as both contribute to the total diversity of the species. *A. bidwillii* is not represented in extensive *ex situ* plantings and thus most of these populations have no buffer against stochastic events. As all populations sampled in this study displayed significant differentiation, the preservation of the remaining genetic material of *A. bidwillii* should remain a priority if we are to adequately protect this economically, sociologically and biologically important southern conifer.

Furthermore, given the significant differentiation revealed among all sampled populations, movement of germplasm should be avoided, even within the Bunya Mountains – a fairly continuous habitat where prior to this study one may have expected increased homogeneity relative to the other populations sampled. In addition, extant population size should not be used as a management indicator for genetic diversity in this species, as all populations displayed similar levels of variation regardless of extant population size.

Potential threats are the same as exist for other rainforest tree species and include anthropogenic processes, fire, and excessive seed predation in the smaller populations. Pathogens may also be an important determinant of population structure. During field collections in the Bunya Mountains, a dieback site of more than five mature trees suggests this threat is more real than perceived. The maintenance and future monitoring of genetic variation within these populations may act to lessen this threat.

7.4 Conservation recommendations for *A. cunninghamii*

While *A. cunninghamii* has been the focus of genetic improvement trials over the past 60 years (Dieters *et al*. 2002), these trials have focussed on traits associated with improved yield and *ex situ* performance in plantations, rather than being indicative of adaptive provenance traits specific to their place of origin. Trees have been highly selected and bred for their superior growth characteristics (Dieters *et al*. 2002) and thus these *ex situ* plantations may be of little assistance in improving our understanding of the natural *in situ* demography of these populations. Thus in a similar manner to that outlined for *A. bidwillii* we immediately require ecological knowledge on the most divergent and economically important populations.

Two of the most economically important provenances, as determined by forestry trials, are Jimna and the Gillies Range (Dieters *et al*. 2000). The former has displayed superior growth traits while the latter has been incorporated in cross-provenance trials with southern Queensland provenances (Dieters *et al*. 2002). This latter trial displayed a superior growth capacity relative to southern provenances (Dieters *et al*. 2000) through extended growth occurring during the southern winter months, which further highlights the importance of this small, isolated population. Further assessment of the genetic diversity of the Gillies Range population should be undertaken, given its significantly lower diversity when compared to all other sampled populations. This assessment should employ a co-dominant molecular marker in order to assess levels of homozygosity in this population, which has the potential to accelerate genetic erosion.

The significant genetic structuring detected among most populations of *A. cunninghamii* sampled also has significant repercussions for the future management of the species. As previously mentioned, population genetic theory, along with the life-history traits of this species, would predict more homogeneity among populations than was detected. However, from these results, the mixing of what are now distinct genotypes should be avoided, particular so among the northern populations. There is some evidence to suggest the population distinction is not as great among the southern populations, however three distinct regions were identified (Lamington, Noosa plus Jimna, Brisbane plus Bunya Mts) which suggests the mixing of genotypes from these regions should also be avoided.

Monitoring for pollen introgression from surrounding plantations, as well as plantation pests is extremely important (Strauss 2001), especially given the significant growth differences among provenances (Dieters *et al*. 2002). Evidence for hybrisation of *Eucalyptus* species from nearby plantations has previously been documented and provides evidence for pollen introgression from nearby plantations (Strauss 2001). Whilst hyridisation is not a threat in this instance, pollen introgression has the potential to severely disrupt the genetic divergence of populations as detected in this study and may be manifested in outbreeding depression (Young *et al*. 1996). The potential for this latter threat may be lessened given the lack of evidence from this study for long-distance pollen dispersal. However, given the economic importance of this species, decisions that are irreversible should not be taken. Plantations in southern Queensland already surround what are now small isolated native Araucarian forest patches (e.g. Jimna

State Forest), so this monitoring may be extremely important, not only for the integrity of forestry trials but also for the maintenance of the genetic integrity of the remaining natural populations. The huge resource availability to pest species in plantation monocultures, such as *A. cunninghamii* plantations, is generally unequalled in native forests. Therefore, the threat to native populations by pest outbreaks is also substantially increased given the proximity of some of the remaining natural populations to plantations (Strauss 2001). Thus ongoing monitoring of pest species within natural populations of *A. cunninghamii* adjacent to plantations should also be conducted.

As was the case for *A. bidwillii*, several markers have been identified in this study which have the potential to greatly inform forestry management of *A. cunninghamii* as well as for the monitoring of native populations for pollen introgression. The majority of these markers appear to show the most potential for the monitoring of the Gillies population, but markers were also isolated that show the potential for ongoing assessment of pollen introgression from northern forestry provenances into native southern populations. For example, one marker from the RAPD primer OPB 17 was specific to the Gillies Range and could be utilised for the above monitoring. Another marker from RAPD primer OPB8 could also be used for this purpose as it displayed fixed absence in the Gillies population. Finally a locus from RAPD marker OPB 20 could be utilised to assess southern population pollen introgression into northern populations, and vice versa, as it was present only in southern populations, and displayed fixed absence in the northern populations. Three ISSR primers also showed utility for future monitoring of population integrity. Primer 857 produced two loci that

displayed fixed absence in all northern populations, while a locus from primer 809 was fixed in all populations but the Gillies. While the utility of these markers for the monitoring of pollen introgression appears promising, they may also be refined and used to investigate the outlined differences in growth traits among provenances.

Further research on the adaptive significance of these results will require a different application of these markers, such as Quantitative Trait Loci (QTLs). The difficulty in developing QTLs may now be overcome given this initial molecular investigation and the fact that some of the population–specific bands detected may now be sequenced and investigated for correlation with adaptive traits.

7.5 Conclusions and future directions

This study has now provided the baseline data on the effects of historical fragmentation on the genetic diversity and divergence in *A. bidwillii* and *A. cunninghamii*. The sampled populations appear to maintain a high degree of diversity, with the exception of the Gillies Range population, which is accompanied by high divergence among extant populations. This historical genetic signal can now be utilised for examining the effects of comparatively recent events, such as the recent exploitation of these species. Heavy exploitation of these species over the past few centuries means that the patterns detected here are likely to be exacerbated and substantial loss of genetic diversity is likely, especially in those populations that historically displayed low genetic variation.

The high divergence among populations sampled means that more effort will be required for adequate conservation of the genetic resources of these species than population theory for wind-pollinated conifers would have predicted. Therefore the conservation of several, if not all, extant populations will be required to maintain the existing evolutionary potential in these species.

Further ecological studies are immediately required on these extant populations to complement the genetic information presented here. Specifically, studies on the genetic pollution of native forests from neighbouring plantations of *A. cunninghamii* are required and can be assessed using the aforementioned markers that have been isolated in this study. Additionally, studies on the effects of recent exploitation can now be conducted and compared with the historical signal presented here for meaningful interpretation of contemporary pressures.

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APPENDIX 1

Binary matrix produced from RAPD analysis of *A. bidwillii*. The format follows that of PopGene v. 1.3.1 (Yeh *et al*. 1999)

```
/* Diploid RAPD data set*/
Number of populations = 6
Number of loci = 50
Locus name :
OPB20-1 OPB20-2 OPB20-3 OPB20-4 OPB20-5 OPB20-6
OPB18-1 OPB18-2 OPB18-3 OPB18-4 OPB18-5 OPB18-6
OPB17-1 OPB17-2 OPB17-3 OPB17-4 OPB17-5
OPB15-1 OPB15-2
OPB10-1 OPB10-2 OPB10-3 OPB10-4 OPB10-5 OPB10-6
OPB9-1 OPB9-2 OPB9-3 OPB9-4 OPB9-5 OPB9-6
OPB8-1 OPB8-2 OPB8-3 OPB8-4 OPB8-5 OPB8-6
OPB1-1 OPB1-2 OPB1-3 OPB1-4 OPB1-5 OPB1-6 OPB1-7 OPB1-8
OPB6-1 OPB6-2 OPB6-3 OPB6-4 OPB6-5

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001101 111110 11110 00 111110 101101 000011 01001110 1..10
001100 111011 11101 00 111101 101100 111011 01011111 11111
010000 111111 11101 00 111100 111010 110010 01011110 01110
011100 111110 11110 00 111101 111100 111001 01001011 11111
...0.. 0111.. 11001 00 110100 101011 101010 01011010 01110
101001 111111 11011 00 111111 101111 101010 01001110 11011
001111 111111 11111 00 111101 101110 001010 11001110 11010
001100 110111 11111 00 110110 101100 110110 01000110 11010
001001 ..01.. 11111 00 110100 111100 111111 01000011 01010
101011 111010 10001 00 110100 111010 011011 01000011 11010
001000 010110 11111 00 110100 101000 100010 01001110 11110
101000 111100 01100 00 110100 101000 011010 01001110 11110
101000 011010 11100 00 110100 101010 011010 01000111 11011
000000 111110 11110 00 110100 101010 101011 01011010 11011
101000 111110 11101 00 110100 111010 011010 01011111 11011
100000 111010 11111 00 010110 101000 101110 01001011 11111
001100 0.1010 11111 00 011101 111010 011010 01011110 11001
001001 111110 01001 00 011100 111100 100010 01010110 11010
```

APPENDIX 2

Binary matrix produced from ISSR analysis of *A. bidwillii*. The format follows that of PopGene v. 1.3.1 (Yeh *et al*. 1999)

```
/* Diploid Bunya ISSR data set*/
Number of populations = 6
Number of loci = 57
Locus name :
17898-1 17898-2 17898-3 17898-4 17898-5 17898-6 17898-7 17898-8
17898-9 17898-10 17898-11 17898-12
808-1 808-2 808-3 808-4 808-5 808-6 808-7 808-8 808-9 808-10 808-
11
810-1 810-2 810-3 810-4 810-5 810-6 810-7 810-8 810-9 810-10 810-
11
807-1 807-2 807-3 807-4 807-5 807-6 807-7 807-8
811-1 811-2 811-3 811-4 811-5
818-1 818-2 818-3 818-4
857-1 857-2 857-3 857-4 857-5 857-6

name = Jimna
010111110100 11111101011 01110101011 01110100 10111 1010 111110
010101011000 10111100011 11111101110 11100000 11011 0110 101110
010111000101 11111101011 11110101110 11110001 10011 0011 111110
110101010000 11111101011 11110101100 01100000 10011 0011 101110
010111011011 10111111011 11111001000 11111000 10111 1101 001111
010111010100 10111101011 01110101110 11110010 11101 1010 101010
110110110100 10110000011 10110101000 11100000 11001 0101 001110
010101010100 10110000011 01111101100 01100010 10100 0010 111110
010111110100 11111101011 11110101001 01110101 11010 1010 111111
011111010010 11111101011 11111001110 01110000 10001 1000 100111
110110111100 11111111011 11111101000 10010011 10101 1010 001010
110111010111 11110000011 11110101000 01100010 11011 1110 111110
110101011000 10111101011 11111101010 01101010 10101 1011 111011
110110011111 10111101011 10111111100 01111100 11001 1010 001111
010110110000 10111101011 11111101000 01001110 10100 1010 111110
111111010101 11111100011 01110101000 00111000 10100 1000 111001
010110010000 10111101011 10110001001 10011100 11010 0010 111110
011110010000 10111101011 11111001001 01111100 10010 1110 001111
011111010111 11101100011 11110101001 10110101 11000 1010 101111
110111010100 00111101000 11110001000 10011111 11010 0010 001010
110111011110 11111101100 11110001010 11110011 10010 1010 .11110

name = Westcott
010111010110 10110000001 11100101001 11101000 11000 1010 011110
0101........ 10000000000 11100000010 ........ ..... .... 0.....
110110110010 10110100011 11110101110 11100000 11010 1010 001110
010101010000 11010111100 11100101110 00111101 11010 1010 001111
010110000111 10110000011 11101101001 11111000 11000 1110 001011
0101......01 10110010011 10100001110 10100000 11010 1110 101001
............ 01000001100 01000000001 ........ ..... .... ......
000110010000 10011010101 11100011000 00100000 10111 0110 111011
010110010100 01110100011 11110101011 10011110 11111 1110 011011
010111010110 10111100010 11111101001 01111110 11111 1011 011111
010111010110 11111100011 11101101001 10111100 11011 1010 011111
```

```
011111010100 10011100011 11100101000 10111101 10010 1010 011110
010110010110 11111100011 11110111001 10111100 11110 1010 001110
010110010000 10000000000 11100111001 11101100 11010 1011 011110
011111000110 11100000011 11110101010 11011010 10010 1010 001011
111101011110 11110101011 11111101000 11011010 10111 1010 011001
111110111100 10110100011 11101101010 10110000 11001 1011 011110
110111010100 10010100011 01111101001 10101000 11000 1010 011110
010100110000 10110100011 11110001000 10100010 11011 0110 011010
010101010000 11010110011 11111011101 11100000 11110 1110 111111
111111010110 11110101011 11100101110 11110100 10010 1110 011110

name = Burtons Well
101100000000 11010000011 00110101000 11100000 01011 1000 111000
100110000100 11111101011 00111101100 11011100 10010 1111 011110
110101001101 11111100011 11110101010 11111100 10100 1111 001111
110110010111 11111100011 11110100000 10100100 11010 1110 111110
010100000000 00001100000 11100001000 10100000 10010 1001 001110
111111001111 11111100001 01111101100 11110100 10011 1110 101001
110111010110 11111110001 01110101000 11111100 11010 1110 110110
110111010010 10111000101 11111101001 10111000 10010 0010 001011
110111010011 11110100000 11100101000 11101100 11010 1100 111011
110111001010 11110100101 01110101010 11110100 11110 1011 011011
111110010010 00010000010 11000001010 10000000 01100 1110 101001
110111111010 11111100011 01110101010 10111110 11110 1110 001110
110101000001 11001110011 01110101100 10101000 01111 1010 0.....
100101001010 11010100011 10110101100 10111000 10110 1010 001110
101110001110 11110110001 10111101100 10111101 10110 1010 001110
001100000000 10010100001 01110101000 10100000 00001 1110 001001
............ ........... ........... ........ ..... .... ......

name = Mt Lewis
............ ........... ........... 00100000 ..... .... ......
110111011010 11111100111 01011101010 11110101 10110 1010 011011
110110011111 11111100111 11010101000 11110001 11011 1011 011011
110110110111 11011000011 10110101100 11110001 10110 1110 001011
101111111110 11011100011 11011101000 01110101 10011 1010 011110
110111011110 11011100011 11110101000 01110100 10001 0010 011111
110111101111 11011000111 01110101010 01110001 11100 1010 011110
111111010110 11111100111 01111101100 11110100 10101 0010 011011
110110111110 10010100111 11011101000 11110001 10101 1110 011110
............ ........... ........... ........ ..... .... ......
111111101111 11111100111 01111101010 11110001 10111 1110 ......
111101111011 10111100111 01111101100 01111111 11010 1110 001011
110101110111 11111000111 11111101100 01111110 10111 1011 011111
111101110111 11011000011 11110101100 01110000 11011 1110 001111
100110010101 11011000011 00010101100 01100000 10011 1010 001110
100001010100 10110000001 01110101100 01100000 10001 0010 000...
110100111101 11010000001 11110101100 01110000 10111 1010 001011
110101011110 10110100011 11110101100 01110000 10110 1110 001011
111101111110 10111010001 01110101100 01110001 10111 1110 001011
100100000000 10010000001 01010101110 01100000 ..... 1010 0.....
110101110110 11111110011 01010101100 01110001 11111 1110 001110
110101110111 11101100001 10110101000 01110000 11110 1110 011110
100100010000 00010000001 00110101010 11100010 00000 0010 011100
100100010000 00010000011 00010101000 01100010 ..... 0100 010100

name = Dandabah
```

```
010111101100 10111100011 11111001010 01101101 10010 1110 011111
111111010101 11111101011 11101011100 10011000 10001 0110 110111
010111110010 11111100001 11101101010 10111000 11000 1010 011011
011110010100 10000000001 11110011000 11111110 11001 1010 001011
110111011100 11111100010 11110101000 11111100 11011 1001 001010
010111010110 11111100011 11111101000 01111100 11111 1110 011111
110111010001 11111100010 11111101010 11111101 11010 1110 001010
110111011110 11101100001 01111101100 11011110 10110 0010 111111
110110001111 11111101110 11111101100 01111000 10111 1110 011111
001111010111 11111101111 10101101000 01111010 11100 1010 101010
111110111100 11111101010 01111011000 11111010 11100 1011 101110
111111001110 10111100001 11111101100 01111010 10011 1111 010110
100111001110 10001101001 11101101100 10011111 00011 1010 011110
110110010110 10111101010 11101111100 10011010 01000 1110 111110
000011000110 11011100011 11111101100 01011010 10100 0111 001010
100111010110 10111100011 11111111010 01111110 10110 1010 011110
010111010111 10111100110 11111001000 01011100 10111 0110 011110
...000010000 00000000100 11100000000 ........ ..... 0001 011000
100110011000 10111100010 01110101001 01000000 01110 0011 111010
001111000100 10011101011 01110101000 01101010 00100 1010 011011
100111110110 10111101011 11101101001 01111011 01111 1111 001110
001100010010 10111100011 11111111000 01111000 10001 0111 101100
010100010100 10111100011 11111101000 01111010 ..... 0010 101011
000100101101 10111110011 01111101011 01111100 11000 0110 001110
000100000110 10111100011 00111101001 01111110 11100 1010 001110
............ ........... ........... ........ ..... 0010 ......

name = Paradise
100101110110 10111100011 01111111001 01110101 11101 1010 011110
010111011110 10101100011 01111001100 11011100 10011 1010 101100
111111011110 10011100011 01100001001 10011101 10111 1010 111011
101101010110 11111100010 11111101010 10110111 10011 1110 011111
............ 00010000001 ........... 10...... ..... .... ......
111101011000 10111110011 11111101100 10110000 10101 1010 101011
100101010110 10111100010 11111101001 01111100 11111 1110 101111
110101010110 11111100011 11101101000 11011100 10010 1110 101111
100011001100 11111110110 11100101001 01111010 11010 1010 101110
000111011100 11111101110 11100101001 11100010 11101 1011 1.....
000111111110 11111101110 00111101001 10111100 11100 1110 001110
101111000010 10011101111 11111011001 10111100 11001 1110 101111
100101110110 10111100111 11100111001 10111000 10101 1110 101111
010101000111 10111110011 11110111001 10011100 11000 1010 001110
101110010010 11011100110 10111111001 01111110 11100 1010 001111
010101000110 10111010011 11111101010 11011110 11100 1010 001111
000101010110 10011100111 01110101000 11111010 11100 1010 101110
100111101110 11111110010 10110111001 01111010 01100 1010 101110
100001010000 10011100010 00100101001 10011000 10110 1010 000110
111110101110 10101101011 11101101010 01011010 11010 1110 001110
100001010000 10110100010 10100101001 10101000 01000 1110 111110
000000010010 00100000011 ........... 01100000 ..... 0011 011110
```

APPENDIX 3

Binary matrix produced from RAPD analysis of *A. cunninghamii*. The format follows that of PopGene v. 1.3.1 (Yeh *et al*. 1999)

```
/* Diploid RAPD data set*/
Number of populations = 8
Number of loci = 75
Locus name :
OPB17-1 OPB17-2 OPB17-3 OPB17-4
OPB10-1 OPB10-2 OPB10-3 OPB10-4 OPB10-5 OPB10-6 OPB10-7 OPB10-8
OPB10-9 OPB10-10 OPB10-11 OPB10-12 OPB10-13 OPB10-14 OPB10-15
OPB10-16
OPB3-1 OPB3-1a OPB3-2 OPB3-3 OPB3-4 OPB3-5 OPB3-6 OPB3-7 OPB3-8
OPB3-9 OPB3-10 OPB3-11 OPB3-12 OPB3-13 OPB3-14 OPB3-15 OPB3-16
OPB20-1 OPB20-2 OPB20-3 OPB20-4
OPB8-1 OPB8-2 OPB8-3 OPB8-4 OPB8-5 OPB8-6 OPB8-7 OPB8-8 OPB8-9
OPB8-10 OPB8-11 OPB8-12 OPB8-13
OPB9-1 OPB9-2 OPB9-3 OPB9-4 OPB9-5 OPB9-6 OPB9-7 OPB9-8 OPB9-9
OPB9-10
OPB12-1 OPB12-2 OPB12-3 OPB12-4 OPB12-5 OPB12-6 OPB12-7 OPB12-8
OPB12-9 OPB12-10 OPB12-11

name = Lamington
0110 0100001110100110 10110111110010110 0101 1101101111111
1010101100 01111111111
0110 0100101001001101 00100011110010110 1110 0101110100010
0110111100 01111110111
0110 0100001111010011 10011111110100100 0101 0100000111110
1010111100 01111111101
0110 0100101000001100 .................. ...0 0000011100110
.......... ..111110010
0110 0100101000101111 10001110110100110 1111 1100000111010
0110101100 01111001101
0100 0100101100110111 10010110110000110 0101 1101011110011
1110111100 11100100101
0100 0100101101001000 .................. 0110 0001010100011
0010...1.. 00101100111
0000 0100101000101010 00000010110000100 1111 1101101111010
0010010100 01011010111
0110 0100001010000011 10111111110000100 1101 0100110110010
1110111100 01101000110
0000 0100001011001010 10000110110100000 0001 1101100100011
0010101100 01111010110
0100 0100..1100000000 .................. 1000 0100101100010
0000100100 00100010110
0100 ................ ................... .... ............
.......... ...........

name = Blencoe
0101 1100111100100001 01111111110000100 1100 1101011011010
1111101110 11111111110
0111 0100111000110101 01011111110001110 1100 1101111110011
1111000110 11001111110
```

```
0111 1100111000100000 00111110110010110 1100 1101011111110
1111001100 11111110110
0101 1000001110100000 01111101110001100 1100 1101111111111
1111011111 11011111110
0101 1100001010110000 00010010110001100 1100 1101111111011
1111111100 01010110110
0110 1100011100010001 11011110110001100 1100 1101011100011
1111011101 11110100110
0111 1110011101101000 11110111110001110 1100 1101110111010
1111011110 11110111110
0111 1000011100100000 00110111110001110 1100 1101111001001
.......... 01101100110
0110 0000001110101000 00110110110010110 0100 1101111111010
1111011110 11100100110
0101 ..............  00010111110001000 1100 1101111111110
1111001100 11001100110
0111 .1101110000000000 00011110110000101 1100 1101111101110
.......... 01101100110
0111 1100001110110001 00110110110011111 1101 1101011111110
1111111110 01011111110
0111 1110011110100111 ..010010110000110 1101 1101011101110
1111001111 01010111110
0101 1100001110110001 11110111111000100 1101 1101111110111
1111001101 01011111110
0100 1010001110100001 11110111110000110 1100 1101011101010
1111101111 11110111110
0111 1110011100110000 11001010100001110 1100 1101011101000
1111111100 11011111110
0101 1110001110100000 01011000110011100 1100 1101111110111
1111011111 11010111110
0111 1110011110100000 10011111111011110 1100 1101111111010
1111011110 01011111110
0110 1110011000000010 01100010110001110 1100 1101011000011
1111001111 01110000110
0101 0100001110100010 01010111110001110 1100 1101011000011
1111011101 11101111110

name = Brisbane
0110 0100001001001011 00011110110000111 1100 1101011101011
0000100100 01110111110
0011 0000101000010011 00111000110000101 1010 0101111000010
0110100100 01101010110
0110 0100001101010000 11011011110000000 1110 0101011101010
1110111101 11110100100
0100 0110011100001011 00011011110000101 0011 1101011101011
1110101100 01110100110
0111 0010001000100111 00110010110001111 0111 1101111111011
1110101100 01111011101
0111 0100111101100111 00000111110001100 1111 1101011100010
1111101100 11111111110
0111 0100101100000011 11011101110011111 1000 1101010101011
0010100101 01110111110
.... ..............  .................. .... ..............
.......... ...........
0101 0100001101000111 00111100110000111 1010 1101111100010
1100101100 01101111110
```

```
0111 0100001100100011 00001001110011000 0010 0101111100011
1011111100 01111111110

name = Festoon
0111 0010001000000011 ................. 1001 1101010100011
.......... 00010100110
.... ................ ................. .... ............
.......... ...........
0100 0100101000001011 00.....0110011100 1001 1101111101111
1010111101 01111110110
0100 0100.....0001010 ............01000 .00. .....11100010
.......... 00011100110
0110 0..0.......0.110 00.....0110010110 1001 1101111101010
.......... 00001111110
001. ...........0.... ................. .... ............
.......... ...........
0101 0101001110001011 10110111110000100 1101 11011111.0111
.......... 01111111110
0110 0...001000010011 ................. 1000 ..01010100010
.......... 00111100100
0010 0........0001001 ............11000 1000 .....10100011
.......... 00111111110
0... ................ ................. .... ............
.......... ...........
0101 0000011011001100 10110010110000101 1011 1101010101110
1110101100 01111111110
0... ................ ................. .... ............
.......... ...........
0101 0000011000000100 00010010110000110 1001 1101111101110
1110101100 01111111110
0101 0100011001100100 00100010110000100 0011 1101100100111
1110101101 01101100110
0110 0110011100000101 ............01001 0001 1101011101110
1010100001 01110110110
0100 0000101100000101 ................. 1000 0001010100110
0010101100 00110000110
0... ................ ................. .... ............
.......... ...........
0101 0110000010010100 ................. 0111 1101110101110
1110111101 01111110110
0110 0100001100001111 00010110110000100 1111 0101110100110
0110111100 01101010110
0101 0010001000110111 00000100110000100 0011 1101111101011
1010000100 01111100110

name = Gillies
0111 1100011100110100 01111101110000100 0000 1101111101100
1111011101 01110100100
0110 0100001100000100 00111100110101110 0000 1101111101000
1111101101 ...........
0111 0100011000110100 01111111110000100 0100 1111111100100
1111100000 11110100100
1111 1100011000110000 01111111110000100 0000 1111111101000
1111111100 11110101100
1101 0100011100100100 01110011110000100 0100 1111111100000
1111001001 11110101100
```

```
.... ..............1.. ................. .... .............
.......... ...........
1101 1100011000100100 00111100110000110 0100 1111111100000
1111110100 01110101100
1110 1100011000100110 01110111110000100 0100 1111111101000
1111100000 01111100100
0101 0100011100000100 01111101110001100 0100 1111111100000
1111010100 01110101110
0111 1100011100101111 11111100110011110 0000 1111111101000
1111111101 11110111110
0110 1100011100000111 10111101110000110 0100 1111111101000
.......... 01110100110
0110 1100011100100110 11110111110001110 0100 1101111100000
1111111100 01110100100
0110 0100011100000110 01111101110001110 0100 1111011100000
1111011101 01110101110
0111 1100011100101110 11111111110000110 0100 1111111100000
0111100101 01110100110
1111 1100001110100110 11111101110011100 0100 1111111100000
1110100001 01110101100
0111 1100011100100110 10111101110011100 0100 1111111100000
1111011101 01110100110
1101 1100011100100110 11111111110001100 0100 1111111101000
1111011000 11110100100
0111 1100011100101110 01111101110011100 0000 1111111100000
1111111101 11110100100

name = Jimna
0100 0100101100001111 10000010010000110 1000 0101100100110
.......... 01111111111
0100 0100101101001110 00011101110100110 1001 1111011101110
1111111100 01111101101
0010 0100101000001011 11010010110001110 0000 1100111101010
1110101100 01111100111
0011 0100011101100111 00011010110001101 1011 1111010101011
1111101100 11011111110
0100 0010011000000111 ..001000110000100 0001 1100011101110
1111101111 11111100110
0101 0100011000010111 10000010110000100 0000 1101110100010
.......... 01111100101
0011 0100011101100011 00011010110010101 1001 1101010101010
1110101100 11011101110
0101 0100001000000111 11000010110001100 0000 1100011001010
.......... 11111101100
0101 0110011000001111 00011101110000101 0001 1100011101110
1111101111 11111101110
0101 0100011000010011 11011010110000110 0000 1100001101011
1111101100 11111001100
0111 0100011111010111 00101100110100100 1001 1101111101010
1111111101 11111011100
0110 0100011101010000 10001110110000110 1011 1101010001110
1010111100 01111000110
0010 0100011100010111 10101101100010101 1001 1100111101110
1111111100 01111011110
0000 0100101011010011 ..000110110000101 1010 1100110100010
1110111101 11111110110
```

```
0101 0110001000010000 00.............. .001 0100010100010
.......... 00111101110
0010 0100011100001011 10010010110001101 0001 1100111101110
0110111100 01101110111
0101 0110011100010111 10000010110000100 0010 1101000100011
..1.101100 11110110110
0101 0100101101011101 10010101110001100 0001 1101010101010
1111111100 01111110101
0111 0100011010111111 10010110110010110 1111 1100011101010
1110101101 01111011101
0001 0100011000011111 10110010110000101 1101 1111011101110
1110101101 01111011101
0111 0100011100001011 10011100110000100 0001 0101101100011
0000100001 01100110111
0001 0100011110000111 11101100110000100 1011 1101101101010
1110111100 01111111110
0111 0110001100010111 10100110110001100 1000 1101111100010
0011101100 01111110110

name = Noosa
0000 0100101000010111 10000000110000110 0000 0001011101010
1110101111 11111000110
0101 0100100100101111 00000011110001101 0010 1101010101010
1111111100 01111111100
0101 0100001011000111 10101111110000100 0111 1101110101110
1111101101 01110010100
0001 0100001010101111 10011000110011101 0000 1100101101010
0111100101 11110000110
0100 0100001111101110 10101101110011110 1001 1101001101110
1000101100 01111110101
0001 0100001101101110 10000111110011100 1100 1101110000010
1010001100 01110111110
0101 0110001000000100 00000010110001101 1000 1101100101010
1110100100 01110110110
0011 0110011110010110 10111111111010100 1111 1101011100010
1110001111 11111101101
0100 0100001110010110 10101100110010110 0011 1101000101110
1111111100 01111010101
0111 0110001000001011 10101101110010100 1011 1101011101110
.......... 11011111101
0001 0010001001110100 10000111110000101 1010 1101010101011
1111101100 11110110110
0100 0110001100010000 ............01000 0011 1100100101010
1110000101 01111010000
0101 0110001111010111 10101111110000110 1011 1101101101010
1110111100 01111101101
0101 0110001101111111 10011101110010111 0001 1101111101110
1111011100 01110011111
0001 0100001001111111 10001110110011100 1100 1101101001010
1110011100 01111111101
0101 0100001000100110 10001000110001100 1000 1101110101010
0010100100 01110010101
0001 0000001000000110 00001001110001100 1001 1101110101010
1010000100 01111111101
0000 0010000100001100 ............01000 1000 1100111000010
.......... 01111110110
```

```
0... ................ ................. .... ............
.......... ...........
0001 0100......010000 ................. 1000 .....10100010
.......... 00111110101
0101 0110011111000111 10100101110000101 1000 1101011100011
1111111100 01111101101
0100 0100101001000111 10000010110001110 1000 1101100101010
.......... 01110000111

name = Paluma
0111 1100001110110010 01111101100001110 1000 1101010101111
1110101101 11110000110
0111 1100011100000010 01101011100000110 0001 0111111101000
1111111110 11110100110
0111 1100001111100000 11111010110000110 1100 1101011101010
1111111110 11010110110
0111 1100111100100100 11111111110001110 0001 1111010101010
1111111110 11110100110
0101 1100001110110000 11111110010001110 1000 1101010101010
1111111110 11110100110
0101 0010111000001000 10101010110001010 1000 0101110101000
1010110100 11010110110
011. 0010010000101000 ..001010010001110 1000 1101010101010
1111100111 01010010110
0111 1100101101001110 00101100010001110 1100 0101011101010
1111101100 01110110110
0111 0110011100101100 00111001110001110 1000 0101010101010
1111011111 01010111110
0100 1100011100000000 00111011110001100 1000 0101011100010
1111111110 01010110110
0111 1100111100101011 01111000100001110 1100 0101010101100
1111101111 01101000101
0111 1100011100110000 01111000011001010 1000 1101010100010
1111111111 11001100101
0111 1001101110001000 10001011100000110 1000 1111011101000
1010010101 11111111110
0111 1100011110101011 10111010110000110 1000 1111011101001
1111111111 11110111101
0101 0100100001100000 ................. 1100 0001010100000
1110111101 11100100110
0... ................ ................. .100 ............
.......... ...........
0... ................ ................. .... ............
.......... ...........
0111 1101001111100000 01011010110001110 1000 0111011001010
1111111111 11010111111
0110 0110111100001000 11100001110001110 1100 1101110101010
1111101100 01101100110
0101 1101101111100000 10101010110001110 1000 0101110101110
1111101111 01100100110
0111 1100001111000000 11101010110001110 1000 1111111101010
1111111111 11110100110
0111 1100101110101000 11001010100001100 1100 1101011001100
1000100001 01010100110
```

APPENDIX 4

Binary matrix produced from ISSR analysis of *A. cunninghamii*. The format follows that of PopGene v. 1.3.1 (Yeh *et al*. 1999)

```
/* Diploid ISSR data set*/
Number of populations = 8
Number of loci = 59
Locus name :
807-1 807-2 807-3 807-4 807-5 807-6 807-7 807-8 807-9 807-10 807-
11
808-1 808-2 808-3 808-4 808-5 808-6 808-7
809-1 809-2 809-3 809-4 809-5 809-6 809-7 809-8 809-9
810-1 810-2 810-3 810-4 810-5 810-6 810-7 810-8 810-9
811-1 811-2 811-3 811-4 811-5 811-6 811-7 811-8 811-9 811-10 811-
11
857-1 857-2 857-3 857-4 857-5 857-6 857-7 857-8 857-9 857-10 857-
11 857-12

name = Lamington
10110100011 1000111 100111011 110101110 01010101000 101000100111
01110110001 0100010 110101101 111101110 11110000000 101000000000
11100110000 0100010 101111111 111111000 11111001000 101001001000
10101000000 0101010 000100101 110111000 01111101010 000001000000
11111010011 1101111 100100101 111011110 11111011011 101100010010
11101100011 1101010 101100110 111101010 01101011100 111100101110
00110000011 0000010 010101101 010101011 00000010010 001000000000
00110000011 1101110 000100100 010101011 01110101110 101000100010
11110000011 1101010 000101100 110111010 11101101100 001000000010
10110010011 1101110 000100100 010111010 11110101000 101100000001
10100000001 0000111 000101000 010100010 00000101000 001001000000
.......... ....... ......... ......... .......... ...........

name = Blencoe
11110111001 1111110 111101101 011111011 10011001000 101000000010
11110111001 1110110 111111111 010111111 10111001000 101000000010
01111111011 1100010 111101111 011011011 10111001000 111000000000
11101111011 1100010 111101110 011111011 11011001000 101000000010
01111110011 1101010 110111111 010111011 11111101000 111000001010
11101001011 1101010 011110111 011110110 11011001000 101000000010
01100110011 1111110 010110111 011111010 11111001000 111000100010
00001110011 1111110 110111110 011111011 10111000000 101000000110
01110110001 1111010 110101101 010111111 10111101100 101000110010
01110000011 1100110 010101110 010111111 10111100000 100000000000
01001111011 1111011 110111111 010111110 10111101000 111000001000
10100110011 1111010 110111111 010111010 10111101100 101000000110
01110111001 1110010 011110010 011011111 10111101000 101000000010
11111111011 1101011 110111100 010111010 10111101000 101010001010
11100111011 1111011 010101000 011011010 10111011000 101000100010
10110111001 1100010 111101111 010011011 00011111100 101000000110
01100110001 1111110 110100110 011001000 10111001000 101000000010
01110110001 1110011 110110100 011111111 00111101000 101000010010
10100111001 1100010 110110100 010011011 11111111000 101010010010
```

```
01100101001 1100010 010111111 010011110 10111101000 101000000000

name = Brisbane
11101110001 1101010 010101000 010111110 11111100001 001010000100
00110110001 1100010 010101100 011101000 10110100000 101000000010
11100110001 1100010 110101111 010100000 11111000000 101000000110
00101110000 1110010 110100111 111101110 00111001000 101001000100
00100110001 1100010 111101100 010001000 11110000000 101001010100
11100000000 1100011 111101001 010011000 11111011000 101101010100
00110110011 1100010 110111001 010100011 10110001000 101001100100
..........  .......  .........  .........  ..........  ..........
01101110001 1100011 110110110 011101100 11111101000 101000000101
01100100001 1101001 000101000 110111001 00000001000 101000010000

name = Festoon
00100000001 1101010 .........  011111010 10111001000 101000010000
..........  .......  .........  .........  ..........  ..........
11100010001 1100010 110101110 110110011 11111001001 101000100100
10000010001 1100010 110101100 010110000 00000000101 011010000000
10000000001 1100010 010100100 010111010 10110001101 000010000110
00000000001 1100110 000100000 011100000 00000000100 110000000011
11101010001 1101010 111111101 010111110 10111001010 101000100000
11100110001 1101010 010101000 010100000 10111001100 101000000100
01000000001 1101010 010101100 010111000 00000001011 010011000000
..........  .......  .........  .........  ..........  ..........
01000110001 1101010 110101100 111011010 10101001010 111000000000
..........  .......  .........  .........  ..........  ..........
00100110001 1101011 010101100 111111000 10111101010 101000110000
01100110001 1100010 110111110 111111000 01110001100 101000110010
01110111001 1100110 010100101 011101110 10111101001 101010000000
01100100000 1100010 010101100 010101000 11111101000 100100000000
..........  .......  .........  .........  ..........  ..........
01110111011 1100011 110100110 011000010 01110100000 101101001100
00100110011 1100010 110101010 011000100 10111011110 101000110000
01101110011 1100010 110111100 010101110 11111001000 101000100001

name = Gillies
01111011001 1110010 111100110 111111010 10111001001 101000000010
01111011001 1110010 111101100 110011000 10111001011 101000000010
11111011001 1110110 111100101 001111001 10111001011 101000110010
01111011001 1111010 111110101 011110001 10111101110 101000110010
01111011001 1110010 111111110 011110000 10111101001 101000000010
..........  .......  .........  .........  ..........  ..........
01111011011 1110110 111110101 001111000 11111101001 101000000010
11111011011 1110111 111010101 001111000 10111001111 111000100100
01111111011 1110110 111100101 011111000 10111011001 101010100010
01111111011 1110110 111111101 011101011 10111111011 111000100010
01111001001 1110010 111110101 011111000 10111000001 101000000010
01111001001 1110100 111101100 011110011 10111111010 101010000010
01111111001 1110000 110110101 110101000 10111101011 101000000010
01111011001 1110000 111110101 .........  10111011000 101000100000
01111011001 1110010 111100100 011111001 10101011000 101000000010
01111011001 1110011 110110101 111111010 10111001001 101000000010
11111011001 1111010 110110101 101100110 11111011001 101000010010
01111011001 1110010 110110100 111111010 10111101001 101000010010

name = Jimna
```

```
010100000000 0001001 100100100 010101110 11001001000 100000000001
00111110011 0101011 010101100 110110000 01111101000 101101101000
00110111011 1000000 110101101 010111000 .......... 101000000001
10110111011 1110010 110101111 010101110 10111001000 101010110000
01010101111 1101010 110111000 110110011 11100001010 101010110001
01110010011 1100010 010100100 011100000 00000001101 100000000000
10110011111 1110010 010101100 010101110 .......1000 101000000000
01110001011 1101010 110100100 011100010 01111001000 101010000000
01110001111 1100011 110111000 100110010 10110001000 101010110001
00110000001 1100010 110111100 011001001 00110001000 101000110000
00111110011 1101111 111110100 011101010 10110001000 101011000110
10110010001 1101000 100101000 110100000 00111001001 101000000000
01110110011 1100010 100101101 010111100 00111101100 101000110000
00010110001 1100110 111111111 010100010 01111001001 101010000110
00110111011 1110000 110101000 110010000 01100000000 101010101000
00110010111 1000111 110111100 011110000 01110000100 101000000000
10110010011 1100010 010100100 011111010 01100001100 101100000000
10110011111 1110010 110100110 111100010 11100001000 101011000000
11011111111 1101000 110101110 010110110 11101101010 101000001000
10010010111 1100010 110101101 111110010 11110011110 101010110001
10110011011 1101010 110101100 011111000 01101101000 101000101000
10110011011 1111111 010101100 011111110 01100101110 101010110100
10110011011 1100010 010101100 111101011 01100100000 101010010000

name = Noosa
10110011111 1111010 100111100 011100010 10110101010 101000110000
00110011011 1110010 010101100 011110000 11110001000 101010101001
10110111011 1101010 000100100 111100010 11111111000 101000110000
00110011011 1100111 110111110 111000011 10110111100 101000000000
01110011011 1101110 110111100 110101010 01111101100 101000010000
00110011011 1100011 110101100 111100000 11101101000 101100110000
10110101011 1100010 100101100 011100010 11111101000 101000110000
10110011011 1100111 000111100 011100010 01011100000 101000100110
01100111011 1100110 000110101 011111010 11111101000 101000010010
01110011011 1110111 010110101 111110010 11111101010 100100100011
00110111011 1110111 110111100 011010010 11111001000 101000101010
10111011011 1101010 100111100 011100000 01111101100 101000101000
10110111011 1101111 100110100 011101011 11111101000 101000110000
10110111011 1110011 100111110 011100010 11110101000 101001110000
00100011011 1110110 010101100 111100010 11101101100 101001000001
00111001011 1100010 000100000 011100010 11001001100 010010000000
00110111011 1110110 000100100 011100010 11011001100 111010000000
00110110101 1111110 000101100 011100010 00111101010 111000100010
00100000000 ....... 000100100 001100000 .......... 110000000000
00110010001 1111010 000101100 010111000 .......1100 001000100000
01010101011 1101011 110111100 010100010 11110101000 101000010000
10110001011 1110010 000111100 011100000 01111101100 101000100000

name = Paluma
01100111111 1110011 110111100 010101000 10111101000 101010001100
01110001011 1110011 110111100 011000000 11111111001 101000010000
01110011111 1101011 110111100 011110010 10111111000 101010010100
01111011011 1101011 111111100 011010000 10110101000 101000010000
01110011111 1110011 111111111 011110001 10110001000 101000010100
10110111011 1110101 111111000 011000001 10010001000 101000000000
00110011111 1110010 010111110 011110010 10011001101 101000010000
11110111111 1111001 110101110 111101010 10111000001 101000000101
```

```
01110111111 1110010 110110111 010110000 10111111000 101000000001
11100001111 1101011 010100101 010110001 10010000000 101000000000
01100101011 1110011 110111111 010000000 10111101100 101000110010
00110111011 1101011 110111100 011010000 10111001000 101000110100
00110101111 1110000 110101100 111010011 11011000000 101000100010
01110011111 1100010 110111111 011011000 10111111101 101000000010
10000001111 1101011 110111101 010101010 10111011000 100000000000
.......... ....... ......... ......... .......... ............
.......... ....... ......... ......... .......... ............
00111111111 1101010 110110101 011010011 11011101000 101000000100
01100111111 1111011 110100101 010101011 10011000001 101000110010
00111001111 1100010 110100101 010000010 10011000000 101000000000
01111001101 1100010 110111101 001001001 11110001000 101000100100
11101000101 1100100 110101100 011011010 10111101001 101000100110
```

APPENDIX 5.

Manuscript produced from material presented in Chapters 2, 3, 5, 6 and 7.

(see attached)

Pye, M. G., and Gadek, P. A. (2004) Genetic diversity, differentiation and conservation in *Araucaria bidwillii* (Araucariaceae), Australia's Bunya pine. *Conservation Genetics* **5**: 619-629.