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Genetics differentiation among and within three Red mahoganies, *Eucalyptus pellita, E. resinifera* and *E. scias*

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Thesis submitted in fulfilment of the requirements

of the Degree of Master of Science at

Southern Cross University

17th, September 2009

Declaration

I, Son Le, certify that the work presented in the following thesis, to the best of my knowledge and belief is original, except as acknowledged in the text, and that the material has not been submitted, either in whole or in part, for a degree at this or any other University.

I acknowledge that I have read and understood the University's rules, requirements, procedures and policy relating to my higher degree research award and to my thesis. I certify that I have complied with the rules, requirements, procedures and policy of the University.

…………………..

Son Le

Date ……………..

Abstract

The red mahogany group (Genus *Eucalyptus* Subgenus *Symphyomyrtus* Series *Annulares*) contains several species of importance to forestry in the tropics and subtropics because of their high productivity and tolerance to pathogens and pests in plantations. Despite recent revisions, and a number of studies to examine genetic and quantitative traits (including morphological and oil composition) variation within and among taxa, the taxonomy of the group remains problematic, and there is no consensus on membership at the species level or below. The taxonomy of three species, *E. pellita*, *E. resinifera* and *E. scias,* has been particularly challenging because of wide spread (in the case of *E. pellita* and *E. resinifera*) but disjunct, overlapping distributions and the high degree of genetic and/or morphological variation leading to the recognition of subspecies or geographic races in each taxon. Using microsatellite markers (n=13), genetic differentiation, among and within these three species, was characterised in order to revisit questions of taxonomy and the influence of geographic factors in determining within-taxon genetic structuring. Despite close geographical proximity and records of natural hybridisation in North Queensland, *E. resinifera* (total of 77 individuals from 8 locations) and *E. pellita* (total of 85 individuals from 12 locations) remain genetically distinct as taxa. Within *E. pellita,* two genetic groups were clearly resolved: one from New Guinea (includes all Indonesia and Papua New Guinea provenances) and one from Queensland (Cape York Peninsula populations were not sampled). Geographic structuring was also evident in *E. resinifera*, with North Queensland populations separating from those from Fraser Island southwards. Ecological factors and species disjunctions were implicated in the genetic substructuring of these two taxa because patterns of geographic variation aligned with biogeographical regions. *Eucalyptus scias* (19 individuals from 5 locations) was indistinguishable from southern *E. resinifera* and its two or three subspecies could not be resolved. At the taxon level, the

three species exhibit levels of genetic diversity within the range of other eucalypts with similar distribution types, widespread in the case of *E. pellita* (H_e= 0.81) and *E. resinifera* (H_e= 0.87), and regional with disjunctions in the case of *E. scias* (H_e= 0.88). A previous trend of increasing genetic diversity with latitude in *E. pellita* evident in isozymes could not be confirmed; however, a strong inverse cline was evident in *E. resinifera* which may indicate a southern centre of origin for this species. Several New Guinea populations of *E. pellita* exhibited high inbreeding coefficients (Goe and Serisa provenances mean inbreeding coefficients of 0.233 and 0.173, respectively) that were thought to have biological origins i.e. either due to inbreeding amongst relatives or selfing.

Publications

The following paper is based on this dissertation:

Le S, Nock C, Henson M and Shepherd M 2009. Genetic differentiation among and within three red mahoganies (Series Annulares), *Eucalyptus pellita*, *E. resinifera* and *E. scias* (Myrtaceae). *Australian Systematic Botany* (In press August 2009)

Le S, Nock C, Henson M and Shepherd M 2009. Genetic relationships of three red mahoganies (Series Annulares), *Eucalyptus pellita, E. resinifera* and *E. scias* (Myrtaceae). Australasian Forest Genetic Conference, Perth 20th – 22nd, April 2009.

Acknowledgements

Firstly, I would like to appreciatively acknowledge the following people involved in my completion of the degree of Master of Science.

- ❖ My supervisors, Dr Mervyn Shepherd and Mr Michael Henson, for the supervision and guidance during my study; and for patience throughout all phases of the project.
- ❖ Professor Robert Henry, Director of Centre for Plant Conservation Genetics (CPCG) for his encouragement and support.
- ❖ Timothy Sexton, CRC Forestry PhD student for giving me beneficial suggestions and checking my writing.
- ❖ CPCG for providing and supporting a laboratory and SCPG (Southern Cross Plant Genomic) for Microsatellite genotyping.
- ❖ All staff and students of the Centre for Plant Conservation Genetics for encouragement and support.
- ❖ Australian Centre for International Agriculture Research (ACIAR) and Southern Cross University for financial support for my study.
- ❖ The Vietnamese Ministry of Agriculture and Rural Development (MARD) for permission.
- ❖ Dr Ha Huy Thinh, Director of Research Centre for Forest Tree Improvement (RCFTI) for encouragement and support.
- ❖ MSc Doan Thi Mai for encouragement and support.

- ❖ Associate Professor Nguyen Hoang Nghia, MSc Le Quoc Huy and Dr Nguyen Viet Cuong for permission.
- ❖ Dang Phuoc Dai (FSIV) for helping in collecting *Eucalyptus pellita* samples in Vietnam.
- ❖ Steve Boyton and Dane Thomas (Forests NSW) for helping me in sampling and field work in Australia.
- ❖ Nigel Slator for collecting Timor *Eucalyptus urophylla* samples.
- ❖ Greg Luker for preparing the Figure 1.
- ❖ My colleagues of the Forest Science Institute of Vietnam (FSIV) for encouragement and support.

Secondly, I am grateful to

- ❖ My parents-in-law for their share in looking after my son with my wife and their support.
- ❖ My parents for their encouragement, sacrifice and support.
- ❖ All the rest of my family for their encouragement and support.
- ❖ Morrie and Kathleen Boath, my Australian family for helping and sharing all of my difficult times in Australia.

Finally, most importantly, and decisively, to my beloved wife Van Thu Huyen for her endless love, encouragement, patience and sacrifice when taking care of my son whilst I was studying at Southern Cross University and to my cherished son Le Dat Son Thinh for giving me more motivation and his tolerance of being far away from his Dad.

I am indebted to all of you.

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Abbreviations used

^{0}C	Degrees Celsius
AMOVA	Analysis of Molecular Variance
bp	Base pairs (nucleotides)
BSA	bovine serum albumin
DNA	Deoxyribonucleic acid
F_{is}	Fixation index, individuals relative to subpopulation
F_{it}	Fixation index, individuals relative to total population
F_{rt}	F statistic, genetic variation between regions
F_{sr}	F statistic, genetic variance between populations within region
F_{st}	Wright's F statistic, subpopulation relative to total population
H_e	Hardy-Weinberg expected heterozygosity
H_o	Observed heterozygosity,
µg	Micrograms
µl	Micro litter
µM	Micro molar
MCMC	Markov Chain Monte Carlo
N	Number of different alleles
Ne	Number of effective alleles
NG	Papua New Guinea and Indonesia
NQ	North (or Northern) Queensland
NQld	North (or Northern) Queensland
NSW	New South Wales
P%	the percentage of polymorphic loci
PCR	Polymerase chain reaction

pers. comm.	personal communication
PNG	Papua New Guinea
Qld	Queensland
SE	Standard error
spp.	Species
SSO	Seedlings Seed Orchard
subsp.	Subspecies
SSR	Simple sequence repeat (alternative for microsatellite)
T_a	Annealing temperatures
T_m	Melting temperature
UH_e	Unbiased heterozygosity
UPGMA	Unweighted pair group mean arithmetic average

Chapter 1. Introduction and Literature Review

1.1. Introduction

The red mahogany series has seven tropical or subtropical forest tree species which are distinguished from other members of the Section *Latoangulatae* Brooker (*Eucalyptus* subgenus *Symphyomyrtus* Schauer) by the persistence of rough bark, usually over the entire trunk (Brooker 2000). The series includes *E. urophylla* S.T. Blake and *E. pellita* F. Muell which are major eucalypts for planting in the low-land humid tropical environments, because of their tolerance to pathogens and pests (Eldridge *et al.* 1994b; Turnbull 1999). These species have been subjected to improvement programs in Australia, Brazil, Indonesia, southern China and Vietnam (Eldridge *et al.* 1994b; Harwood 1998; Kha *et al.* 2000).

The taxonomy of the red mahoganies remains problematic despite a number of studies of genetic, morphological and oil composition variation in *E. urophylla*, *E. pellita* and *E. scias* (Doran *et al.* 1995; House *et al.* 1994; Payn *et al.* 2008; Payn *et al.* 2007; Pinyopusarerk *et al.* 1993; Pryor *et al.* 1995; Singh *et al.* 1988). Several contemporary classifications exist and there remains no consensus on membership or classification at species level and below (Brooker 2000; Hill *et al.* 2000; Johnson *et al.* 1990). Describing the geographic distribution of genetic variation for a species can also be of value in management of the species for conservation outcomes, where the goal can be the determination of management units (Allendorf *et al.* 2007; Moritz 1994; White *et al.* 2007a; White *et al.* 2007b). This can be important for conservation by determining risk management strategies, where planting of translocated species or provenances which have the potential for gene flow into nearby native forests (Barbour *et al.* 2008; Barbour *et al.*

2002) and for species such as *E. urophylla*, which is increasingly under threat from the conversion of forest for agriculture (Payn *et al.* 2008). Finally, knowledge of genetic relationships among species and population structuring can be used in decisions for tree improvement (Eldridge *et al.* 1994a). *Eucalyptus pellita* exemplifies the conundrum of how breeding populations should be structured because of the strong differences in silvicultural and performance characteristics among its diverse provenances (Harwood 1998). Patterns of genetic diversity, revealed with genetic markers, can inform on the relative importance of evolutionary forces, such as drift and adaptation, that cause racial and ecotype differentiation and therefore assist in avoiding the use of maladapted material for regionalized breeding and planting programs (White *et al.* 2007b).

The objective of the present study was to describe the distribution of genetic variation in three closely related red mahoganies, *E. pellita*, *E. resinifera* and *E. scias*. More specifically, as there has been no previous study of genetic diversity in *E. resinifera*, it was of interest to see whether *E. resinifera* formed a genetically cohesive taxonomic unit, given its extensive and disjunct distribution and its close proximity with *E. pellita* which it naturally hybridises in the north of its range (Chippendale *et al.* 1984). Additionally, the uniqueness and affinities of *E. scias* were studied. Historically *E. scias* has been classified as *E. pellita,* yet it is distributed wholly within New South Wales and is geographically closer to southern populations of *E. resinifera* (Johnson *et al.* 1990; Hill *et al.* 2000).

1.2. Study Objectives and Rationale

The motivation for this study was to allow tree improvement and forest management to proceed in a more informed and efficient manner, by clarifying patterns of genetic diversity within and among three red mahoganies of emerging importance for forestry in the tropics. In the case of the three taxa investigated here, *E. pellita*, *E. resinifera* and *E. scias,* clarification of the gene pool for breeding was first necessary because of the uncertainty over delineation of taxa. A more complete understanding of geographic variation within the species (breeding population) could then be addressed. Historically geographic variation has been largely studied in provenances trials but, increasingly, genetic markers have also been used to characterise genetic variation as they may be quicker and cheaper than long term provenances studies (White *et al.* 2007b). Genetic markers also offer the potential to investigate relationships among tree species.

The study also has the potential to provide insight into the evolutionary processes shaping diversity in this group. This information may also be useful in the management of genetic resources for conservation and tree improvement, especially for *E. pellita,* which had strong differences in silvicultural and performance characteristics (Harwood 1998). This information is also important for conservation by determining risk management strategies when planting translocated species or provenances which have the potential for gene flow into nearby native forests (Barbour *et al.* 2008; Barbour *et al.* 2002) and for species such as *E. urophylla*, which is increasingly under threat from the conversion of forest for agriculture (Payn *et al.* 2008).

Specifically the study will address the following questions:

1. As there has been no previous study of *E. resinifera*; does it form a cohesive genetic unit given its extensive and disjunct distribution?
2. Is there a genetic basis for recognition of two subspecies of *E. resinifera*? Do they align with current taxonomic subdivision into subsp. *resinifera* and *hemilampra*?
3. Is *E. resinifera* genetically distinct from *E. pellita* in northern Australia despite their close proximity?
4. Is there genetic evidence for splitting *E. scias* into three subspecies?
5. Historically *E. scias* has been classified as *E. pellita* despite it being geographically closer to southern populations of *E. resinifera*. Is *E. scias* genetically closer to *E. resinifera* than *E. pellita*?
6. As Hill and Johnson 2000 proposed, is there a genetic basis for subdivision of *E. pellita* into two species based on New Guinea and North Queensland divisions?

1.3. Study Approach

Genetic variation at microsatellite markers will be used to investigate patterns of within and among taxa differentiation of the three red mahoganies. In the first stage, genetic diversity and population structure within each taxon will be investigated based on a current taxonomic classification (Brooker 2000). In a second stage, genetic relationships both within and among the three species will be addressed simultaneously using Bayesian model-based approach of STRUCTURE (Pritchard *et al.* 2000). This approach is appropriate for this situation as it does not assume an *a priori* grouping of individuals. In this analysis structure is determined entirely by the data. The STRUCTURE analysis is reported in Chapter 3.

For this study, microsatellite markers will need to be transferred from related *Eucalyptus* species (focal taxa) and initially be evaluated for their suitability for estimation of diversity parameters in the non-focal taxa. This evaluation will be performed in stage 1 along with population parameter estimation and reported in Chapter 2. The traditional genetic diversity and population structure analysis within species analysis will be carried out by using GenAlEx V6 (Peakall *et al*. 2006) and FSTAT software (Goudet *et al.* 2004). Microsatellite markers were thought most suitable for this study because: (1) they are suitable for study of both genetic diversity and population structure within species as well as among closely related species. (2) Many SSR are available in eucalypts and from closely related species to the study species, for examples *E. grandis* and *E. urophylla*, therefore they are expected to transfer well and provide reliable estimates of diversity parameters (Barbara *et al.* 2007).

Final conclusions and synthesis across the two results chapters are presented in Chapter 4. The current Chapter continues with a review of the literature on the taxonomy, distribution and genetic variation on the three red mahoganies of interest, *E. pellita*, *E. resinifera* and *E. scias*, microsatellite markers and statistical approaches for estimating genetic diversity and structure in forest trees.

1.4. Taxonomy

In the classification of Brooker (2000), the Red mahogany group (*Eucalyptus* subgenus *Symphyomyrtus* section *Latoangulatae* series *Annulares*) comprises seven species, *E. urophylla*, *E. pellita*, *E. scias*, *E. notabilis* Maiden., *E. resinifera*, *E. robusta* Sm. and *E. botryoides* Sm. Two subspecies of *E. scias* are recognised by Brooker, subsp. *scias* and *apoda*, as are two subspecies of *E. resinifera*, subsp. *resinifera* and *hemilampra* (Centre for Plant Biodiversity Research 2006). There is, however, uncertainty with respect to classification as the red mahoganies have been alternatively treated as nine species in the Series *Resiniferae* (Subgenus *Symphyomyrtus*, Section *Transversaria*) with *E. botryoides* and *E. robusta* relegated to Series *Salignae*, the addition of two new species raised from *E. urophylla*, *E. orophila* L.D. Pryor and *E. wetarensis* L.D. Pryor, and the splitting of *E. biterranea* L. Johnson & K. Hill and *E. macta* L. Johnson & K. Hill, from *E. pellita* and *E. resinifera,* respectively (Table 1 in Hill *et al.* (2000)). These authorities also recognise a third subspecies of *E. scias* subsp. *callimastha* from the south of the species range (Johnson *et al.* 1990). Table 1 summarises the distinguishing taxonomic features, the distributions, flowering time and records of hybridisation for the three red mahoganies studied here.

Table 1. Distinguishing taxonomic characteristics, flowering times and hybrids of the three red mahoganies, *Eucalyptus pellita*, *E. resinifera* and *E. scias* (Data from EUCLID - Centre for Plant Biodiversity Research which follows (Brooker 2000) for taxonomy, unless otherwise indicated).

Species	**Subspecies**	**Distribution**	**Flowering time**	**Description**	**Hybrids** (Natural (N) or artificial (A))
resinifera	*hemilampra* (Johnson *et al.* 1990)	Near Taree NSW to about Gladstone Qld	Unknown	Intergrades form amongst the two subspecies. Longer, narrower operculum with stamens mainly erect	*E. saligna* and *E. pellita (N); E. urophylla. E. alba* and *E. tereticorn*is (A) (Griffin *et al.* 1988) (in species level)
	resinifera (Johnson *et al.* 1990)	Two disjunct regions – Jervis Bay to Kempsey NSW then Eungella to Atherton Tablelands NQ according to EUCLID. Johnson *et al.* (1990) do not consider NQ population as *E. resinifera* but rather as an undescribed species	Dec.	Short conical operculum, stamens regularly to irregular flexed	Intergrades with *E notabilis, E. botryoides* (N); Intersectional hybrids with *E. amplifolia, E. saligna and E. propinqua* (Johnson *et al.* 1990)

Species	Subspecies	Distribution	Flowering time	Description	Hybrids (Natural (N) or artificial (A))
pellita		Irian Jaya (Indonesia), PNG, CYP and NQ – wetter forests in NQ from Mission Beach area north through to PNG	Feb, Oct	*E. pellita* is closely related to *E. scias*, *E. urophylla* and *E. notabilis* – all these species have short fat buds with operculums wider at base – *E. pellita* differs by larger adult leaves, buds and fruit (includes *E. biterranea*).	*E. grandis, E. resinifera, E. punctata* and *E. brassiana (N); E. urophylla. E. alba* and *E. tereticornis* (A) (Griffin *et al*. 1988)
scias (previously *pellita* (Johnson *et al.* 1990))	*apoda* (could been named as *tanyula* by Hill *et al.* (2000))	High elevations near Tenterfield NSW	Unknown	Buds mostly in 3s	
	scias	Coastal and subcoastal ranges from Narooma NSW to Cessnock NSW		Robust buds in 7s	
	callimastha (combined with subsp. *scias* in EUCLID)				

New species by Hill and Johnson 2000

Species	Subspecies	Distribution	Flowering time	Description	Hybrids (Natural (N) or artificial (A))
biterranea (Hill *et al.* 2000), recognized as *E. pellita* in EUCLID		New Guinea and Australian endemic restricted to the Cairns region.		Distinguished from *E. pellita* by the smaller buds, fruits and leaves	

Species	**Subspecies**	**Distribution**	**Flowering time**	**Description**	**Hybrids** (Natural (N) or artificial (A))
macta (Hill *et al.* 2000) recognized as *E. resinifera* subsp. *resinifera* in EUCLID		Highland areas of North Queensland, the MacIlwraith range, Atherton Tableland and Eungella range		Distinguished from *E. resinifera* by the broader, shorter, more rostrate calyptra, and the larger, broader and relatively shorter fruit with a descending disc and heavier, more prominently exserted valves	

1.5. Distributions

The collective distribution of *E. pellita*, *E. scias* and *E. resinifera* spans from New Guinea (NG) along the east coast of Australia to near Nowra in southern New South Wales. *Eucalyptus pellita* is the most northerly occurring of the taxa and is found in the southern lowlands of NG but also in the wetter forests of the east coast of Australia from Iron Range on the Cape York Peninsula (CYP), south to Coen and from north of Cooktown to south of Ingham, with a small population of *E. pellita* in Cape Melville National Park (Boland 2006). Recently it was proposed that the NG and North CYP populations of *E. pellita* be recognised as a separate species, *E. biterranea* (Hill *et al.* 2000), based on their distinctive morphology and isozyme differences (Pinyopusarerk *et al.* 1993; House *et al.* 1996).

Eucalyptus resinifera overlaps in distribution with *E. pellita* in the northern part of its range. The northern distribution is fragmented with sporadic occurrences in the Lankelly Creek-McIlwraith range region in the CYP and near Mareeba and Ravenshoe in North Queensland (NQ), Australia, but its distribution is more continuous in the south from near Gladstone in Queensland to Huskisson on Jervis Bay on the south coast of New South Wales (Centre for Plant Biodiversity Research 2006). Two subspecies of *E. resinifera* are recognised. *Eucalyptus resinifera* subspecies *resinifera* (herein subsp. *resinifera*) occurs in two disjunct regions, a northern population in the CYP and NQ and a second population in coastal areas from Jervis Bay north to Kempsey in New South Wales (Centre for Plant Biodiversity Research 2006). The second subspecies, *E. resinifera* subspecies *hemilampra* (herein subsp. *hemilampra*) ranges from near Taree on the north coast of New South Wales to Gladstone in Queensland, thus it is more or less central to the two disjunct populations of subsp. *resinifera* (Hill *et al.* 2000). Recently it was proposed that the populations of *E.*

resinifera from the CYP, the Atherton Tablelands and Eungella be recognised as a separate species, *E. macta* (Hill *et al.* 2000), although this is not universally accepted (Centre for Plant Biodiversity Research 2006).

Eucalyptus scias is regarded as having two (Centre for Plant Biodiversity Research 2006) or three subspecies (Hill *et al.* 2000) and was previously included under *E. pellita* (Johnson *et al.* 1990). It has a sporadic and disjunct distribution in New South Wales in coastal ranges, from Tenterfield in the north to Cessnock and Batemans Bay in the south (Pinyopusarerk *et al.* 1993), where it may grow in close proximity with *E. resinifera* (Binns D pers. comm.).

1.6. The Red Mahoganies – Breeding and Uses

Eucalyptus urophylla and *E. pellita* are increasingly important for forestry for low-land humid tropical environments such as South America, Indonesia or Vietnam because of their economic values and tolerance to pathogens and pests (Dickinson *et al.* 2005; Dickinson *et al.* 2004; Eldridge *et al.* 1994; Harwood 1998; Pino *et al.* 2002; Turnbull 1999). These species are recommended for planting only on well-drained, sandy soils where, at an early stage, it forms a dense crown that shades out weeds once it is established (Turnbull 1999; Harwood 1998). The red mahogany group are also a suitable source of firewood and charcoal. The heartwood is red to dark red, strong and durable, moderately heavy with a density of 775-1150 kg/m^{-3} (Harwood 1998). Although the grain is somewhat interlocked, the wood is not difficult to work. It has a wide range of uses for buildings, heavy construction and heavy ornamental work. *Eucalyptus pellita* is being used for pulp in Brazil as well as a general construction timber in both Brazil and New Guinea (Harwood 1998). *Eucalyptus pellita* is also an important source of fuel for tobacco curing in Cuba and has been used for solid wood in Queensland (Harwood 1998). *Eucalyptus pellita* was introduced to Cuba in the 1970s and this species became one of the most important forest species in that country with thousands of hectares of plantation established (Harwood, 1998).

Since the mid 1990s, *E. pellita* has been the main hardwood species planted in North Queensland (Lott *et al.* 2005). It is also the most planted species in the Community Rainforest Reafforestation Program (CRRP) in Queensland with about 100 hectares of forest plantation; but is currently established as a monoculture (Lott *et al.* 2005).

Improvement programs for *E. urophylla* and *E. pellita* are carried out in Brazil, Indonesia, southern China, Vietnam (Eldridge *et al.* 1994; Kha *et al.* 2000*).* A number of

provenance/progeny trials were established in Brazil in the mid-late 1980s. These trials were conducted based on 10 families from CSIRO seedlots, collected in Helenvale, Queensland and 18 seedlots collected in Coen, Queensland (Harwood 1998). They will be selected and thinned in order to convert trials into seedling seed orchards. In northern Australia seedlings seed orchards have been established with material from NG and Queensland (Harwood 1998).

Within 4 years of planting in 2 SSOs (Melville and Kairi), at 200 trees per hectare, the orchards have produced about 10kg ha^{-1} of seed (Harwood 1998). In 1996, second – generation progeny trials and seed orchards of *E. pellita* were established in North Queensland (Harwood *et al.*1997). The material used was seeds collected from the best individuals from a former SSO. After one year, orchard families showed that growth increased 10% in mean height over those from natural provenance controls.

Eucalypts species were introduced to Vietnam about 30 years ago (Kha *et al.* 2000). In some provenance/species trials *E. urophylla, E. camaldunesis*, *E. tereticornis* and *E. pellita* have displayed the higher potential in growth and wood quality than other species. It has become the major tree species in forest plantations. To 2001, about 348,000 ha of eucalypts were planted and established in reafforestation programs. This area was about 30% of the total of Vietnamese plantation areas (Kha *et al.* 2000).

Breeding programs for *E. pellita, E. scias* and *E. resinifera* are also being tested in family/provenance and hybrid trials in NSW. In these trials *E. resinifera* has shown high potential for growth and wood quality (Dang *et al.* 2009).

1.7. Geographic Variation

Provenances tests of *E. pellita* have been carried out in Australia, Brazil, Malaysia, China, Laos and Vietnam. From these trials the variation between provenances and regions (New Guinea and Australia) has been clearly recognized. Some provenances/family tests were established in the late 1980s for NG populations. Material from this region has shown better growth, survival and disease resistance than those from Australia in lowland tropical environments with short dry seasons (0-4 months) (Harwood 1998). However, Queensland populations showed better performance in the longer dry season regions (5-6 months) located in southern China and Laos (Harwood 1998).

In 2-3 year old trials at Luasong, Sabah, Malaysia, it was clear that NG provenances showed a higher ability to resist leaf fungal pathogens compared to Queensland provenances. New Guinea provenances also had significantly greater straightness in stem form than those from Queensland in 3 year old Melville Island trials. The Landkelly Creek and Tozers Gap provenances from Queensland had poor straight stem form compared to other provenances. Significant difference in growth traits, form and survival were demonstrated between families within provenances. In this trial, *E. pellita* also displayed better growth and survival than tropical provenances of *E. urophylla* and *E. grandis*. However, *E. pellita* was inferior to *Acacia mangium* in growth and crown health (Harwood *et al.* 1997).

Seven north Queensland provenances of *Eucalyptus pellita* were also tested in a provenances/species trial at four different sites in north and north-west Minas Gerais Brazil (Harwood 1998). This trial was based on 112 provenances of 13 eucalypts species. After 29 months, *E. pellita* was one of the best performing species in this trial (the mean volume

of this species reached to 36.1 - 49.2 m^3ha^{-1}). The difference between provenances in volume production was remarkable; however, the provenance–by–site interaction was not significant for this species (Harwood 1998).

There is no available data on provenances/species tests of *E. resinifera* and *E. scias*. Genetic variation in *E. urophylla* (an other member of this series) have also been tested in provenance and progeny trials throughout the tropical and subtropical world (Darrow *et al.* 1983; Dianese *et al.* 1984; Camphinos *et al.* 1989; Havmoller P 1989; Ngulube MR 1989; Pegg RE 1989; Mori *et al.* 1990; Clegg *et al.* 1991; Aradhya *et al.* 1993; Eldridge *et al.* 1994; Baril *et al.* 1997; Wei *et al.* 1997).

Hybrid studies of some red mahogany species have been carried out and some hybrids established in plantations. The understanding of genetic relationship among species could be of importance for hybrid breeding programs (Potts *et al.* 2004). Hybrid research has been carried out for some red mahogany members (mainly *E. urophylla*) (Ikemori *et al.* 1986; Cremiere L 1988; van Wyk *et al.* 1988; Lambeth CC 1990; Brouard *et al.* 2000). Interspecific hybrid seedlings from control pollination of *E. urophylla*, *E. grandis* and *E. pellita* have been tested in species/progeny trials in Aracruz, southern-eastern Brazil (Camphinos *et al.* 1989). In Mindanao Island, Philippines, the production of interspecific hybrids between *E. pellita* and *E. urophylla, E. deglupta* by control pollination has commenced. Control pollination between *E. pellita and E. urophylla* has also been started in Congo. In some species/provenances/families trials in Brazil, Cuba, Australia and Vietnam, putative natural hybrids between *E. pellita* and other eucalypts (*E. tereticornis, E. grandis, E. brassiana*) grew very well and were better than the pure parents (Harwood 1998; Kha *et al.* 2000).

1.8. The Red Mahoganies – Genetic Studies

Genetic, morphological and variation in oil composition have been studied in *E. urophylla, E. pellita* and *E. scias* (House *et al.* 1994; Pryor *et al.* 1995; Pinyopusarerk *et al.* 1993; Payn *et al.* 2007; Singh *et al.* 1988; Doran *et al.* 1995). Genetic diversity, mating system and systematic relationship in *E. pellita* and *E. scias* have been carried out by House *et al.* (1996). The isozyme studies on six New Guinea (NG) and eleven Australian provenances of *E. pellita* showed clear separation of these two seed sources based on Nei's 1978 unbiased genetic distance (D = 0.09). Two out of 16 *E. pellita* provenances had lower outcrossing rate in comparison with other eucalypts. They are Bupul –Muting, NG (t= 0.49 ± 0.08) and Lankelly Creek, Cape York (t= 0.45 ± 0.06) provenances. The other population, Kuranda from Queensland (with t= 0.73 ± 0.05) was closer to the overall mean of the outcrossing rate for natural populations of the genus (t = 0.74 by Eldridge *et al.* 1993). There was a significant positive correlation between latitude and the observed heterozygosity in *E. pellita*. However, a correlation between latitude and other genetic diversity parameters (A, P, H_e) was not evident.

In their study, House *et al.* (1996) also examined 400 samples in 8 populations of three different defined subspecies of *E. scias* (50 seedlings per population). While *E. scias* was clearly differentiated from *E. pellita,* no patterns relating to the subspecies division were found. *Eucalyptus scias* and *E. pellita* were believed to have a common ancestor that has spread and changed through genetic drift and selection from southern coastal parts of NSW (House *et al.* 1996).

A study of morphology in *E. urophylla*, *E. pellita* and *E. scias* was done by Pinyopusarerk *et al.* (1993). In their study, a difference in morphology between some species was found,

as well as between provenances within *E. urophylla*. The results from glasshouse trials showed that the stem and petiole colour of seedlings collected from NG and Cape York were paler than those from Southern Queensland (southern provenances) and their leaves also were smaller (Pinyopusarerk *et al.* 1993). These differences in colour were also found in 1-4 year old trees of *E. pellita* in trials in Australia and Malaysia. The north eastern Queensland provenances had reddish-purple upper twigs; the NG provenances were a green colour, while the Cape York provenances were intermediate in colour. Although not confirmed by comprehensive population sampling, a difference in fruit and leaf size between NG/CYP and north eastern Queensland provenances was also recognized; NG/CYP seedlings seem to be smaller in fruit and leaf size than those in other populations (Harwood 1998). Leaves of *E. scias* were generally shorter and narrower than those in *E. urophylla* and *E. pellita*. Seedlings, morphologically characterised in *E. urophylla,* were highly variable compared to other species, due to the distinctive features of Wetar populations (Pinyopusarerk *et al.* 1993).

Wood properties variation of 3 year old trees from Kerala, India also has been carried out by Bhat *et al.* (1987). In this study, fibres in *E. pellita* were longer than in *E. camaldunesis* and *E. tereticornis*. Wood density in *E. pellita,* however, was the lowest in four species studied.

1.9. Microsatellites

1.9.1. Definition

Microsatellites, or simple sequence repeat (SSR) markers, are short tandemly repeated sequences, consisting of repeated units of 1- 6 bp in length, that are found in every organism (Hancock 1999). The most common repeat types are: (1) Dinucleotide repeats, (2) Trinucleotide repeats and (3) Tetranucleotide repeats. Dinucleotide repeats occur most frequently in genomes and their density varies widely with species, hence they have been used frequently to study genetic diversity. In plants, dinucleotide repeats differ between species and are rich in either TA or GA repeats (Depeiges *et al.* 1995). In *Eucalyptus*, the majority of dinucleotide repeats are GA (Byrne *et al.* 1996). This type is also one of the most frequent repeat varieties in *Pinus* species; the most common repeats in this genus are AC and AG (Smith *et al.* 1994; Echt *et al.* 1997).

The important features of microsatellite markers for genetic studies are that they are codominant, and display high heterozygosity among and within individuals, populations and species. They are believed to be typically selectively neutral and randomly distributed across the entire genome (Jarne *et al.* 1996).

1.9.2. Advantages and disadvantages of microsatellites

In general, like other DNA markers, microsatellites have some powerful aspects:

1. Potentially, large numbers of polymorphisms can be identified.
2. Widely differing levels of polymorphism can be studied.
3. Both Mendelian and non-Mendelian inheritance can be identified, because microsatellite markers reside in the nucleus and the cytoplasmic organelles.

4. They are highly variable codominant loci.
5. They are PCR (polymerase chain reaction) based markers, so they are easy to automate and are relatively cheap to assay large numbers of individuals.

A disadvantage, however, at least in plants, has been the considerable time taken in the development of microsatellite loci. This will not be a problem for the current study, however, as they are expected to transfer efficiently from very closely related species. A large number of SSR are available in *E. grandis* and *E. urophylla* (Brondani *et al.* 1998; Brondani *et al.* 2002). Transfers of microsatellite markers from amongst related species has been demonstrated to be relatively efficient in eucalypts (Byrne 2008; Butcher *et al.* 2008; Shepherd *et al.* 2008; Payn *et al.* 2007).

1.9.3. Application of the microsatellite markers in forest tree studies

Microsatellites were first found to be abundant in plants by Morgante and Oliveieri in 1993, since then many have been developed for forest tree species. In 1994, Smith and Devey first developed two microsatellites from *Pinus radiata*, these markers showed high level of polymorphism with six alleles detected and observed heterozygosity from 0.6 – 0.65. Recently, SSR markers were developed widely in many forest tree species: *Pinus* species (Echt *et al.* 1997; Karhu *et al.* 2000), *Quercus* spp. (oaks) (Dow *et al.* 1995), Eucalypts (Byrne *et al.* 1996; Butcher *et al.* 2008; Shepherd *et al.* 2006; Shepherd *et al.* 2008; Payn *et al.* 2008; Stokoe 2002), *Acacia mangium* (Butcher *et al.* 2000) and some tropical forest species (Chase *et al.* 1996; Ujino *et al.* 1998).

Microsatellites can be used for quantification of genetic diversity; genotype verification, gene mapping and marker-assisted selection (MAS). The most important application of

microsatellite markers is the study of genetic structure and genetic diversity in population studies (Wang *et al.* 2001). SSR markers have been used to investigate the level of genetic variation and genetic differentiation between and within populations, especially for populations that reveal low levels of diversity detected by others types of markers. Analysis of the genetic structure of forest tree species gives insight into an ongoing process of genetic differentiation among populations (Wang *et al.* 2001).

Microsatellites have also been widely used for germplasm identification, mating system and taxonomic studies. The power of microsatellite markers to genetically discriminate between individuals or groups of individuals is typically high (Nybom *et al.* 1990; Scala *et al*. 1999). Microsatellites can also be used in seed orchard management for pollen contamination from outside sources, as well as for studying mating patterns and male fertility variation. For example, six microsatellite loci were used for estimating outcrossing rates in six different seed orchards of *Acacia mangium* in Vietnam (Butcher *et al*. 2002). The results indicated four seed orchards had high outcrossing rates; no selfing was detected in the Dong Ha orchard, whereas the Ba Vi Fortip seed orchard had the highest selfing rate (87%).

Quantitative traits, such as wood yield, wood quality, or pulp yield, are usually controlled by many genes located at quantitative trait loci (QTL). By using molecular markers closely linked to, or located within one or more QTL, information at the DNA level can be used for early selection. The potential benefit of MAS is greatest for traits that are difficult, time-consuming or expensive to measure (for example wood quality traits and pulp yield) and for traits which only appear under certain conditions, such as resistance to a particular pest, pathogen or abiotic factors such as salinity. However, mapping and MAS tend to be

used only in species which have a high economic value and have the best potential in clonal breeding programs.

Microsatellites also have been used to establish genetic linkage maps in some economical and commercial forest species such as *Eucalyptus grandis* and *E. urophylla* (Brondani *et al.* 1998), *Pinus radiata* and *P. taeda* (Devey *et al.* 1999), *Acacia mangium* (Butcher *et al.* 2000) and *Eucalyptus cloeziana* (Stoke 2002) in combination with other markers, for example isozyme, RFLPs and RAPDs.

1.10. Structure Analysis

In order to investigate genetic relationships without imposing preconceived taxonomic or geographic relationships upon the taxa in this study, genetic groupings will be determined using the Bayesian model-based approach of the STRUCTURE program (Pritchard *et al.* 2000).

The STRUCTURE approach uses a clustering method upon multilocus genotype data to infer population structure and assign individuals to genetic groups. Importantly it imposes no groupings, taxonomic, geographic or otherwise, upon the data during the determination of the most-appropriate model of structure. Individuals are assigned to a population based on their genotype, whilst simultaneously estimating population allele frequencies and model optimisation by maximising Hardy-Weinberg and linkage equilibrium. A hierarchical approach will be used to successively analyse structure, with the entire dataset examined initially to identify the major sources of structure, then the analysis is iterated for each subpopulation identified to identify any further substructure (Prichard *et al.* 2000; Evanno *et al.* 2005).

Chapter 2. Genetic Differentiation Within Three Red Mahoganies, *Eucalyptus pellita, E. resinifera* and *E. scias* – A Taxonomic-based Analysis

2.1. Abstract

This chapter reports the population structure and genetic diversity within each taxa. Population parameters are based on 13 microsatellite markers and the application of standard F statistic and AMOVA analyses in the GenAlEx V6 software program. The suitability of the microsatellite markers for this purpose was first evaluated by testing linkage disequilibrium (LD) and checking for null alleles and other artifactual effects that might influence genetic diversity. The lack of LD amongst loci was consistent with the independence of the selected loci based on genetic mapping studies in other species indicating the loci represented independent samples of the genome of each taxon. A lack of evidence for null alleles favoured the reliability of diversity estimates in this study.

The study found that *E. resinifera* had more genetic diversity than the two other red mahoganies studied, *E. pellita* and *E. scias*. Geography was a major factor for dividing *E. resinifera* into two subspecies or geographic races, one from North Queensland (includes Mareeba and Ravenshoe populations) and the other from Fraser Island southward with most populations in NSW and will be referred to as Southern *E. resinifera*. There was strong evidence for splitting *E. pellita* into two subspecies or species, one from New Guinea (includes Papua New Guinea and Indonesia) and one from North Queensland, Australia (Cape York population is not included). There was no support for the separation of *E. scias* into two or three subspecies. These relationships among taxa in these three red mahoganies at the species level and below are explored further in Chapter 3 using a

STRUCTURE analysis, where the analysis proceeds without the *a priori* assumptions regarding taxa and population groups that restrict this analysis.

2.2. Introduction

This chapter aimed to verify and complement the genetic variation and population structuring in *E. pellita* and *E. scias* that was found using isozyme markers by House *et al.* (1996) and provide the first study of genetic variation and population structuring in *E. resinifera*. The following questions were addressed:

1. As there has been no previous study of *E. resinifera*; does it form a cohesive genetic unit given its extensive and disjunct distribution?
2. Is there a genetic basis for recognition of two subspecies of *E. resinifera*? Do they align with current taxonomic subdivision into subsp. *resinifera* and *hemilampra*?
3. Is there genetic evidence for splitting *E. scias* into three subspecies?
4. As Hill and Johnson 2000 proposes, is there a genetic basis for subdivision of *E. pellita* into two species based on New Guinea and North Queensland divisions?

2.3. Materials and Methods

2.3.1. Plant material and DNA isolation

The study was based on 192 individuals sampled from family-within-provenance trials in Vietnam and New South Wales. These trials utilised individual-tree seedlot collections made by the Australia Tree Seed Centre (CSIRO) and family identity was maintained for all trees in the trial. Provenance, CSIRO seedlot numbers (which represent a provenance and year of collection rather than individual-tree seedlot numbers) and number of seedlings sampled per seedlot are detailed in Table 2 and mapped in Figure 1. Foliage samples were collected from one tree per family for each of *E. pellita*, *E. resinifera* and *E. scias* from a Forests NSW species and provenance-within-species trial near Woolgoolga in Northern NSW in April 2008. This trial was largely composed of material of Australian origin but included a small number of families sourced from a seedling seed orchard (SSO) on Melville Island (Northern Territory, Australia). The Melville Island SSO was established jointly by CSIRO and the Conservation Commission of the Northern Territory in 1991 and was composed of a mix of Queensland and Papua New Guinea (PNG) families (Harwood *et al.* 1997). This included samples from three *E. pellita* seedlings planted in the Woolgoolga trial that were from an open pollinated single-tree seedlot from a tree of PNG origin in the Melville Island SSO in this current study. To expand the representation of NG *E. pellita* from native forest NG sources, one tree per family was also sampled from a Forest Science Institute of Vietnam (FSIV) trial in Vietnam. This material along with *E. urophylla* material from another FSIV trial was collected, stored dried, and shipped to Australia in early 2008. Samples from two additional individuals of *E. urophylla* from East Timor, collected from natural stands by Nigel Slator in July 2008 were also included in the study (see details in Table 2).

Table 2. Natural location, number of trees sampled and seedlot numbers for red mahoganies used for this study. NB. Location 19 for *E. pellita* was a mixed-provenance seed orchard and therefore the geographic coordinates are not provided.

Index	Species	Location Identifier	Natural Location	Latitude and Longitude	No. Ind.[1]	Seedlots no. (CSIRO)
1	*resinifera*	RAVE	Ravenshoe	17^{0}42’S, 145^{0}28’E	5	*12411*
2	*resinifera*	MARE	SW Mareeba	17^{0}10’S, 145^{0}33’E	8	*14421*
3	*resinifera*	FRAS	Fraser Island	25^{0}37’S, 153^{0}00’E	2	*13573*
4	*resinifera*	POMO	NW Pomona	26^{0}15’S, 152^{0}48’E	7	*13982*
5	*resinifera*	BEER	WNW Beerburrum	26^{0}56’S, 152^{0}50’E	7	*13981*
6	*resinifera*	STRA	Stradbroke Is	27^{0}25’S, 153^{0}25’E	1	*13980*
7	*resinifera*	EWIN	Ewingar SF	29^{0}10’S, 152^{0}29’E	2	*16896*
8	*resinifera*	COFF	N Coffs Harbour	29^{0}52’S, 153^{0}15’E	7	*13571*
9	*resinifera*	WOOL	N Woolgoolga	29^{0}53’S, 153^{0}13’E	4	*13978*
10	*resinifera*	COFF	SW Coffs Harbour	30^{0}12’S, 153^{0}30’E	6	*13977*
11	*resinifera*	WOOL	W of Woolgoolga	30^{0}40’S, 153^{0}10’E	7	*13322*
12	*resinifera*	KEND	NE of Kendall	31^{0}34’S, 152^{0}47’E	7	*13318*
13	*resinifera*	KEND	NNE of Kendall	31^{0}43’S, 152^{0}47’E	7	*13975*
14	*resinifera*	NOWR	Nowra	35^{0}20’S, 150^{0}36’E	7	*13953*
15	*pellita*	BUPU	Bupul Muting Indonesia	7^{0}21’S, 140^{0}36’E	8	*17854*
16	*pellita*	GOE	Goe PNG	8^{0}20’S, 141^{0}32’E	6	*19207*
17	*pellita*	KIRI	Kiriwo PNG	8^{0}25’S, 141^{0}30’E	6	*18197*
					6	*19206*
18	*pellita*	SERI	Serisa PNG	8^{0}36’, 141^{0}26’E	16	*18199*
19	*pellita*	SSO	SSO		3	*18602*
20	*pellita*	HELE	Helenvale	15^{0}49’S, 145^{0}14’E	5	*18774*
21	*pellita*	STAR	Starcke Stn	15^{0}05’S, 145^{0}12’E	5	*18313*
22	*pellita*	MOSS	Mossman	16.26’S, 145^{0}24’E	3	*18748*

Index	Species	Location Identifier	Natural Location	Latitude and Longitude	No. Ind.[1]	Seedlots no. (CSIRO)
23	*pellita*	KURA	NW Kuranda	$16^0$41'S, $145^0$32'E	6	*17861*
24	*pellita*	ELAR	El Arish	$17^0$50'S, $146^0$03'E	7	*18314*
25	*pellita*	KIRR	Kirrama Range	$18^0$12'S, $145^0$46'E	8	*18773*
26	*pellita*	CARD	S Cardwell	$18^0$25'S, $147^0$56'E	9	*18597*
27	*scias* subsp. *apoda*	BOON	Boonoo Boonoo	$28^0$54'S, $152^0$09'E	5	*18282*
28	*scias* subsp. *apoda*	MALA	Malara	$29^0$08'S, $152^0$18'E	2	*18283*
29	*scias* subsp. *apoda*	MtBA	Mt Banda Banda	$31^0$09'S, $152^0$27'E	2	*18295*
30	*scias* subsp. *callimastha*	BROO	South Brooman SF	$35^0$30'S, $150^0$15'E	6	*18320*
31	*scias* subsp. *scias*	MtBO	Mt Bouddhi NP	$33^0$31'S, $151^0$24'E	4	*18294*
32	*urophylla*	WETA	Utak, Wetar	$7^0$39'S, $124^0$31'E	3	*17836*
33	*urophylla*	ALOR	Waikul Central Alor	$8^0$14'S, $124^0$44'E	1	*17840*
34	*urophylla*	TIMO	E Timor	$8^0$30'S, $126^0$00'E	2	
35	*urophylla*	FLOR	Egon, Flores	$8^0$38'S, $122^0$58'E	2	*17567*
	Total				***192***	

[1]: Number of individuals

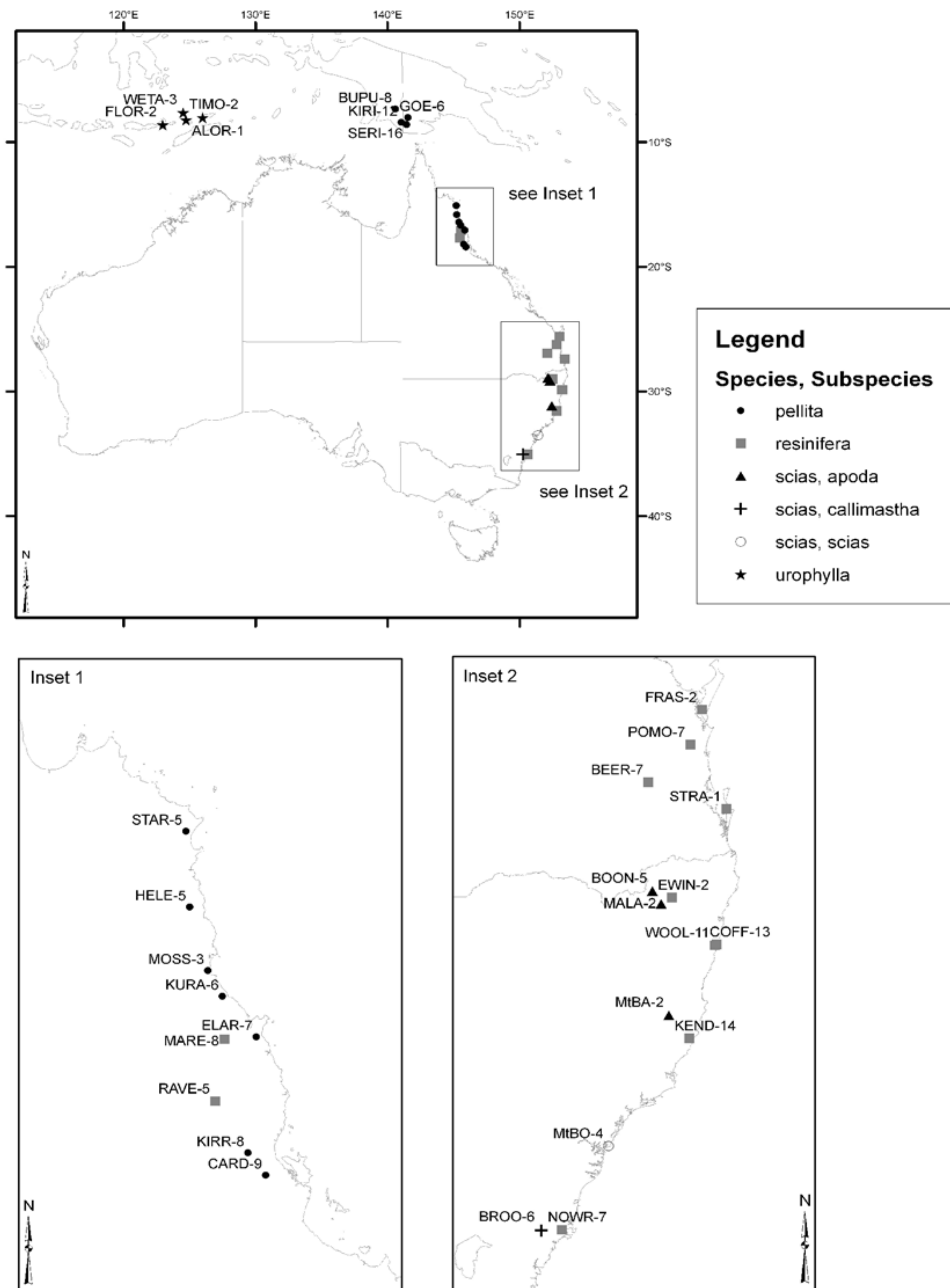

Figure 1. Natural origins of materials used in this study. Samples were obtained from planted sources, tree breeding trials at Woolgoolga (NSW Australia) or Vietnam (Sampling source locations not shown). See Table 1 for explanation of Locality code. Number following locality code is the number of trees sampled (e.g. CARD-9: 9 samples were collected from Cardwell, Qld).

Total genomic DNA was extracted from 30 - 40mg of dried leaf tissue using a Qiagen DNeasy 96 Plant Kit (Qiagen Pty Ltd, Doncaster, Australia) according to the protocol described in Shepherd *et al.* (2006). DNA quality and quantity were determined by agarose gel electrophoresis and comparison with standard amounts of DNA using ethidium bromide staining and UV visualisation.

2.3.2. Microsatellite analysis

2.3.2.1. Candidate primer information

Microsatellite genotyping was conducted as described by Shepherd *et al.* (2008). For this study, 48 informative SSR markers isolated from *E. grandis* and *E. urophylla* (Brondani *et al.* 2001; Brondani *et al.* 1998; Brondani *et al.* 2006) (Appendix 1) were initially evaluated for transfer on a set of 32 samples from the target species, 8 samples from each of the Queensland *E. pellita*, NG *E. pellita*, *E. scias* and *E. resinifera* collections. Fluorescently labelled primers were synthesized by Proligo (VIC, Ned, PET and 6-Fam) in which fluorophores were added to the 5' end of the oligonucleotides for detection of SSR amplicons on the ABI capillary electrophoresis instruments.

2.3.2.2. PCR amplification

PCR was carried out using the amount of reagents as described in Table 3 below. Microsatellite loci were amplified using a series of touchdown programs with annealing temperatures (T_a) spaning 10°C. The annealing temperature range was centred on the optimal T_a for each primer-pair. Optimal T_a was determined as 5°C less than the primer

with the lowest T_m. For example, a primer-pair with an optimal T_a of 50°C was assigned a program with an initial annealing temperature of 55°C which decreased to 45°C over 20 cycles, achieved by decreasing the temperature by 1°C every second cycle. Cycling was composed of a 95°C hold for 1 minute, annealing temperature for 1 minute and 72°C for 1 minute followed by a further 20 cycles using the final annealing temperature. Cycling was preceded by a hold at 95°C for 5 minutes to provide a "hot start" and finished with a final hold of 3 minutes at 72°C.

Table 3. PCR reaction composition. (NB amounts include 20% extra to allow for loss during pipetting)

Reagents	Working solutions	Volume for a PCR reaction (12 µl)	Final concentration
Forward primer	20 µM	0.12 µl	0.24 µM
Reverse primer	20 µM	0.12 µl	0.24 µM
$MgCl_2$	50 mM	0.48 µl	2.4 mM
dNTPs	20 mM	0.48 µl	0.96 mM
Buffer	10 x	1.2 µl	1.2 x
Taq	5 U	0.05 µl	0.02 U
DNA template	0.2 ng/µl	5.0 µl	1 ng
BSA	10X	1.2 µl	1.2X (0.72µg/ µl)
H_2O		3.35 µl	

2.3.2.3. Microsatellite and genotyping detections

Microsatellite PCR products were first run on agarose to check the amplification of the PCR, using DNA MW Marker XIV (100-1500bp) (Cat#11 721 933 001, Roche Applied Science, Germany) as a ladder. Marker rating from Prescreen using low resolution agarose gel data as follows:

0= no amplification in focal

1= amplification in focal

2 = amplification in non-focal

3=amplification and poly

Raw electro-pherogram data was processed using GenMapper V3.7 software (Applied Biosystems). Marker rating was also repeated for the AB 3730 data as follows:

0 = no amplification in focal taxa

1 = amplification in focal taxa only

2 = amplification in focal but monomorphic

3 = amplification in focal, polymorphic but can’t score

4 = amplification in focal, polymorphic, manual score

5 = amplification in focal, polymorphic, assisted-automated score

6 = amplification in focal, polymorphic, automated score.

Automated fluorescent scanning detection of DNA enables separation and quantification of DNA fragments. Fluorescent dyes attached to the fragments via labelled primers emit light at specific wavelengths which could be separated by a spectrograph according to emission frequency. The ABI 3730 DNA analyser uses a 48 capillary array and 36 cm capillary length and the samples were run in two hours at 8,000 volts.

2.3.2.4. Loci selection

Based on ease of automated scoring, transferability to all taxa, gene diversity, a lack of null alleles, and minimum linkage disequilibrium, 13 loci were selected for further analyses with full samples (Table 4).

Table 4. Genetic diversity, allele range and amplification conditions for 13 microsatellite loci summarised over a sample of 32 individuals from three taxa

Index	Marker	H_e [C]	Allele size range (bp)	PCR Cycling program (T_a)[A]	Marker score [B]
1	EMBRA 006	0.84	62-150	52	5
2	EMBRA 011	0.84	72-150	50	6
3	EMBRA 020	0.95	120-150	45	5
4	EMBRA 028	0.92	175-226	52	5
5	EMBRA 042	0.94	107-149	50	5
6	EMBRA 068	0.94	77-106	50	5
7	EMBRA 081	0.90	86-114	52	6
8	EMBRA 120	0.90	131-161	50	6
9	EMBRA 175	0.92	58-98	50	5
10	EMBRA 209	0.89	113-151	52	5
11	EMBRA 210	0.95	205-227	48	5
12	EMBRA 214	0.90	110-156	50	6
13	EMBRA 242	0.88	138-170	52	5

[A] A standardised touchdown PCR cycling program was used for all primers which varied according to the starting annealing temperature. See Shepherd *et al.* (2006).

[B] Marker score is a rating from 0 – 6 assessing the transferability to non-focal taxa, the polymorphism and amenability of the locus for scoring using software for automated scoring of marker phenotype generated on a AB 3730 Genetic Analyser. 5 = amplification in focal, polymorphic, assisted-automated score, 6 = amplification in focal, polymorphic, automated score.

[C] H_e = Hardy-Weinberg expected heterozygosity (Nei 1973) based on a mix of 32 individuals from *E. pellita*, *E. resinifera* and *E. scias*.

Linkage groups nomenclature for these markers were based on *E. grandis* and *E. urophylla* maps and adapted from Brondani *et al.* (1998, 2002 and 2006) and are shown in the Table 5 below.

Table 5. Linkage group for 13 SSR markers based on *E. grandis* and *E. urophylla* by Brondani *et al.* (1998, 2002 and 2006)

Linkage groups	**Makers name**	**Distance to the next marker (centiMorgan - cM)**
Group 1	EMBRA 006	52.2
	EMBRA 011	
Group 2	EMBRA 068	
Group 5	EMBRA 242	15.1
	EMBRA 120	74.7
	EMBRA 209	1.2
	EMBRA 214	
Group 6	EMBRA 028	22.2
	EMBRA 175	38.5
	EMBRA 020	2.6
	EMBRA 081	
Group7	EMBRA 042	
Group 9	EMBRA 210	

2.3.3. Population genetic analysis

2.3.3.1. Test for linkage disequilibrium

Linkage disequilibrium is the non-random association between alleles of different loci on the same or different chromosomes. Linkage disequilibrium (LD) describes a situation in which some combinations of alleles or genetic markers occur more or less frequently in a population than would be expected from a random mating of haplotypes from alleles based on their frequencies. Linkage disequilibrium can be created and maintained, by selection favouring one combination of alleles over another or simply by close association of genetic

markers in the same linkage group. Linkage disequilibrium can also arise from intermixture of populations with different gene frequencies, or from change in small populations (or subdivision of populations).

The null hypothesis (Ho) for the LD test was that the genotypes at one locus are independent from those at other loci. The analysis of LD was carried out for each species using FSTAT software (Goudet *et al.* 2004) with significance levels adjusted for multiple comparisons using Bonferroni correction. This indicates the number of independent sample points in the genome.

2.3.3.2. Tests for Hardy-Weinberg Equilibrium and null alleles

In large random-mating populations without selection, mutation or migration, the gene frequencies and the genotype frequencies are constant from generation to generation. This state is called Hardy – Weinberg principle or Hardy-Weinberg Equilibrium (HWE). So, a population in HWE has constant gene and genotype frequencies.

The testing for Hardy Weinberg Equilibrium (HWE) at each locus was carried out for each species by using Genepop V4 (Rousset 2008) with the parameters, dememorisation set to 10000, batches 20 and interactions per bath 5000. A Bonferroni correction was applied for an experiment-wise error rate of 5% (Rice 1989). The χ^2 test for HWE test was also conducted by GenAlEx V6 software for each locus in each population (Peakall *et al.* 2006).

The Microchecker program V 2.2.3 (Oocterhout *et al.* 2004) was also used to test for null alleles. This program detects possible null alleles based on Monte Carlo simulation (bootstrap) methods to generate expected homozygote and heterozygote alleles size difference frequencies. It is also based on the Hardy – Weinberg theory of equilibrium to calculate expected alleles frequencies and the frequencies of any null alleles was detected. These modules were also used for the identification of errors that might due to the PCR process, such as large-allele dropout effects (large alleles do not amplify as efficiently as small alleles) and stuttering - the slight changes that occur in the allele size during PCR (Oocterhout *et al.* 2005).

2.3.3.3. Population genetic parameters detection

Genetic diversity parameters for each species were obtained using GenAlex V6 (Peakall *et al.* 2006), in which the Frequency module and the codominant allele frequencies option were applied. Diversity criteria included: percentage of polymorphic (P%), number of different alleles (N), number of effective alleles (Ne), observed heterozygosity (H_o), expected heterozygosity (H_e), unbiased expected heterozygosity (UH_e) and inbreeding coefficients (F). The UH_e may be a more reliable measure of expected heterozygosity when sample size is small (Nei 1978).

Population structuring in each taxa was studied using standard F statistics and an AMOVA. F statistics were derived from GenAlex V6 using the Frequency module, the codominant allele frequencies option and with 99 permutations. For each species, all populations were pooled and F statistics were obtained for each individual locus to examine locus specific as well as genome-wide inbreeding effects (Peakall *et al*. 1995). In addition, a model with a further level of structure was generated using a region (or in the case of *E. scias* a

subspecies) level. These analyses were conducted using the AMOVA module of GenAlex. This analysis was used to partition genetic variation among and within regions (or subspecies in *E. scias*) and test the significance of these partitions (99 permutations).

2.4. Results

2.4.1. Genetic diversity and population structure in *Eucalyptus pellita*

The mean genetic diversity (H_e) for *E. pellita* was 0.68 (Table 6). The unbiased measure of gene diversity (UH_e) for *E. pellita* was notably higher (0.74), however, suggesting that the small sample sizes of some provenances downwardly biased gene diversity of this taxon and this unbiased estimate may be more appropriate when comparing gene diversity to other studies (Table 6).

Table 6. Genetic diversity in 12 populations of *E. pellita (n=85)*

Location	P (%)	N	Ne	H_o	H_e	UH_e	F
Bupul Muting	100.00	5.231	3.68	0.668	0.671	0.716	-0.009
South of Kiriwo	100.00	6.615	4.53	0.758	0.730	0.782	-0.045
Serisa	100.00	8.231	4.74	0.627	0.745	0.774	0.173
Kirowo	100.00	4.462	3.70	0.769	0.680	0.777	-0.115
Goe	100.00	5.077	3.78	0.524	0.651	0.724	0.233
Kuranda	100.00	5.000	3.44	0.654	0.673	0.734	0.026
Starcke	100.00	5.615	4.15	0.846	0.748	0.831	-0.133
El Arish	100.00	5.231	3.56	0.615	0.651	0.702	0.034
Cardwell	92.31	6.154	4.02	0.684	0.673	0.713	-0.019
Mossman	92.31	3.538	2.87	0.641	0.594	0.713	-0.091
Kirrama	100.00	5.077	3.38	0.625	0.611	0.654	-0.041
Helenvale	92.31	5.538	4.50	0.719	0.701	0.783	-0.031
Over loci and populations							
Mean per race	98.08	5.48	3.86	0.678	0.677	0.742	-0.001
SE		*0.161*	*0.127*	*0.020*	*0.015*	*0.016*	*0.021*
Across species							
Mean	***100.00***	***18.01***	***6.623***	***0.670***	***0.806***	***0.811***	***0.172***
SE		***1.201***	***0.827***	***0.035***	***0.033***	***0.033***	***0.026***

N= the number of different alleles, P = polymorphic percentage, Ne= the number of effected alleles, H_o = observed heterozygosity, H_e= Expected heterozygosity, UH_e = Unbiased heterozygote, F= fixation index.

The level of genetic diversity was relatively even across provenances, ranging between 0.59 and 0.75 (Table 6). Most provenances had low or negative fixation coefficients (F) values, except for two New Guinea (NG) provenances Serisa and Goe, which had F values of 0.17 and 0.23, respectively which may indicate some inbreeding (selfing or neighbour) in these populations (Table 6). Almost all markers were polymorphic in all populations of *E. pellita*, with only one marker out of the thirteen fixed in three Northern Queensland (NQ) populations (i.e. P = 92.3 %; Table 6).

These relatively high values across all markers studied suggested a genome-wide influence structured this species. Comparison with the other three species studied showed that *E. pellita* had the highest population structuring (i.e. largest mean F_{st} value (Tables 7).

Table 7. Hierarchical F statistic analysis for three red mahoganies. Between regions (F_{rt}) (regions in *E. pellita, E. resinifera* and subspecies in *E. scias*), among populations within regions (F_{sr}), (F_{st}), (F_{is}) and F_{it}

Species	F_{rt}	F_{sr}	F_{st}	F_{is}	F_{it}
E. pellita (between regions)	0.079	0.039	0.115	0.168	0.263
E. resinifera (between regions)	0.041	0.019	0.059	0.108	0.161
E. scias (subspecies)	-0.019	0.088	0.071	0.027	0.096

The individual locus F_{st} values were uniformly high, ranging between 0.12 and 0.29, and averaging 0.16 across all 13 loci (Table 8).

Table 8. Individual locus F statistics for 13 SSR loci for *E. pellita* (n=85)

Locus	F_{is}	F_{it}	F_{st}
EMBRA006	-0.038	0.228	0.257
EMBRA011	0.020	0.131	0.113
EMBRA020	-0.023	0.138	0.157
EMBRA028	0.048	0.193	0.153
EMBRA042	0.044	0.181	0.144
EMBRA068	-0.052	0.152	0.194
EMBRA081	-0.122	0.035	0.140
EMBRA120	-0.001	0.146	0.146
EMBRA175	-0.024	0.115	0.135
EMBRA209	0.044	0.158	0.119
EMBRA210	0.123	0.276	0.175
EMBRA214	-0.106	0.030	0.123
EMBRA242	0.108	0.370	0.294
Mean	0.002	0.166	0.165
SE	0.020	0.025	0.015

Most of this among population differentiation was attributable to differentiation among regions (Figure 2). When *E. pellita* provenances were classified into NG or NQ regions, an AMOVA with a regional level partition indicated 8% of variance was attributable to the variation among regions component (F_{rt}) and 4% among populations within regions (F_{st}) (Table 8 and Figure 2). Most variation resided within individuals (73 %), with 15 % among individuals within a population.

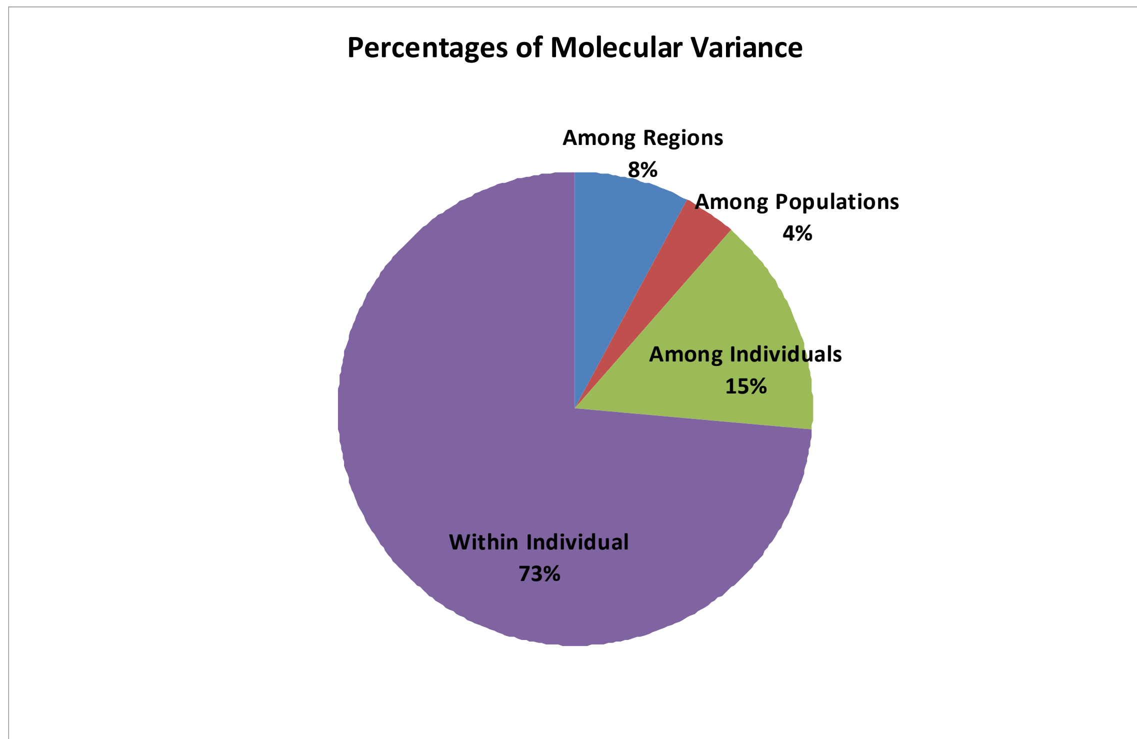

Figure 2. An AMOVA with a regional level for *E. pellita*

Testing for HWE for each locus on a species wide basis indicated all but three loci were significantly disturbed from equilibrium (Appendix 3). This suggested there was a genome-wide rather than locus-specific effect (such as selection) contributing to genetic structure in *E. pellita* and was consistent with the relatively large among population differentiation (F_{st}) and among regions (F_{rt}) components observed in the F statistics analysis. The test for null alleles using Microchecker analysis, which also uses disturbance from HWE, indicated four loci (EMBRA 242, 210, 028 and 020) may have null alleles (Appendix 4). However, because disequilibrium at the species level was expected due to the substructure in this species, this was not thought to be due to null alleles in this case.

2.4.2. Genetic diversity and population structuring of *Eucalyptus resinifera*

Eucalyptus resinifera was the most genetically diverse of the three species studied with a mean genetic diversity (H_e) of 0.73 (Tables 9). It was also the most heterogeneous in terms of levels of variation across provenances, as H_e ranged between 0.42 for Stradbroke Island and 0.84 for Woolgoolga and Kendall (Table 9).

Table 9. Genetic diversity for 11 provenances of *E. resinifera* (n=77)

Locations	**P**[1]	**N**	**Ne**	**H_o**	**H_e**	**UH_e**	**F**
Ravenshoe	100.00	5.077	3.827	0.742	0.640	0.712	-0.178
Mareeba	100.00	7.000	5.322	0.757	0.768	0.831	0.016
Fraser Island	100.00	3.538	3.385	0.962	0.692	0.923	-0.395
Pomona	100.00	7.538	5.870	0.820	0.788	0.856	-0.024
Beerburrum	100.00	7.385	5.570	0.718	0.760	0.819	0.063
Stradbroke	92.31	1.846	1.846	0.846	0.423	0.846	-1.000
Ewingar	100.00	3.308	3.195	0.808	0.615	0.821	-0.311
Coffs Harbour	100.00	10.923	7.279	0.839	0.834	0.867	-0.009
Woolgoolga	100.00	9.231	6.766	0.823	0.835	0.887	0.023
Kendall	100.00	10.308	6.645	0.873	0.835	0.866	-0.043
Nowra	100.00	8.615	6.612	0.866	0.827	0.891	-0.049
Over loci and populations							
Mean	*99.23*	*6.797*	*5.120*	*0.823*	*0.729*	*0.847*	*-0.161*
SE	*1.50*	*0.287*	*0.221*	*0.019*	*0.015*	*0.016*	*0.027*
Across species							
Mean	*100.00*	*19.692*	*9.943*	*0.821*	*0.876*	*0.882*	*0.067*
SE		*1.195*	*1.125*	*0.038*	*0.020*	*0.020*	*0.031*

[1] See Table 6 for explanation of column headings

Some of this variation however, was likely due to the small sample sizes in this species, and inspection of unbiased H_e (UH_e) (which adjust for small sample sizes (Nei, 1978)) indicates this was likely the case as UH_e for these provenances are more close to the species average. Number of alleles in each provenance also fluctuates widely in this

species but again, this parameter is more sensitive to small sample sizes than H_e and therefore likely reflects the small sample size in some provenances (White *et al.* 2007).

Population structuring was also relatively high for this abundant and widespread eucalypt species. The mean F_{st} value was 0.13 with a range of 0.08 to 0.25 (Table 10).

Table 10. Estimated value of F statistics for 13 SSR loci analysis in populations of *E. resinifera*

Locus	F_{is}	F_{it}	F_{st}
EMBRA006	-0.142	-0.017	0.109
EMBRA011	-0.163	-0.023	0.121
EMBRA020	-0.053	0.077	0.123
EMBRA028	-0.059	0.041	0.094
EMBRA042	-0.139	-0.048	0.080
EMBRA068	0.203	0.404	0.252
EMBRA081	-0.121	-0.016	0.093
EMBRA120	-0.115	0.029	0.129
EMBRA175	-0.104	0.096	0.181
EMBRA209	-0.072	0.046	0.110
EMBRA210	-0.107	0.032	0.125
EMBRA214	-0.032	0.067	0.096
EMBRA242	0.033	0.172	0.144
Mean	-0.067	0.066	0.128
SE	0.027	0.032	0.013

These relatively high values across all markers studied suggested a genome-wide influence structuring in this species. However, comparison with the other three species studied showed that *E. resinifera* had higher population structuring than *E. scias* and lower than *E. pellita* (Table 7).

Further, partitioning of structure variance using an AMOVA analysis and the fitting of a regional level for North Queensland and Southern provenances indicated most of the differentiation among populations was due to differentiation among regions (F_{rt}). Four

percent of variance was attributable to among regions, two percent among populations within regions, ten percent among individuals within a population and 84 % within individuals (Figure 3).

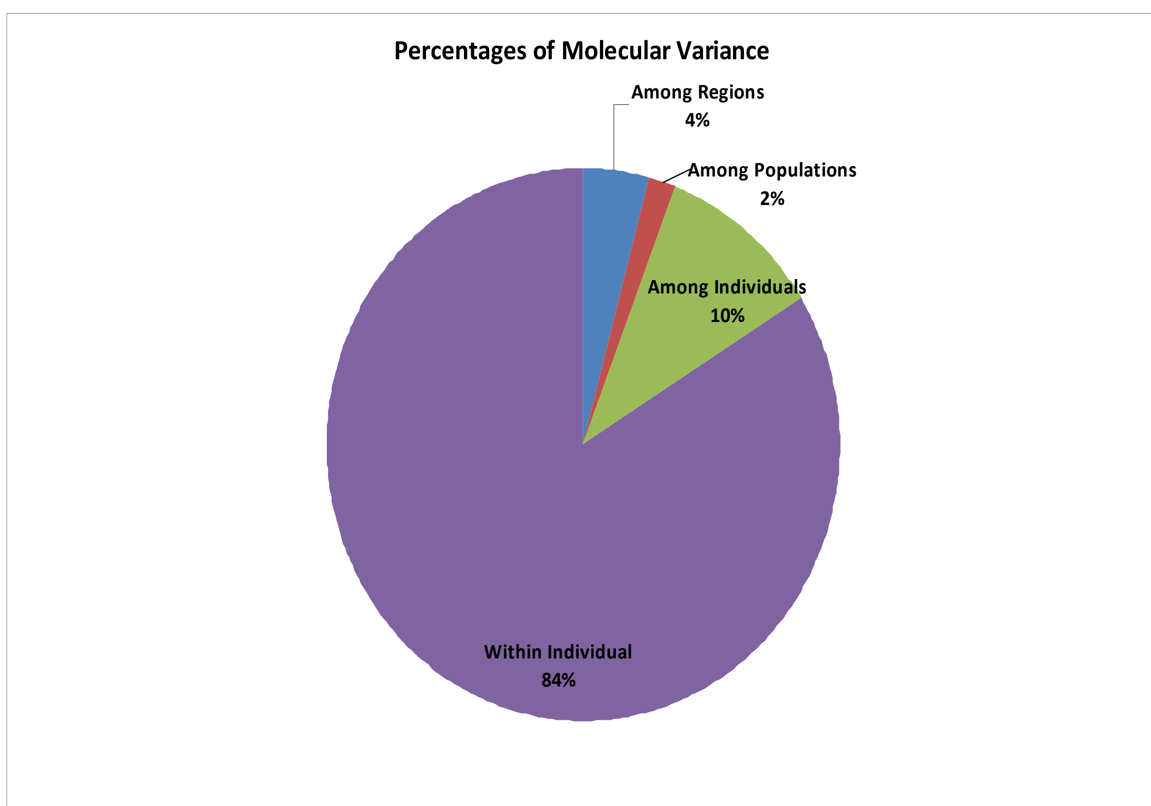

Figure 3. The AMOVA, input as allelic distance matrix for F-statistics analysis result in *E. resinifera*

Ten out of the thirteen loci studied were significantly disturbed from HWE when tested on a species wide basis (Appendix 3). This suggested a genome-wide factor was causing the disturbance in this species and was consistent with the strong structure indicated by the AMOVA analysis, as population substructure (Walhuld effect) produces a deficit of heterozygotes in the overall population (White *et al.* 2007; Hartl, 2000).

A Microchecker analysis for each locus at the species level indicated the potential for three loci to have null alleles (Appendix 4). However, given that this approach uses disturbances to HWE to detect nulls (Van Oosterhout *et al.* 2006), the substructure in this species probably accounts for the significance of these tests.

2.4.3. Genetic diversity and population structuring of *Eucalyptus scias*

The analysis of genetic diversity in *E. scias* was based on a small sample size (n=19) hence caution was needed in interpretation of diversity parameters. Assuming the sample size of 19 was adequate for a species-wide estimate of diversity the H_e value was 0.7 was higher than *E. resinifera* (0.67) but lower than *E. pellita* (0.73) for the same set of genetic markers (Tables 6, 9 and 11).

Table 11. Measures of genetic diversity in five populations of *E. scias (n=19)*

Locations	P[1]	N	Ne	H_o	H_e	UH_e	F
Boonoo	100.00%	5.538	4.240	0.781	0.740	0.826	-0.061
Malara	100.00%	3.231	3.015	0.769	0.635	0.846	-0.210
Banda	92.31%	2.692	2.497	0.692	0.529	0.731	-0.333
Brooman	100.00%	6.462	5.023	0.897	0.786	0.858	-0.136
Bouddhi	100.00%	5.615	4.900	0.942	0.783	0.898	-0.208
Over loci and populations							
Mean	98.46%	4.708	3.935	0.816	0.695	0.832	-0.187
SE	1.54	0.228	0.185	0.030	0.019	0.021	0.035
Across species							
Mean	100.00	13.615	8.563	0.840	0.875	0.899	0.042
SE		0.615	0.652	0.027	0.009	0.009	0.026

[1]See Table 6 for explanation of column headings.

Adjusting for the small sample size and comparing UH_e, the genetic diversity of *E. scias* (0.83), a regional species with disjunction, was similar to a widespread but disjunct species

E. pellita (0.85). Inbreeding coefficients for each population in *E. scias* as well as the overall value for the taxon were negative, indicating the observed heterozygosity was greater than expected in all populations; hence there was no evidence for inbreeding in this species despite its relatively restricted and disjunct distribution (Table 11).

Population structuring in this species was intermediate to that detected in the other two red mahoganies. The mean F_{st} from a standard F statistic analysis was 0.2 ± 0.02 (SE) for this species and was uniformly high across all loci suggesting genome-wide structuring in this species (Table 12).

Table 12. Estimated value of F statistics for 13 SSR loci analysis in populations of *E. scias*

Locus	F_{is}	F_{it}	F_{st}
EMBRA006	-0.122	0.039	0.144
EMBRA011	-0.057	0.103	0.152
EMBRA020	-0.155	0.164	0.276
EMBRA028	-0.036	0.159	0.188
EMBRA042	-0.368	-0.131	0.173
EMBRA068	-0.083	0.152	0.217
EMBRA081	-0.299	0.127	0.328
EMBRA120	-0.301	-0.107	0.149
EMBRA175	-0.286	0.101	0.301
EMBRA209	-0.140	0.079	0.192
EMBRA210	-0.154	0.014	0.146
EMBRA214	-0.208	-0.001	0.171
EMBRA242	-0.103	0.134	0.215
Mean	-0.178	0.064	0.204
SE	0.029	0.027	0.017

An AMOVA analysis with a subspecies level fitted indicated that most of the differentiation among populations was not due to an among subspecies component (Figure

4). The differentiation among subspecies component was zero, whereas the genetic differentiation among populations within subspecies component was 9% (Figure 4).

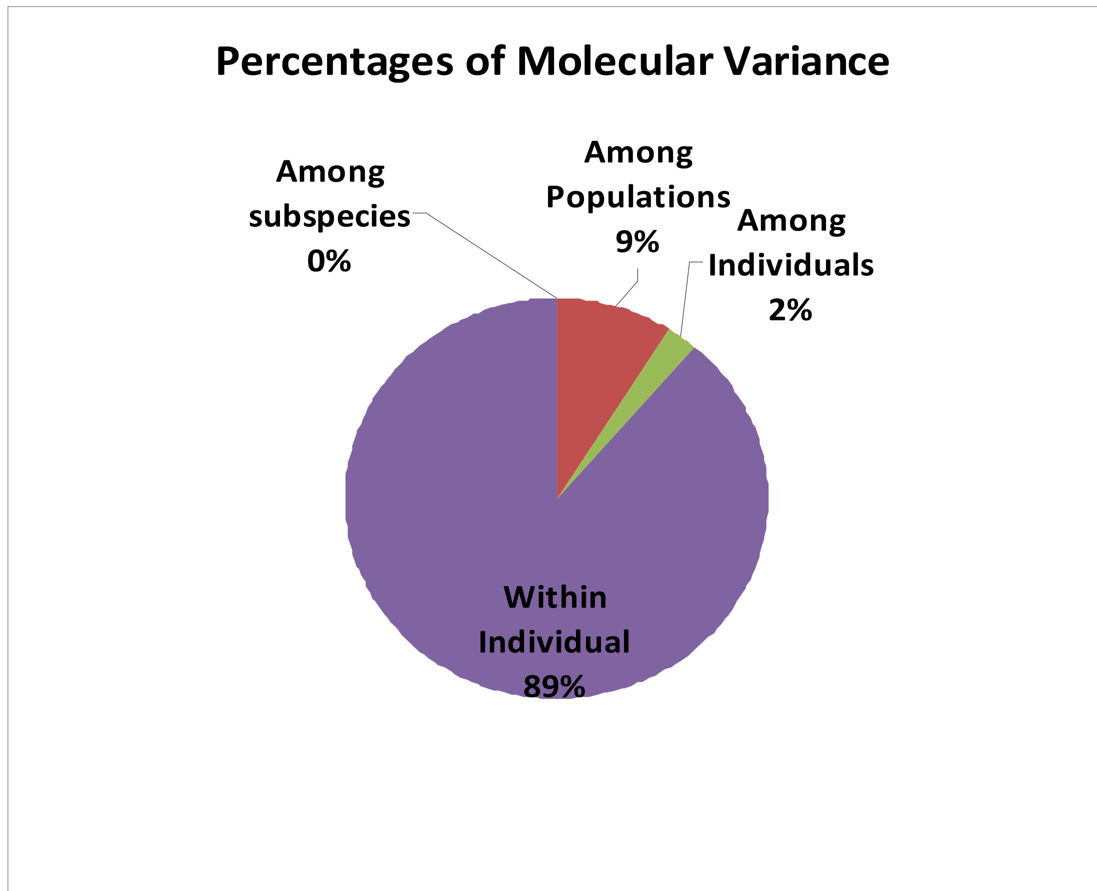

Figure 4. The AMOVA, input as allelic distance matrix for F-statistics analysis result in *E. scias*

Unlike the other two red mahoganies studied, relatively few loci (3) were disturbed from HWE in *E. scias* (Appendix 3). This suggested substructure was adequately defined with a provenance (population) level, with no cryptic substructure (i.e. Walhuld effect), unlike the other two species where substructure was not adequately defined without taking into account regional structure.

2.5. Discussion

2.5.1. Genetic diversity in three red mahoganies relative to other eucalypts

Genetic diversity of all three taxa was within the range expected for eucalypts with similar distribution types suggesting the breeding systems and life history characteristics of these species are typical as gene diversity is a function of a species' distribution type, breeding system, life history traits, population size and migration (Moran 1992, White et al. 2007). The distribution of a tree species can be a major determinant in the case of forest trees (Hamrick et al. 1989) and eucalypts are no exception (Moran 1992). Widespread species are typically more diverse within population than among populations. Because forest species are widespread they may have large population sizes and disjunct distributions and the patterns of genetic diversity often relate to their geographic distributions. The present study indicated that the three species examined have gene diversities and structures similar to other eucalypts with similar distributions. Generally, these results showed a lack of evidence for inbreeding in these species.

The expected heterozygosities were moderate to high in *E. resinifera* and *E. pellita* (0.88 and 0.81 respectively), they were within the range of other species in the genus *Eucalyptus* with similar geographic distribution types (Appendix 5). For example, they were slightly higher than another red mahogany (0.739 in *E. urophylla* by Payn *et al.* 2007) and other eucalypt species with widespread distributions (0.790 over 6 species by Byrne 2008). As expected, the H_e in *E. pellita* was lower than the two other species studied. This estimate of diversity for *E. pellita* may be an underestimate because the CYP population was not included in this study.

Despite the small sample size the number of alleles per locus in *E. scias* (13.61) was higher than for other regionally distributed *Eucalyptus* species assessed using microsatellites according to Byrne (2008) (5.45 over 6 species). The analysis also showed that the expected heterozygosity in *E. scias* was high in comparison with other *Eucalypts* with regional distributions (0.875 and 0.66 respectively).

The F_{st} value estimated for *E. scias* (without a subspecies level in the model) (F_{st} =0.2) (Table 12) was approximately or slightly lower than the mean for 6 other eucalypts with regional distributions (0.205; Byrne 2008), however, it was higher than *E. pellita* (0.17) and *E. resinifera* (0.13), suggesting more structuring between populations in this species (Appendix 5). Regional species with disjunction typically have higher F_{st} (or G_{st}) than widespread species (Moran 1992). This high F_{st} value may reflect the effects of drift or adaptation that have lead to greater differentiation among the small isolated populations of *E. scias*.

2.5.2. *Eucalyptus pellita* was distinct from *E. resinifera* but has two distinct geographically-based populaitons

In this study, *E. pellita* exhibited the strongest structure at the regions/species level with a F_{st} = 0.115 (F_{st} value from analysis with regional level included; Table 7). This high level of differentiation among regions was consistent with the isozyme research by House *et al.* (1996). The genetic differential between the NG and NQ populations of *E. pellita* (Figure

2) and the F_{st} estimate of this species (0.115) was greater than that for *E. resinifera* (0.058) and *E. scias* (0.071) (Table 7). This suggests that, based on the geographic distribution, *E. pellita* could be divided into two races or subspecies. This conclusion was consistent with the isozyme results from House *et al.* (1996) (despite the Cape York Peninsula (CYP) population not being included in this current research), who also found two groups within *E. pellita* and proposed the recognition of two subspecies. Their study was based on variation at 10 isozymes in a set of 16 *E. pellita* seedlots from 16 locations (6 NG and 10 Queensland seedlots). They found that *E. pellita* from NG was clearly separated from those in Queensland (including two predefined populations from NQ and CYP) (House *et al.* 1996). Although this study included many of the same seedlots (including 3/7 seedlots from Queensland and 3/5 seedlots from NG), it did not have any representation of CYP material. This may explain why genetic diversity estimate in this study was slightly lower. In a recent, taxonomic revision of *E. pellita*, Hill *et al.* (2000) regarded *E. pellita* as an Australian endemic restricted to the Cairns region. *Eucalyptus pellita* from CYP and NG was split-off as a new species, *E. biterranea*, distinguishable morphologically from *E. pellita* by smaller buds, fruit and leaves. Genetic differentiation at microsatellite markers would also seem consistent with this division.

2.5.3. Two populations of *Eucalyptus resinifera* were identified

The AMOVA analysis of *E. resinifera* also showed that this species has considerable regional level structure, as the genetic difference between regions (4%) was twice that of populations within regions (2%) and F_{st} value in this species reached 0.059 (table 7). This indicated relatively strong geographically based differentiation within *E. resinifera*, and

suggested two subspecies may need to be recognized, one from North Queensland (including Ravenshoe and Mareeba provenances - referred to here as NQ *E. resinifera*) and one from Fraser Island southward (referred to here as Southern *E. resinifera*).

2.5.3. There was no genetic difference between three subspecies of *Eucalyptus scias*

The genetic difference among three defined subspecies of *E. scias* (Johnson *et al.* 1990) where subspecies level is fitted in analysis was very low compared to the other taxa studied (F_{rt} = -0.019) (Table 7). In addition the genetic difference between subspecies in AMOVA analysis was zero (Figure 4). This showed that, there was no evidence for splitting *E. scias* species into three subspecies. A limitation of this study; however, was the small sample size used for *E. scias*. The congruence of this result with the findings of the isozyme study of this species by House *et al.* (1996), however, tends to support a biological rather than methodological explanation for the lack of subspecies structure. This finding highlights the difficulty of relying on taxonomic groupings for the study of genetic relationships in some plant groups. Phenetic similarity may not be appropriate diagnostic information for the formation of gene pools for breeding, as groups of individuals with different geographical locations, behavioural pattern or phenotypes are not necessarily genetically differentiated (Evanno *et al.* 2005). Conversely, highly polytypic species may be interbreeding (Ridley 2004). The affinities of the three subspecies of *E. scias* will be revisited in the STRUCTURE analysis in the following chapter.

2.5.4. *Eucalyptus pellita* does not appear to increase in genetic diversity at southern latitudes, but a latitudinal cline in genetic diversity was evident in *Eucalyptus resinifera*

Based on isozymes *House et al.* (1996) observed a trend of increasing observed heterozygosity in southern latitude provenances. One hypothesis may be that if the species has a southern origin the migration to NG may be more recent. This trend toward a southern increase in diversity was not clear in this study for *E. pellita* but a cline of increasing genetic diversity with latitude was evident in *E. resinifera*. Out of the 11 populations of *E. resinifera* used in this study, four populations from Coffs Harbour southwards (includes Coffs Harbour, Woolgoolga, Kendall and Nowra populations) had higher genetic diversity parameters (number of alleles, number of effected alleles and expected heterozygosity) than their northward counterparts. This was consistent with a Southern or Central coastal region of NSW origin for the species and a more recent migration northward.

2.5.5. Some *Eucalyptus pellita* provenances had high F values

Some NG *E. pellita* provenances (Goe and Serisa) had higher F values relative to other populations, which may indicate selfing or sib-mating in these populations. Low outcrossing rates were also noted for some *E. pellita* provenances by House *et al.* (1996) (they were Bupul Miting, Iran Jaya and Lankelly Ck, Qld). In provenance/family trials in Vietnam and China, Goe provenances produced significantly higher volume at 3-4 years than other provenances, as well as showing high fungal resistance. Other explanations for

the high fixation index in these provenances are: limited pollen movement, unusual weather condition at the time of flowering, asynchronous flowering within and between populations (House *et al.* 1996) or the samples may have been biased and non-representative of the provenances.

Chapter 3. Genetic Differentiation Among Taxa

(This chapter was published in part as *"Genetic differentiation among and within three red mahoganies (Series Annulares), Eucalyptus pellita, E. resinifera and E. scias (Myrtaceae)"* Son Le, Catherine Nock, Michael Henson and Mervyn Shepherd. *Australian Systematic Botany* [in press August 2009])

3.1. Abstract

The red mahogany group (*Eucalyptus* ser. *Annulares* Blakely) includes some of the most important commercial species (i.e. *Eucalyptus urophylla* S.T. Blake) worldwide for forestry in the subtropics and tropics. However, the taxonomic status of some species in this group is unclear and the relationship between, and genetic structuring within, some species is unresolved. This study examined genetic variation at 13 microsatellite loci in *E. pellita* F. Muell., *E. resinifera* Smith and *E. scias* L. Johnson & K. Hill. Despite sympatry across part of their range in North Queensland, *E. resinifera* and *E. pellita* remain genetically distinct as taxa. Within *E. pellita,* however, two genetic groups were clearly resolved: one from New Guinea and one from Queensland (Cape York Peninsula populations were not sampled). Geographic structuring was also evident in *E. resinifera*, with North Queensland populations separating from those from Fraser Island southwards, although subdivision was less distinct than in *E. pellita*. The alignment of genetic subdivision with biogeographical regions implicated ecological factors in the substructure of both *E. resinifera* and *E. pellita*. *Eucalyptus scias* was indistinguishable from southern *E. resinifera* with which it is largely sympatric and there was no support for the recognition of three subspecies within *E. scias*.

3.2. Introduction

The taxonomy of the red mahoganies remains problematic despite a number of studies of genetic, morphological and oil composition variation in *E. urophylla*, *E. pellita* and *E. scias* (Pinyopusarerk *et al.* 1993; House *et al.* 1994; Doran *et al.* 1995; Pryor *et al.* 1995; Singh *et al.* 1988; Payn *et al.* 2007; Payn *et al.* 2008). Several contemporary classifications exist and there remains no consensus on membership or classification at species level and below (Johnson *et al*. 1990; Brooker 2000; Hill *et al.* 2000). In the previous chapter, the genetic diversity and population structure within species have been carried out; however, the genetic relationship and population structuring between these closely related species has not been resolved.

The objective of the chapter was to describe the distribution of genetic variation across three closely related red mahoganies, *E. pellita*, *E. resinifera* and *E. scias*. More specifically, as there has been no previous study of population structure in *E. resinifera*, this current study were interested in whether *E. resinifera* formed a genetically cohesive taxonomic unit, given its extensive and disjunct distribution and its close proximity with *E. pellita,* with which it naturally hybridises in the north of its range (Chippendale *et al.* 1984). Additionally, the uniqueness and affinities of *E. scias* were studied. Historically, *E. scias* has been classified as *E. pellita* yet it is distributed wholly within New South Wales and is geographically closer to southern populations of *E. resinifera* (Johnson *et al.* 1990; Hill *et al.* 2000). Analysis of genetic structure among and within these three taxa, based on genotypes at 13 microsatellite loci and using the STRUCTURE approach of Pritchard *et al.* (2000), confirmed the genetic uniqueness of *E. pellita* and *E. resinifera* but failed to differentiate *E. scias* from *E. resinifera*.

3.3. Methodology

Materials and laboratory methods have largely been described in Chapter 2.

3.3.1. Determining optimal clustering with STRUCTURE software

Genetic groupings were determined using the model-based approach in STRUCTURE (Pritchard *et al.* 2000) and followed closely the hierarchical method used in Shepherd *et al.* (2008) as recommended by Evanno *et al.* (2005). In addition to strict hierarchical analysis, a study of structure was conducted on taxonomic units to examine whether groupings were influenced by including multiple taxa in STRUCTURE analyses.

STRUCTURE uses a Bayesian model-based approach to group individuals by multi-locus genotypes (Pritchard *et al.* 2000). Importantly, this approach imposes no groupings, taxonomic, geographic or otherwise upon the data during the determination of the most-appropriate model of structure. Determining the optimal number of populations (K) is an *ad hoc* process based on testing a range of models with different K values and inspecting the estimated log probability of data Pr(X|K) to identify the most likely model (Pritchard *et al.* 2000). Individuals are assigned to a population based on their genotype, whilst simultaneously estimating population allele frequencies and model optimisation seeking to maximise Hardy-Weinberg and linkage equilibrium. In simulation studies it has also been observed that as the real K is reached, likelihoods for larger Ks plateau and the variance increases. The approach of Evanno *et al.* (2005) which uses an alternate optimisation criterion - delta K, related to the second-order rate change in the log probability of the data, was also used in this study to choose the most-likely model. A hierarchical approach was used to successively analyse structure, with the entire dataset examined initially to identify

the major sources of structure, then the analysis is iterated upon to identify further substructure. Both "admixture" and "no admixture" models were used for investigating sub-structuring within primary populations, however both models gave similar results so only the admixture models are reported. Ten runs, with a burn-in and replicates of 10,000 runs each, for each K (for K = 1–10), were used to calculate means and standard deviations for posterior probability of the data for a given K (L(K)) and delta K. These parameters were found to provide stable optimisation criteria for replicate runs of in the dataset when detecting primary populations. Ancestry assignments generated by STRUCTURE were used to classify individuals into primary populations. Individuals with population admixture were assigned to a single population if they possessed >90% ancestry from one population; otherwise they were considered to be population hybrids (Evanno *et al.* 2005).

3.3.2. Genetic distance analyses

Distance matrices based on population allele frequencies were generated in Arlequin (Excoffier *et al.* 2005) or MSA v4.05 for Dos (Dieringer *et al.* 2003) for pairwise F_{st} and Nei's *DA* (Nei *et al.* 1983) distances respectively. A dendrogram based on Nei's *DA* distance was used to visualise relationships among the genetic groups because this distance has been found to provide the best chance of obtaining a true tree from microsatellite allele frequency data, unless very large numbers of microsatellite loci are used (Takezaki *et al.* 2008). Neighbour joining amalgamation was carried out using Phylip release 3.68 module "Neighbor" with no outgroup (Felsentein J; available at http://evolution.genetics.washington.edu/phylip.html). An unrooted majority rule consensus tree was constructed from 1000 bootstrapped trees using Geneious Pro software (Drummond *et al.* 2008).

3.3.3. Genetic diversity and suitability of microsatellite markers

The following locus characteristics determined in the 'within taxa analysis' presented in Chapter 2 supported the reliability of allele frequency estimates used during the STRUCTURE analysis.

For each of the three species studied, genetic diversity was typical of widespread or regional eucalypt species at microsatellite loci (Mean H_e = 0.88, 0.88 and 0.81 for *E. resinifera*, *E. scias* and *E. pellita* respectively (Appendix 5). *Eucalyptus pellita* consistently exhibited lower H_o than H_e across all loci suggesting a genome-wide effect such as hierarchical population structuring leading to a homozygous excess when diversity is assessed on a species-wide level p 73 (Hartl 2000).

A total of 1560 permutation in pair wise comparisons on LD test were done. The P-values obtained for locus-to-locus combination ranged from zero to one (0.00-1.00). To test the null hypothesis, a confidence level of 5% was set, the test results showed that all P-values were larger than the adjusted P-value (was 0.00061 in this study) (Appendix 2), the null hypothesis was accepted. Therefore all the genotypes at one locus are independent from the genotype at the other locus.

Departure from Hardy-Weinberg equilibrium (HWE) was found for most loci (10/13) in *E. pellita* and *E. resinfera* but only 3 out 10 for *E. scias* (Appendix 3). Again this may suggest genome-wide effects leading to disequilibrium in the first two taxa, possibly a consequence of population structuring (i.e. Wahlund effect). There was no strong evidence for null alleles at any locus in any species evident from Microchecker analysis (Appendix 4).

3.4. Results

3.4.1. Hierarchical analysis of genetic groups

3.4.1.1. Entire data set

To identify the major sources of genetic structure across this current study selection of red mahoganies, the entire set of 192 samples was subjected to analysis with models of K ranging from 1 to 10. Using the delta K criteria of Evanno *et al*. (2005), a major peak at K = 2 (Figure 5) was evident for this sample set, suggesting a division into two genetic groups.

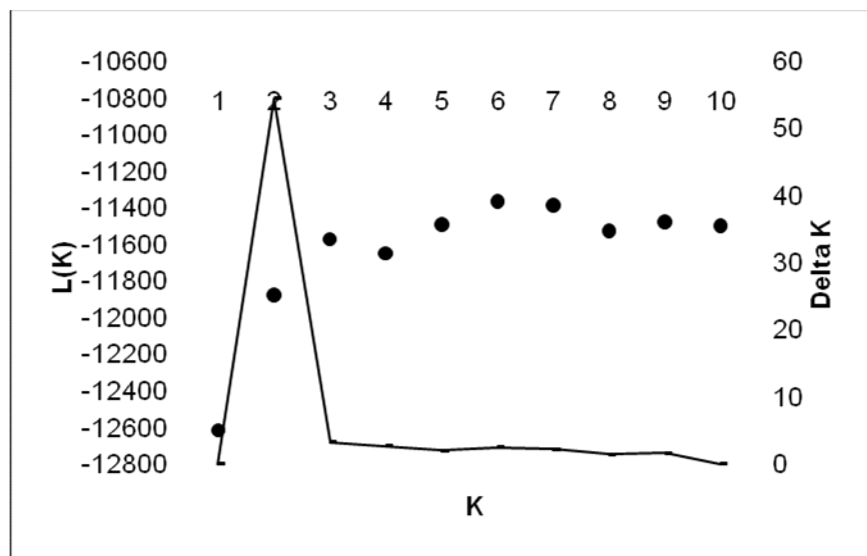

Figure 5. Magnitude of delta K (solid line) and L(K) (dots) as a function of K (mean of 10 replicates) for K = 1-10 for the entire dataset of 192 individuals. A peak at K = 2 suggested two genetic groups was optimal for this dataset.

On the base of examination of the ancestry assignments for the model K = 2 and the use of the criteria of >90% of ancestry in a single population as a threshold for inclusion, most individuals (93%) were assigned to one or other of the two genetic groups (Appendix 6 and Figure 6).

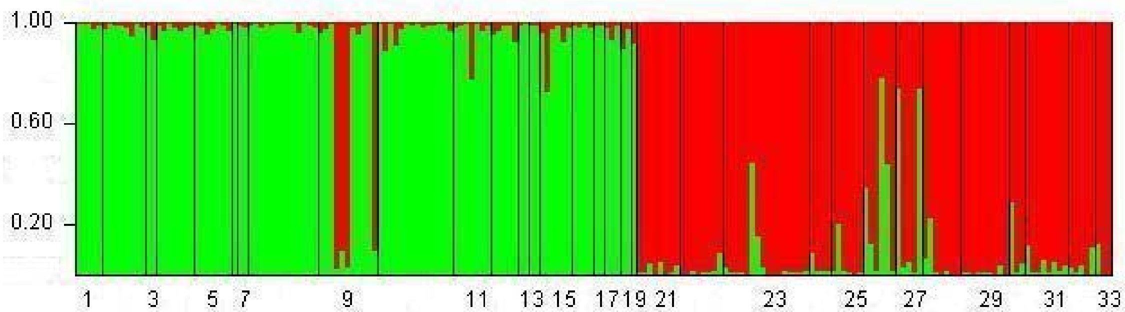

Figure 6. Ancestry assignment for entire dataset of 192 inviduals taxa using a K = 2 model. Individuals are arranged in taxa and within taxa by location and ordered by latitude. The number on the x axis represents a sampling locality and was coded as follows: *E. resinifera* (1, Ravenshoe, Qld; 2, SW Mareeba, Qld; 3, Fraser Island, Qld; 4, Stradbroke Is, Qld; 5, WNW Beerburrum, Qld; 6, NW Pomona, Qld; 7, Kendall; 8, Woolgoolga; 9, Coffs Harbour; 10, Nowra; 11, Ewingar SF), *E. scias* (12, Boonoo Boonoo; 13, Malara; 14, Mt Banda Banda; 15, South Brooman SF; 16, Mt Bouddhi NP); *E. urophylla* (17, E Timor; 18, Utak, Wetar; 19, Egon, Flores; 20, Waikul Central, Alor) and *E. pellita* (21, Bupul Muting, Indonisea; 22, S of Kirowo, PNG; 23, Serira, PNG; 24, Kiriwo, PNG 25, Goe, PNG; 26, Helenvale, Qld; 27, Starcke Stn, Qld; 28, Mossman, Qld; 29, NW Kuranda, Qld; 30, El Arish, Qld; 31, Kirrama Range, Qld; 32, S Cardwell, Qld; 33, SSO, Melville Is. NT). Localities without state or country details are NSW Australia.

The first genetic group consisted of almost entirely *E. pellita* individuals from both Queensland and NG. This group will herein be referred to as the “Pellita genetic group”. The second group comprised almost all the *E. resinifera* individuals as well as the *E. scias* and *E. urophylla* individuals, and herein is referred to as the “Resinifera genetic group”.

There were three cases where individuals labelled as *E. resinifera* grouped into the Pellita group (RES6021WOON, RES6022WOON and RES6023WOON) suggesting that these individuals were misclassified or incorrectly labelled. In addition, 10 out of 192 individuals were of mixed population ancestry (i.e. having less than 90% ancestry from a single population). These included two *E. resinifera* (RES6043NOWN, RES6025COFN), a single *E. scias* (SCIC3011BRON) and seven *E. pellita* samples (PEL438SERPN, PEL501KURQL, PEL504KURQL, PEL507KURQL, PEL512STAQL, PEL546MOSQL,

and PEL549KIRQL) (Appendix 6). In the case of the *E. resinifera* RES6025COFN, the major portion (89%) of its ancestory was assigned to the Pellita genetic group, suggesting that most likely the parent tree was mistakenly classified in the field or that seeds or foliage material was subsequently mislabelled. As all putatively mis-classified *E. resinifera* derived from the same region, the Woolgoolga/Coffs Harbour district, this suggests there may be difficulties in distinquishing the two taxa at these locations.

3.4.1.2. Genetic structuring of the Pellita genetic group

To test for substructuring within the Pellita genetic group, the 89 samples with >90% ancestory assignment to this group, including almost all of *E. pellita* samples (except PEL504KURQL, PEL507STAQL, PEL512STAQL) and the four *E. resinifera* samples, were subject to the same analysis as the entire dataset. In this case a major peak was evident at K = 2 but there were also minor peaks at K = 5 and K = 7 (Figure 7).

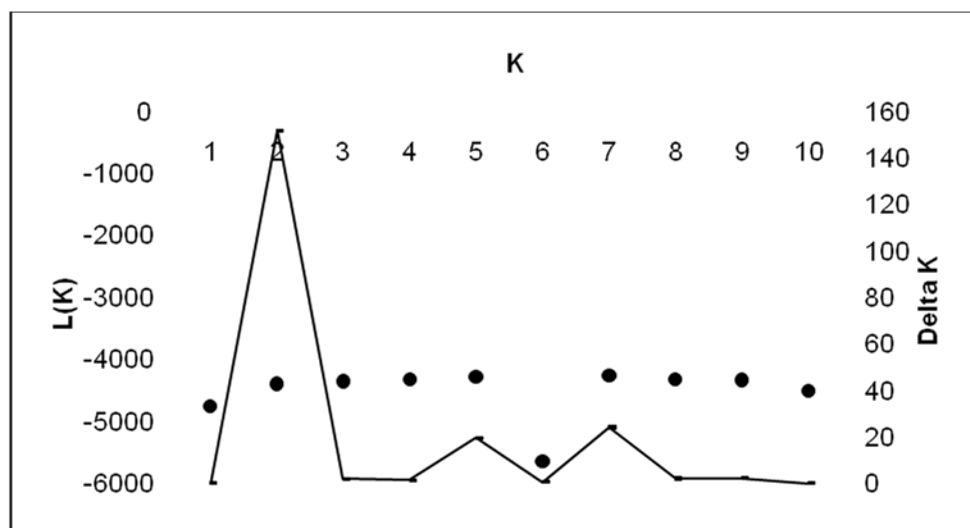

Figure 7. Magnitude of delta K (solid line) and L(K) (dots) as a function of K (mean of 10 replicates) for K = 1-10 for the Pelleta genetic group of 89 individuals. A large peak at K = 2 suggested a major level of substructuring into two genetic groups.

This may indicate a major level of structure at K = 2 but there may also be more subtle substructuring giving rise to the peaks at K = 5 and 7 as the peak size can be an indicator of the degree of structure (Evanno *et al.* 2005). Excluding the four *E. resinifera* samples did not affect this result significantly hence the analysis was essentially equivalent to an analysis of the taxonomic group *E. pellita*.

From the examination of ancestory assignments at the major level of structure where K = 2, the assignments of individuals into two sub-groups indicated one group comprised *E. pellita* from NQ plus the four presumably misidentified NSW *E. resinifera* samples, and herein referred to as the "Queensland Pellita genetic group". The second group comprised all *E. pellita* samples from NG and the material derived from the Melville Island SSO (which originated from PNG) and was herein referred to as the "NG Pellita genetic group" (Figure 8).

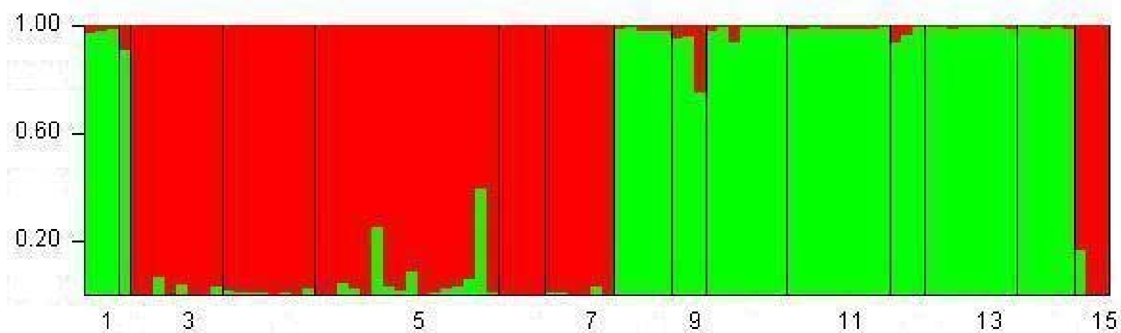

Figure 8. Ancestry assignment for 86 individuals in the Pellita genetic group using a K = 2 model. Individuals are ordered according to the scheme described for Figure 2; numbers on the x axis correspond to: *E. resinifera* (1, Woolgoolga; 2, Coffs Harbour) and *E. pellita* (3, Bupul Muting, Indonisea; 4, S of Kirowo, PNG; 5, Serira, PNG; 6, Kiriwo, PNG; 7, Goe, PNG; 8, Helenvale, Qld; 9, Starcke Stn, Qld; 10, Mossman, Qld; 11, NW Kuranda, Qld; 12, El Arish, Qld; 13, Kirrama Range, Qld; 14, S Cardwell, Qld; 15, SSO, Melville Is. NT). Localities without state or country details are NSW Australia.

It was clear from this analysis that substructuring within *E. pellita* was geographically based with a major division corresponding to material from the two different land masses, NG and continental Australia. The grouping of the Melville Island SSO derived material was consistent with the documented origins of the parent tree as PNG. However, as the orchard was a mix of near equal numbers of both Queensland and PNG families, there was potential for inter-provenance hybridisation. The lack of evidence for infusion of Queensland ancestry into the open pollinated offspring studied, however, was consistent with observations on flowering in the orchard which was dominated by trees of PNG origin (Harwood CE pers. comm.).

3.4.1.3. Genetic structuring of NG Pellita genetic group

No substructuring was detectable within the NG Pellita genetic group. Although the analysis indicated a peak and maximal likelihoods for a model with K = 3, examining ancestry assignments indicated nearly equal proportioning of ancestry to each indivdiual (analyses not shown). This pattern of equal proportioning of ancestry into the number of populations is a signature of a lack of structure (Pritchard *et al.* 2000) and therefore indicates minimal population subdivision within NG *E. pellita*.

3.4.1.4. Genetic structuring of Queensland Pellita genetic group

In the Queensland Pellita genetic group, a major peak was evident at K = 2, but there were also some minor peaks at K = 4, K = 6 and K = 7 (plot not shown). Examining ancestry assignments at K = 2 indicated that only 9/44 individuals were assigned to one or other of the two sub-populations at the applied threshold of 90% (Figure 9)

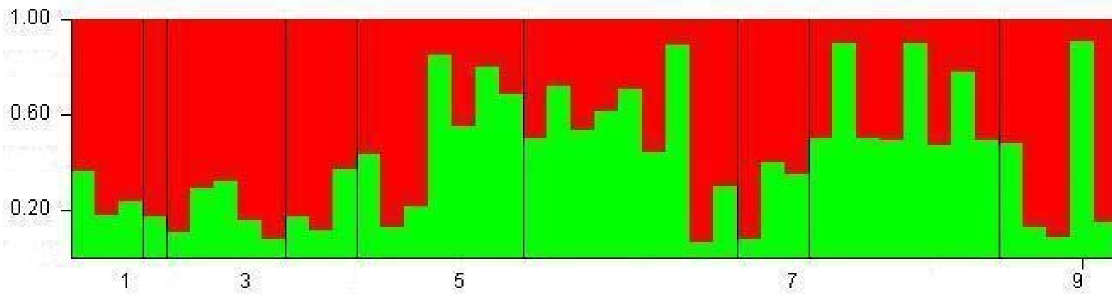

Figure 9. Ancestry assignment for 44 individuals in the Queensland Pellita group determined using STRUCTURE with a K = 2 model. Labels on x axis correspond to localities as follows; *E. resinifera* (1, Woolgoolga; 2, Coffs Harbour) and *E. pellita* (3, Helenvale, Qld; 4, Starcke Stn, Qld; 5, Mossman, Qld; 6, NW Kuranda, Qld; 7, El Arish, Qld; 8, Kirrama Range, Qld; 9, S Cardwell, Qld). Localities without state or country details are NSW Australia.

The majority of Queensland Pellita genetic group samples were of mixed population ancestry suggesting limited genetic subdivision within the group (Figure 9). No further analysis for sub-structure was conducted as groups of “pure” population ancestry were too small.

3.4.1.5. Genetic structuring of the Resinifera genetic group

The test for sub-structure in the Resinifera genetic group (n = 103) suggested the major level of structure was at K = 3 but there were several other minor peaks at K = 6 and 8 (Figure 10). In accordance with Pritchard *et al*. (2000), the numerically smallest value of K but with the largest peak height was examined as the major level of substructure.

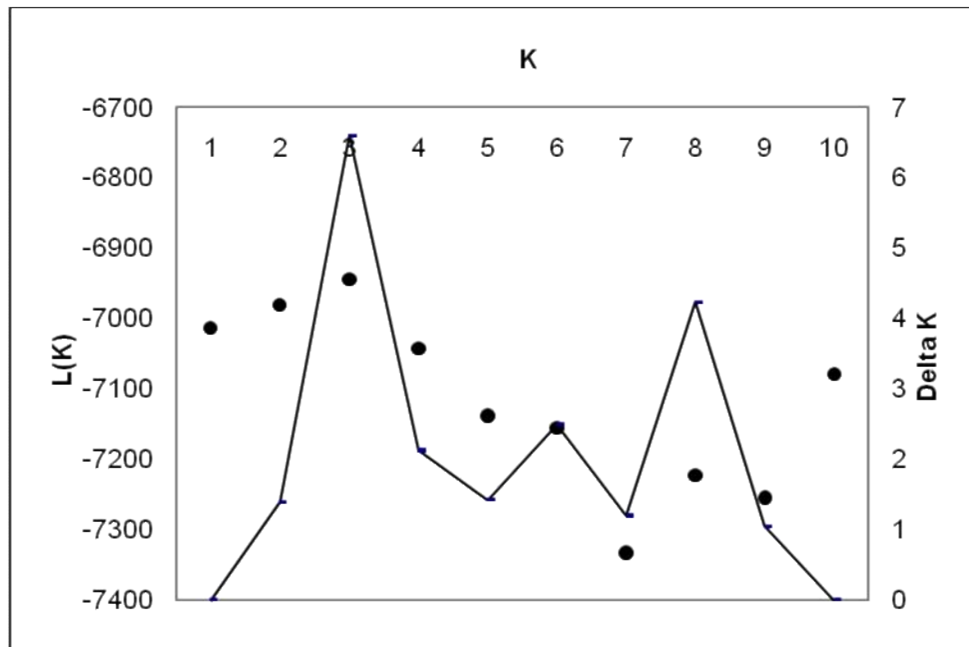

Figure 10. Magnitude of delta K (solid line) and L(K) (dots) as a function of K (mean of 10 replicates) for K = 1-10 for the dataset of 103 individuals in the Resinifera genetic group.

Ancestry assignments for the K = 3 model indicated few (28) of the 103 samples could be assigned to a population at the choosen threshold of >90% for membership to a single population, most individuals (75) therefore were of population admixture (Figure 11).

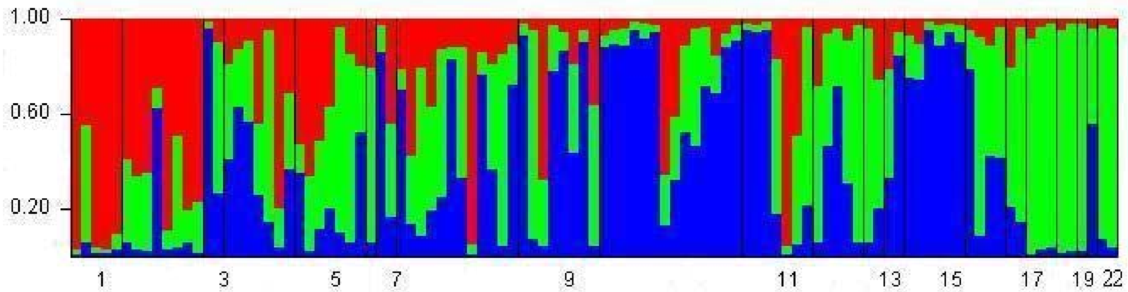

Figure 11. Ancestry assignment for 103 individuals in Resinifera group determined by STRUCTURE analysis using a K = 3 model. See Figure 2 for explaination of x axis label arrangement. Numbers correspond to *E. resinifera* (1, Ravenshoe, Qld; 2, SW Mareeba, Qld; 3, Fraser Island, Qld; 4, Stradbroke Is, Qld; 5, WNW Beerburrum, Qld; 6, NW Pomona, Qld; 7, Kendall; 8, Woolgoolga; 9, Coffs Harbour; 10, Nowra; 11, Ewingar SF), *E. scias* (12, Boonoo Boonoo; 13, Malara; 14, Mt Banda Banda; 15, South Brooman SF; 16, Mt Bouddhi NP); *E. urophylla* (17, E Timor; 18, Utak, Wetar; 19, Egon, Flores; 20, Waikul Central, Alor) and *E. pellita* (21, Starcke Stn, Qld; 22, NW Kuranda, Qld). Localities without state or country details are NSW Australia.

One group included five *E. resinifera* individuals orginating from Ravenshoe or Mareeba in NQ, herein referred to as the "northern Resinifera group". A second group consisted of nine *E. resinifera* individuals from sites south of Fraser Island (mainly in Coffs Harbour and Nowra) and two *E. scias*, and is herein referred to as the "southern Resinifera group". The last sub-group (n = 11) comprised largely the *E. urophylla* samples, two NQ *E. pellita* and four *E. scias* samples (ancestry assignments not shown).

By excluding the sample of *E. urophylla* and the northern Resinifera group, the grouping of the remaining individuals from the Resinifera genetic group supported the conclusion that the group of remaining individuals from the Resinifera genetic group was better explained by geographic proximity than taxonomic grouping. For example, some *E. scias* samples collected from Sth Brooman grouped into the same population as the *E. resinifera* from the nearby Nowra location, suggesting gene flow between these two populations of these species may be extensive.

Hence in summary, within the Resinifera genetic group, genetic structure was concordant with taxonomy in respect to *E. urophylla* but not *E. scias*. Within the *E. resinifera* and *E. scias* material there was evidence of a structuring along geographic rather than taxonomic lines. A northern and southern population were evident, but a large number of individuals were of population admixture.

3.4.2. Genetic structuring strictly within taxonomic groups

Structure was also examined using samples assigned strictly to taxonomic groups to test for hybridisation and misidentification of individuals. In the case of *E. pellita*, the sample set corresponded closely with the Pellita genetic group, differing only in that four odd *E. resinifera* were included. Exclusion of these four *E. resinifera* did not significantly affect the outcome of the STRUCTURE analysis (data not shown). Testing for substructure with the data set of 19 *E. scias* individuals suggested a model with K = 2 was optimal but that minor peaks were evident at K = 6 and 8 (data not shown).

Examining ancestry assignments for the K =2 model, however, indicated that there was no detectable substructure within *E. scias*, as all individuals were assigned equal proportions of population ancestry, a signature of no structure (Pritchard *et al.* 2000). Further study, with increased sample size per population should increase the reliability of detecting structure within *E. scias* (Evanno *et al.* 2005; Latch *et al.* 2006). Testing for structure within the taxonomic group of *E. resinifera* (n = 77) indicated peaks for models where K = 2, 7 or 9 (data not shown). Examining the ancestry assignments on a locality basis for the *E. resinifera* taxonomic group suggested that the two genetic groups had a geographic basis with one group containing low latitude material from Ravenshoe and Mareeba, and

the second group containing material from Fraser Island southwards (including mostly NSW provenances). In this regard the outcome was similar to the analysis of the Resinifera genetic group. However, some localities were not strongly assigned to one or other of the subgroup (i.e. the proportion of membership ranged from 67.1 to 86.6%). Again in this regard the analysis mirrored the analysis of the Resinifera genetic group which also indicated a large number of individuals of population admixture.

3.4.3. Relationships amongst genetic/taxonomic groups

Genetic distances (*DA* and F_{st} values) were estimated to explore relationships amongst a composite of taxonomic and genetic groupings identified by the STRUCTURE analysis. Six genetic groups or taxa were defined, Queensland *E. pellita*, NG *E. pellita*, Northern *E. resinifera*, Southern *E. resinifera*, *E. scias* and *E. urophylla*. These groups did not correspond exactly with genetic groups defined above as potentially mis-classified individuals were excluded. The grouping of *E. scias* as a separate taxonomic unit was retained to allow illustration of its relationship with other groups, despite the evidence of this study showing it was largely indistinguishable from Southern *E. resinifera* (Table 13).

Table 13. Pairwise population F_{st} or Nei's genetic distance values (DA above diagonal) for six taxa/genetic groups (below diagonal). All F_{st} values were significant at p-value < 0.05. Group names are NG Pell = New Guinea *E. pellita*, QLD Pel = Queensland *E. pellita*, NQ Res = North Queensland *E. resinifera*, Sth Res = Southern *E. resinifera,* Scias = *E. scias,* Urophy = *E. urophylla*

	NG Pel	*QLD Pel*	*NQ Res*	*Sth Res*	*Scias*	*Urophy*
NG Pel		0.30	0.52	0.36	0.41	0.45
QLD Pel	0.089		0.43	0.27	0.31	0.44
NQ Res	0.150	0.144		0.31	0.43	0.51
Sth Res	0.100	0.107	0.044		0.17	0.34
Scias	0.094	0.102	0.055	0.014		0.45
Urophy	0.104	0.108	0.074	0.050	0.032	

An unrooted neighbour joining tree based on Nei DA distance was used to summarise the relationships amongst groups defined above (Figure 12).

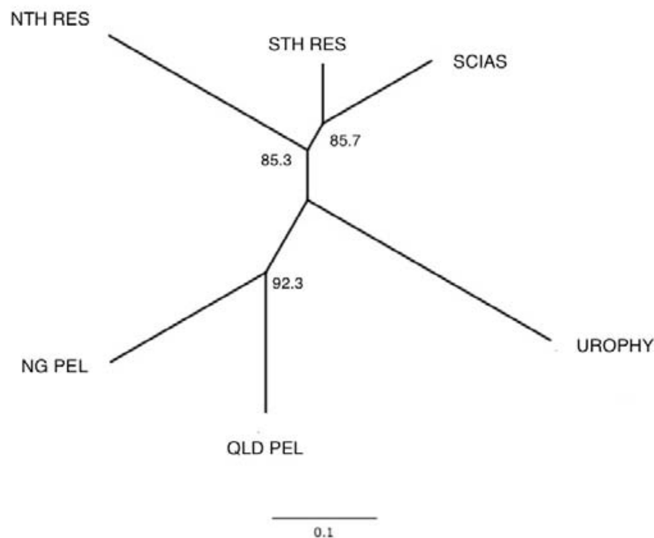

Figure 12. Majority rule, unrooted consensus tree of 1000 bootstrapped neighbour joining trees for six populations based on Nei's *DA* distance. Percentage bootstrap support values are given at each node. See Table 13 for explanation of group label.

It showed strong support for the grouping of *E. scias* with the Southern *E. resinifera* (bootstrap value 85.7%) and also for a cluster that included Northern *E. resinifera* (85.3%). There was also strong support for a group containing both northern and southern *E. pellita* (92.3%).

3.5. Discussion

This study of genetic differentiation among and within three red mahoganies allowed testing of two main hypotheses; firstly whether *E. resinifera* forms a genetically cohesive taxonomic unit despite its extensive and disjunct distribution and the potential for hybridisation with *E. pellita;* and, secondly, whether *E. scias* showed stronger affinity with *E. resinifera* with which it is geographically more proximal, than *E. pellita,* under which it was previously classified. As a result of this study it was concluded that *E. pellita* and *E. resinifera* are both distinct, genetically cohesive taxonomic units but that each shows significant geographic substructuring. In contrast, *E. scias* was not genetically distinct from southern populations of *E. resinifera*, suggesting these taxa may either be morphotypes, and a consequence of polymorphism within a species, or instead, an example of incipient speciation. Taxonomic implications, the congruency of the current study results with previous genetic studies, and potential factors contributing to the observed genetic structuring are discussed further below.

The genetic differentiation evident in this study using microsatellites was consistent with the isozyme study of House *et al.* (1996) in finding two genetic groups within *E. pellita* and supports their proposition for the recognition of two subspecies. The House *et al*. 1996 study was based on variation at 10 isozymes in a set of 16 *E. pellita* seedlots from 16 locations (6 NG and 10 Queensland seedlots) and 8 seedlots of *E. scias* from 8 locations (including the 3 proposed subspecies). They found that *E. pellita* from NG was clearly separated from those in Queensland (included two predefined populations from NQ and CYP). Further, they found evidence that the CYP material was subtly distinct (Nei's genetic distance 0.039) from NQ material, although a population-based phylogram was not monophyletic for each region (House *et al.* 1996). Although the current study included

many of the same seedlots (including 3/7 seedlots from Queensland and 3/5 seedlots from NG), but did not have any representation of CYP material. This may be one reason that this study did not find any structure within Queensland *E. pellita*, and it is likely that similar subtle differentiation would be evident if CYP had been studied here using microsatellite markers.

For *E. pellita* there seems to be a convergence of genetic and systematic classifications probably because both align with groups based on geographic locations. In a recent taxonomic treatment of *E. pellita*, Hill *et al.* (2000) regarded *E. pellita* as an Australian endemic restricted to the Cairns region. *Eucalyptus pellita* from CYP and NG was raised as a new species, *E. biterranea*, distinguishable morphologically from *E. pellita* by smaller buds, fruit and leaves. Genetic differentiation at microsatellite markers would also seem consistent with this division.

Genetic structuring in *E. pellita* aligned with distinct biogeographic zones and major barriers to gene flow. North Queensland has been separated from NG by the Torres Strait sea at least since the Holocene (last 10,000 yrs) (Ladiges *et al.* 2003). The drier and hotter Cape York Peninsular region is separated from the mesic Atherton plateau by the Normanby gap (Ford 1987; James *et al.* 2000). Changes in flora composition as a consequence of geological and edapthoclimatic variation in the landscape has created "ecological gaps" that represent inhospitable habits for many fauna species, including birds and invertebrates and restricts their movement between biogeographic zones (Christidis *et al.* 2008; Cracraft 1991; Crisp *et al.* 1995; Ford 1987; James *et al.* 2000). Disjunctions in the distribution of *E. pellita,* as well as limitations to movement of its dependent pollinator

fauna between biogeographic zones, may explain the observed genetic structuring in this species.

Despite being sympatric through part of their ranges in NQ, *E. resinifera* was genetically distinct from *E. pellita*. In this current study, the two closest locations where *E. resinifera* and *E. pellita* were sourced were East of Mareeba (MARE) and El Arish (ELAR), respectively, sites which are estimated to be around 110 km apart. Although a large distance over which to detect the direct effects of gene flow between two taxa, it is a relatively small distance compared to that between the northern and the southern Resinifera groups, which show a higher degree of genetic connectivity. This suggests there are strong reproductive barriers isolating *E. pellita* and *E. resinifera* in NQ. Natural interspecific hybrids are possible but are rare (Chippendale *et al.* 1984; Griffin *et al.* 1988), hence relatively strong pre-zygotic barriers probably exist, perhaps due to flowering asynchrony or floral structural incompatibles. Fruit size (and hence flower size which is strongly correlated with fruit size) in *E. pellita* is generally larger than *E. resinifera* (Hill *et al.* 2000). This may limit crossing between the two species as style length incompatibles are known to cause unidirectional crossing in eucalypts (Gore *et al.* 1990). Although the two species can flower in Spring, Summer or Autumn, there is no recorded overlap in the flowering times of the two species (Hill *et al.* 2000). The strong genome-wide differentiation evident from this study also suggests that post-zygotic isolation factors have also probably evolved between these two species and may also help maintain species integrity.

Within the species studied, *E. resinifera* in particular, has been subject to few previous genetic studies and its taxonomy continues to be revised. *Eucalyptus resinifera* is described

as a species complex including two subspecies; subsp. *hemilampra* and subsp. *resinifera* (Centre for Plants Biodiversity Research 2006). Subspecies *hemilampra* occurs from Taree (NSW) to near Gladstone (Central part of Queensland) whereas subsp. *resinifera* has a distribution restricted to NSW, from Jervis Bay in the south to around Kempsey in the north of its range. An additional population occurs in far northern Queensland on higher ground in the Nebo-Eungella, the Mount Spec, Paluma, and the Tablelands regions as well as the Lankelly Creek–McIlwraith range region of CYP and has been described as a different species, *E. macta* (Hill *et al.* 2000). This current study included samples from all proposed taxa within the *E. resinifera* complex. Only two genetic sub-groups were identified in the current study however, a NQ group (represented by 2 provenances (namely Ravenshoe and Mareeba) that probably correspond with *E. macta,* and a southern group consisting of all the remaining *E. resinifera* material from Fraser Island southwards. Because this southern group should represent both subsp. *hemilampra* and subsp. *resinifera*, this current study found no evident of genetic differentiation between these subspecies. Like *E. pellita*, however, the major geographic subdivision aligned with an ecological barrier, the Burdekin gap which occurs between the Atherton and the Central Queensland (Cracraft 1991; Ford 1987). A disjunction in the species range and restrictions in movement of the dependent pollinator fauna may therefore also contribute to the genetic structuring in *E. resinifera*.

Microsatellite markers were unable to distinguish *E. scias* from southern *E. resinifera* which was largely in accord with isozymes data (House *et al.* 1996). In earlier studies, *E. scias* was found to be distinct from *E. pellita* by isozymes (House *et al.* 1996) and in patterns of seedling leaf oils and morphology (Doran *et al.* 1995; Pinyopusarerk *et al.* 1993). The question of whether *E. scias* is genetically distinct from *E. resinifera* has not

been previously addressed. The current study found that within the southern Resinifera genetic group (including both *E. resinifera* and *E. scias*) geographic isolation seems to be a greater factor in determining genetic structure than division along taxonomic lines. Populations from these two taxa that were geographically proximate tended to show the highest relationship (e.g. *E. scias* from Sth Brooman and *E. resinifera* from nearby Nowra). Furthermore, this study found no genetic evidence to support sub-division of *E. scias,* into three subspecies, which was also in accordance with a previous isozyme study (House *et al.* 1996). Caution was required, however, with this conclusion because of the small sample sizes for each *E. scias* subspecies which potentially reduces the power to resolve population differences (see discussion below on *E. urophylla*).

Eucalyptus scias may represent a nascent species, early in its evolutionary divergence from *E. resinifera*. The two species separate on ecological grounds inhabiting environments with different edaphic and topographical conditions (Bevege DI pers. comm.). Hence they likely experience different adaptive pressures and may be undergoing divergent selection. *Eucalyptus scias* and *E. resinifera* may represent ecotypes or genic species: i.e. species that are divergent at a relatively few adaptive or speciation loci and share widespread genome similarity because of ongoing gene flow (Wu 2001; Wu *et al.* 2004). Genic species may not have undergone chromosomal rearrangements or more extensive genic differentiation that leads to reproductive isolation evident in later stages of speciation. The current evidence, however, is insufficient to distinguish the genic species hypothesis and an alternative, morphotypic or ecotypic variation. Morphological or anatomical variation may arise in the two entities because of segregation at one or a few polymorphic loci, and may or may not be adaptive. The production of hybrids and growth with parental taxa in common garden trials would likely help resolve between these possibilities. Testing for

segregation of taxonomic characters may reveal the degree and architecture of genetic control of these characters and examining potential fitness loss in hybrids may be indicative of the development of species isolation factors.

In this study, a small number of samples of *E. urophylla* this current study were included as a method controls and to test the power of resolving among the *Annulares*. *Eucalyptus urophylla* was expected to be the most genetically distinct taxon and free from the influence of recent hybridisation with the remaining taxa studied. *Eucalyptus urophylla sensu lato* is geographically remote from the other three taxa studied; the closest known populations of *E. pellita* are around 1400 km distant (Payn *et al.* 2008; Pryor *et al.* 1995). Reticulation, following recontact amongst closely related species, limits power to resolve among the taxa and otherwise confounded measures of relationship (Zink *et al.* 2008). Hence a major split between *E. urophylla* and the remaining three taxa was expected but, instead, the most distinct genetic group was the Pellita genetic group (primarily *E. pellita* individuals). Furthermore the affinity of *E. urophylla* with the Resinifera genetic group, and particularly Southern Resinifera material and *E. scias* rather than *E. pellita,* was also surprising, as based on geographic proximity, a stronger affinity between *E. pellita* and *E. urophylla* might be expected.

The most likely explanation for the clustering of *E. urophylla* within the Resinifera genetic group appears to be an anomaly of the small number of *E. urophylla* samples in this study. A small number of individuals may have resulted in unreliable estimates of population allele frequencies and thus the genetic relationships derived from them. High-frequency private alleles were found for *E. urophylla* at three of the thirteen loci studied (Data not shown) which suggested some genetic differentiation at microsatellite loci for *E.*

urophylla. However, the sample size may have been too small to provide reliable allele frequency estimates for the STRUCTURE analyses and distance metrics, both based on population allele frequency estimates.

Genetic distances derived from microsatellite loci for these taxa might not reflect those expected based on biogeography or evolutionary history for other reasons. Although microsatellites are sometimes found to retain a phylogenetic signal across more divergent groups (e.g. Ritz *et al.* 2000; Schlotterer 2001), they are usually thought to lose their phylogenetic information over longer time spans that separating more divergent taxa because of high mutation rates (Feldman *et al.* 1997; Goldstein *et al.* 1995). Difficulties in modelling variable mutation rates amongst lineages, homoplasy, and maximal allele size constraints render genetic distance estimates from microsatellite loci non-linear over longer time scales (Ellegren 1995; Goldstein *et al.* 1995).

Further study, including all members of *Annulares,* will be required to determine the extent which microsatellite markers are useful for analysis of relationships across the taxon. A phylogeographic study of *Annulares* and sympatric *Exertaria* is suggested, using an approach more suitable for phylogenic inference, such as chloroplast haplotyping (Freeman *et al.* 2001; Jones *et al.* 2006) or nuclear gene sequencing. Increasingly it has been shown that the correct inference of management groups and genealogies often requires analysis of a number of genes (Burg *et al.* 2004; Muir *et al.* 2006; Zink *et al.* 2008).

Chapter 4. General Conclusions

This study aimed to verify and complement previous genetic diversity and population structure analysis in *E. pellita* and *E. scias*, and describe genetic diversity and population structure in *E. resinifera* for the first time. In addition, this study allowed analysis of the genetic relationship between these three closely related red mahoganies.

For each of the three species studied, genetic diversity was typical of widespread or regional eucalypt species at microsatellite loci (with mean expected heterozygosity by 0.88, 0.88 and 0.81 for *E. resinifera, E. scias* and *E. pellita* respectively). This suggested the breeding system and life history characteristics of these species are typical of eucalypts. Although not conclusive, the congruence in observed and expected diversity parameters, also suggested that these estimates were probably reliable and not unduly biased due to the locus discovery and transfer processes associated with microsatellites (Barbara *et al.* 2007). In this case, problems associated with transfer (e.g. null alleles) may have been circumvented by close affinity of the focal (*E. urophylla* and *E. grandis*) with nonfocal taxa (*E. pellita, E. resinifera* and *E. scias*)

The genetic differentiation evident in this study using microsatellites was consistent with the isozyme study of House *et al.* (1996) and morphology of Hill *et al.* (2000) in finding two genetic groups within *E. pellita,* and supported their proposition for the recognition of two subspecies. Disjunctions in the distribution of *E. pellita,* as well as limitations to movement of its dependent pollinator fauna between biogeographic zones, may explain the observed genetic structuring in this species.

At the regional scale, genetic drift rather than adaptation appeared to better explain genetic differentiation in microsatellites in *E. pellita*. Material from both regions of *E. pellita* can show good performance when planted both on sites similar and different to their native environments (Harwood 1998). Provenance trials of *E. pellita* have shown Queensland and NG provenances perform well in a similar range of planting environments (at least for early growth) but, as might be expected, NG provenances have a better tolerance to prolonged hot and wet conditions, largely because of fungal tolerance (Harwood 1998). Climate profiling of suitable planting regions for NG provenances showed conditions that are only slightly different (lower minimum mean annual temperature range and increased dry season length) to those for Queensland, which were: a mean annual precipitation range of 1200 to 3000 mm in a summer or uniform/bimodal pattern; MAT (Mean of Annual Temperature) 19-29^{0}C; and dry season of 1-5 months (i.e. <40mm) (Harwood 1998).

Such plasticity often reflects a lack of adaptation to local conditions in a forest tree and suggests genetic differentiation may largely be a consequence of genetic drift in isolation, rather than adaptation (White *et al.* 2007). In the case of *E. pellita*, genetic differentiation at effectively neutral microsatellites in geographic regions is also congruent with this hypothesis as it suggests factors affecting the whole genome have contributed majorly to differentiation. Evidence of selection leading to adaptive variation (growth performance) would not generally be expected to align with patterns of neutral marker variation.

Adaptation at the regional level also occurs, no doubt, and probably contributes to the recognised morphological and flowering time differences between the regions (Harwood 1998). But it is heterogeneity in environmental conditions within each region that might better explain the apparent plasticity of *E. pellita* to a range of planting sites. The natural

distribution of *E. pellita* spans climates which have no effective dry season (NG) to those with a prolonged dry season (i.e. less than 40mm per month for at least four consecutive months) (Queensland) (Harwood 1998). However, with each region (NG and Queensland) there is considerable heterogeneity in conditions, for example *E. pellita* around Tully in NQ has no effective dry season and southern parts of *E. pellita* range in NG have a marked dry season. This heterogeneity within each region, may account for the apparent plasticity of material when performance is compared at the regional level. This hypothesis suggests predicting performance on a regional level may not be reliable, and better matching of *E. pellita* germplasm to planting site might be achieved at a provenance level.

Breeding of *E. pellita* faces a dilemma, whether to manage material from the two regions separately or combine and perhaps achieve complementation of desirable characters such as fungus resistance and wind firmness (Harwood 1998). One hypothesis, as a result of this study, is that genetic differences in the two regions may largely reflect random drift; hence inter-regional hybrids may not suffer too much from later generation hybrid breakdown (Potts *et al.* 2001). This is because the two forms may not have developed the strongly interacting gene complexes that can sometimes be associated with geographical races (White *et al.* 2007; Ridley 2004). This is testable, with a comparison of advanced generation inter-regional and intra-regional cross performance. If there are strongly co-adapted gene complexes in each region, advanced generation hybrid breakdown may be apparent in inter-regional crosses.

Differentiation at adaptive loci at both scales, regional and within region, nonetheless probably exists and may be associated with patterns of genetic differentiation at microsatellite loci (although this was not shown). It was shown in this study, however, that

planted *E. pellita* could be correctly identified to region using microsatellite markers (Chapter 3 Melville Island SSO derived material). This shows the potential of markers for studying genealogy of inter-regional hybrids and seed orchard dynamics in *E. pellita*. Markers may be particularly important for monitoring hybridisation and infusion in this species where large differences in flowering times between regions are known.

Some NG *E. pellita* provenances (Goe and Serisa) had relatively high F values, which may indicate selfing or sib-mating in these populations. Limitations to pollen movement, unusual weather condition at the time of flowering, asynchronous flowering within the population, and samples that may not accurately represent the genetic character of these provenances may explain the higher F values (House *et al.* 1996). Family to family variation in performance may be high in such situations and an overall reduction in provenance means is expected (Eldridge *et al*. 1994; Harwood 1998).

Despite being sympatric through part of their ranges in NQ, *E. resinifera* was genetically distinct from *E. pellita*. This suggests there are strong reproductive barriers isolating *E. pellita* and *E. resinifera* in NQ. Natural interspecific hybrids are possible but rare (Chippendale *et al.* 1984; Griffin *et al.* 1988), hence relatively strong pre-zygotic barriers probably exist, perhaps due to flowering asynchrony or floral structural incompatibles and flower size. This may limit crossing between the two species as style length incompatibles are known to cause unidirectional crossing in eucalypts (Gore *et al.* 1990).

Within the species studied, *E. resinifera* in particular has been subject to few previous genetic studies and its taxonomy continues to be revised. This current study included

samples from all proposed taxa within the *E. resinifera* complex. Only two genetic sub-groups were identified, however, a NQ group (including 2 provenances, Ravenshoe and Mareeba) which probably corresponding with *E. macta,* the new species identified by Hill *et al.* (2000), and a southern group consisting of all the remaining *E. resinifera* material from Fraser Island southwards. Like *E. pellita*, the major geographic subdivision aligns with a transition in biogeographic regions from the Atherton to the Central Queensland biogeographic zone and an ecological barrier, the Burdekin gap (Cracraft 1991; Ford 1987). It is likely that a disjunction in the species range and restrictions to the dependent pollinator fauna also contribute to the genetic structuring of *E. resinifera*. Should they show significant adaptive differences, these two regions may be considered geographical races or subspecies. Closer examination of this aspect may be pursued by study of quantitative variation of this material planted in provenance trials such as the Forests NSW trials in northern NSW.

In this study, a cline in genetic diversity of *E. resinifera* occurred, with the highest level of diversity in the south of species' range. This suggests the origin of this species was in the south or Central coast of NSW, which was followed by a migration northward and subsequent differentiation through drift and/or adaptation. Differentiation in this species was likely enhanced by ecological isolation due to the Burdekin gap.

Microsatellite markers were unable to resolve *E. scias* from southern *E. resinifera* which was largely in accord with isozymes data (House *et al.* 1996). It was found that within the southern *E. resinifera* group (including both *E. resinifera* and *E.scias*) geographic relationship seemed to be a better predictor of genetic relationship than taxonomic

grouping. Populations from these two taxa that were geographically proximate tended to show the highest relationship (e.g. *E. scias* from Sth Brooman and *E. resinifera* from nearby Nowra).

Furthermore, the current study found no genetic evidence to support sub-division of *E. scias* into three subspecies, which was also in accordance with a previous isozyme study (House *et al.* 1996). Because of the small sample sizes for each *E. scias* subspecies, however, the power to resolve population differences was low and larger samples sizes are required to be confident of this. *Eucalyptus scias* may represent a nascent species, early in its evolutionary divergence from *E. resinifera*. The two species differentiate on ecological grounds inhabiting environments with different edaphic and topographical conditions (Bevege DI pers. comm.). Hence they likely experience different adaptive pressures and may be undergoing divergent selection, in which case they may represent genic species, species which are divergent at a relatively few adaptive or speciation loci, but may share widespread genome similarity due to ongoing gene flow or only very recent isolation (Wu 2001; Wu *et al.* 2004).

The study demonstrates the power of the Bayesian model based approach and microsatellites in resolving lower order classification in groups like the eucalypts. A major advantage of the approach is that preconceived groupings, taxonomic, geographic or otherwise are not imposed upon the data, thus the data determine the most-appropriate model of structure. This is particularly useful for studies when the taxonomy is not clear, such as these red mahoganies. The delineation of gene pools for breeding and forest management decisions may be better served based on genetic affinities. For example, managing *E. scias* material with a *E. resinifera* gene pool for breeding would seem more

appropriate than with *E. pellita* despite their historic relationship, and may help avoid issues with outbreeding depression in hybrids.

Further study, including all members of *Annulares* will be required to determine the extent which microsatellite markers are useful for analysis of relationships across the taxon. A phylogeographic study of *Annulares* and sympatric *Exertaria* is suggested, using an approach more suitable for phylogenic inference, such as chloroplast haplotyping (Freeman *et al.* 2001; Jones *et al.* 2006) or nuclear gene sequencing. Increasingly it has been shown that the correct inference of management groups and genealogies often requires analysis of a number of genes (Burg *et al.* 2004; Muir *et al.* 2006; Zink *et al.* 2008).

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Appendices

Appendix 1 List of 48 candidate SSR primers for the pre-screen experiment

Index	Marker	Fluoro[A]	Org[B]	Focal Expected	SSR Describe	T_m	PCR Program
1	EMBRA 066F	6Fam	EG	155	(AG)18A(AG)4	50.7	PtTX50
2	EMBRA 195F	Ned	EG	240	(GA)24	51.3	PtTX50
3	EMBRA 182F	VIC	EG	211	(GA)22	51.9	PtTX50
4	EMBRA 210F	PET	EG	213	(TC)25	52.1	PtTX50
5	EMBRA 062F	6Fam	EG	139	(AG)18	52.3	PtTX50
6	EMBRA 116F	6Fam	EG	104	(TC)20	53.5	PtTX50
7	EMBRA 107F	PET	EG	123	(GA)20	53.7	PtTX54
8	EMBRA 103F	Ned	EG	116	(AG)24	54	PtTX54
9	EMBRA 183F	6Fam	EG	183	(GA)18	54.2	PtTX54
10	EMBRA 055F	PET	EG	178	(AG)26	54.4	PtTX54
11	EMBRA 034F	VIC	EG	104	(AG)21	54.5	PtTX54
12	EMBRA 169F	PET	EG	127	(CT)17	54.7	PtTX54
13	EMBRA 068F	6Fam	EG	108	(AG)20	54.8	PtTX54
14	EMBRA 214F	Ned	EG	151	(TC)22	54.9	PtTX54
15	EMBRA 054F	VIC	EG	144	(AG)17	54.9	PtTX54
16	EMBRA 094F	PET	EG	200	(GA)21	55	PtTX54
17	EMBRA 081F	PET	EG	70	(CT)20	55.2	PtTX54
18	EMBRA 098F	PET	EG	197	(AG)8(G)6(AG)3AA...(GA)20	55.3	PtTX54
19	EMBRA 175F	6Fam	EG	81	(CT)16	55.4	PtTX56
20	EMBRA 042F	PET	EG	127	(AG)15	55.5	PtTX56
21	EMBRA 043F	VIC	EG	145	(AG)14	55.6	PtTX56
22	EMBRA 011F	Ned	EG	97	(AG)4GG(AG)13	55.7	PtTX56
23	EMBRA 007F	6Fam	EG	115	(AG)15	55.8	PtTX56
24	EMBRA 092F	6Fam	EG	178	(GA)24	55.8	PtTX56
25	EMBRA 204F	PET	EG	147	(TC)25	55.9	PtTX56
26	EMBRA 083F	6Fam	EG	65?	(CT)22	55.9	PtTX56
27	EMBRA 095F	6Fam	EG	110	(CT)19	55.9	PtTX56
28	EMBRA 053F	6Fam	EG	130	(AG)17GT(AG)5	56	PtTX56
29	EMBRA 001F	VIC	EG	127	(AG)33	56.1	PtTX56
30	EMBRA 070F	6Fam	EG	169	(CATA)9CAT(AC)17	56.2	PtTX56
31	EMBRA 242F	PET	EG	144	(TC)4CC(TC)3TT(TC)6CC(TC)9TCC...(CA)18AA(CA)3	56.2	PtTX56
32	EMBRA 008F	Ned	EG	127	(AG)21	56.2	PtTX56
33	EMBRA 028F	6Fam	EG	178	(AG)25	56.2	PtTX56
34	EMBRA 039F	VIC	EG	146	(AG)9(CA)11	56.2	PtTX56
35	EMBRA 040F	6Fam	EG	141	(AG)19(CA)4	56.3	PtTX56
36	EMBRA 088F	Ned	EG	84	(TC)21	56.4	PtTX56
37	EMBRA 080F	VIC	EG	55	(GA)14GC(GA)7	57.2	PtTX58
38	EMBRA 209F	6Fam	EG	153	(TC)22	57.3	PtTX58
39	EMBRA 006F	VIC	EG	98	(AG)19	57.4	PtTX58
40	EMBRA 104F	PET	EG	99	(CT)17	57.4	PtTX58
41	EMBRA 206F	PET	EG	316	(GA)8AA(GA)11	57.4	PtTX58
42	EMBRA 187F	6Fam	EG	193	(GA)9CAGG(GA)20	57.7	PtTX58
43	EMBRA 164F	Ned	EG	127	(CT)18	57.9	PtTX60

Index	Marker	Fluoro[A]	Org[B]	Focal Expected	SSR Describe	T_m	PCR Program
44	EMBRA 012F	6Fam	EG	98	(AG)22	60.3	PtTX60
46	EMBRA 2002F	PET	EG	263	CCACCACCACCAC CACCACCACCACC	62.2	PtTX60
47	EMBRA 2000F	PET	EG	238	CTCTCTCTCTCTCT CTCTCTCTC	62.4	PtTX60
48	EMBRA 941F	Ned	EG	235	TCTCTCTCTCTCTC TCTCTCTCTCTCTC TCTCT	62.9	PtTX60

[A]: Fluorescently labelled
[B]: Original
EG: *Eucalyptus grandis*

Appendix 2 P- value for genotypic disequilibrium based on 1560 permutation. Adjusted P-value for 5% nominal levels is: 0.00061

Locus_A	Locus_B	P - Value	Locus_A	Locus_B	P - Value
EMBRA006	EMBRA011	0.00192	EMBRA028	EMBRA210	0.00064
EMBRA006	EMBRA020	0.00064	EMBRA028	EMBRA214	0.00064
EMBRA006	EMBRA028	0.00064	EMBRA028	EMBRA242	0.08526
EMBRA006	EMBRA042	0.01026	EMBRA042	EMBRA068	0.38846
EMBRA006	EMBRA068	0.04551	EMBRA042	EMBRA081	0.2359
EMBRA006	EMBRA081	0.57436	EMBRA042	EMBRA120	0.11603
EMBRA006	EMBRA120	0.00449	EMBRA042	EMBRA175	0.02692
EMBRA006	EMBRA175	0.14423	EMBRA042	EMBRA209	0.05321
EMBRA006	EMBRA209	0.06859	EMBRA042	EMBRA210	0.07885
EMBRA006	EMBRA210	0.00064	EMBRA042	EMBRA214	0.00128
EMBRA006	EMBRA214	0.00833	EMBRA042	EMBRA242	0.00128
EMBRA006	EMBRA242	0.98782	EMBRA068	EMBRA081	0.09487
EMBRA011	EMBRA020	0.23974	EMBRA068	EMBRA120	0.10962
EMBRA011	EMBRA028	0.05385	EMBRA068	EMBRA175	0.09167
EMBRA011	EMBRA042	0.03654	EMBRA068	EMBRA209	0.46859
EMBRA011	EMBRA068	0.30962	EMBRA068	EMBRA210	0.03846
EMBRA011	EMBRA081	0.09038	EMBRA068	EMBRA214	0.08846
EMBRA011	EMBRA120	0.04487	EMBRA068	EMBRA242	0.09615
EMBRA011	EMBRA175	0.00449	EMBRA081	EMBRA120	0.40897
EMBRA011	EMBRA209	0.13077	EMBRA081	EMBRA175	0.06218
EMBRA011	EMBRA210	0.00128	EMBRA081	EMBRA209	0.03269
EMBRA011	EMBRA214	0.00128	EMBRA081	EMBRA210	0.3859
EMBRA011	EMBRA242	0.38974	EMBRA081	EMBRA214	0.00321
EMBRA020	EMBRA028	0.00064	EMBRA081	EMBRA242	0.00385
EMBRA020	EMBRA042	0.01474	EMBRA120	EMBRA175	0.00064
EMBRA020	EMBRA068	0.28269	EMBRA120	EMBRA209	0.01731
EMBRA020	EMBRA081	0.07308	EMBRA120	EMBRA210	0.00064
EMBRA020	EMBRA120	0.00385	EMBRA120	EMBRA214	0.00513
EMBRA020	EMBRA175	0.00385	EMBRA120	EMBRA242	0.03846
EMBRA020	EMBRA209	0.01282	EMBRA175	EMBRA209	0.01859
EMBRA020	EMBRA210	0.0109	EMBRA175	EMBRA210	0.00064
EMBRA020	EMBRA214	0.00128	EMBRA175	EMBRA214	0.00962
EMBRA020	EMBRA242	0.10577	EMBRA175	EMBRA242	0.49808
EMBRA028	EMBRA042	0.00064	EMBRA209	EMBRA210	0.00064
EMBRA028	EMBRA068	0.00128	EMBRA209	EMBRA214	0.00064
EMBRA028	EMBRA081	0.1359	EMBRA209	EMBRA242	0.00064
EMBRA028	EMBRA120	0.00385	EMBRA210	EMBRA214	0.00128
EMBRA028	EMBRA175	0.00064	EMBRA210	EMBRA242	0.06859
EMBRA028	EMBRA209	0.00064	EMBRA214	EMBRA242	0.00192

Appendix 3 Test for HWE**.** Departures from HWE may indicate substructure or null alleles. Test each locus in each species.

Locus	***E. pellita***		***E. resinifera***		***E. Scias***	
	Pro	**Significant**	**Pro**	**Significant**	**Pro**	**Significant**
EMBRA 006	0.000	<0.001	0.013	<0.05	0.073	ns
EMBRA 011	0.006	<0.01	0.905	ns	0.040	<0.05
EMBRA 020	0.000	<0.001	0.003	<0.01	0.699	ns
EMBRA 028	0.000	<0.001	0.000	<0.001	0.189	ns
EMBRA 042	0.000	<0.001	0.011	<0.05	0.869	ns
EMBRA 068	0.000	<0.001	0.000	<0.001	0.003	<0.05
EMBRA 081	1.000	ns	0.124	ns	0.724	ns
EMBRA 120	0.703	ns	0.074	ns	0.861	ns
EMBRA 175	0.045	<0.05	0.000	<0.001	0.948	ns
EMBRA 209	0.000	<0.001	0.000	<0.001	0.360	ns
EMBRA 210	0.000	<0.001	0.000	<0.001	0.822	ns
EMBRA 214	0.482	ns	0.000	<0.001	0.029	<0.05
EMBRA 242	0.000	<0.001	0.000	<0.001	0.389	ns

Appendix 4 Null allele test**.** Locus by locus on a taxa basis

Locus	***E. pellita***	***E. resinifera***	***E. scias***
EMBRA 006	No	No	No evidence
EMBRA 011	No	No	No evidence
EMBRA 020	Yes	Yes	No evidence
EMBRA 028	Yes	No	Yes
EMBRA 042	No	No	No evidence
EMBRA 068	No	Yes	Yes
EMBRA 081	No	No	No evidence
EMBRA 120	No	No	No evidence
EMBRA 175	No	Yes	No evidence
EMBRA 209	No	No	No evidence
EMBRA 210	Yes	No	No evidence
EMBRA 214	No	No	No evidence
EMBRA 242	Yes	No	No evidence

Appendix 5 Genetic diversity for widespread and regionally distributed eucalypts based on microsatellite

Species	A	H_e	F_{st}	No. Pop[A]	No. Indi[B]	Loci	References
Widespread species							
C. citriodora subsp. *citriodora*	14.3	0.87		5	24	9	Shepherd *et al.* 2009 (unpub.)
C. citriodora subsp. *variegata*	15.0	0.89		5	24	9	Shepherd *et al.* 2009 (unpub.)
C. maculata	8.7	0.76		4	24	9	Shepherd *et al.* 2009 (unpub.)
E. brownii/E. poplnea	16.1	0.88	0.02	10		5	(Holman *et al.* 2003)
E. camaldulensis	8.4	0.83	0.08	29	990	15	(Butcher *et al.* 2008)
E. cloeziana	22.6	0.89	0.13	16	259	5	Stokoe 2002
E. globulus	9.5	0.75	0.09	34		8	(Steane *et al.* 2006)
E. sieberi	18.9	0.87			100	8	(Glaubitz *et al.* 2001)
E. grandis	19.8	0.86			192	6	(Kirst *et al.* 2005)
E. loxophleba	10.2	0.74		1		5	Byrne unpub. in (Byrne, 2008b)
E. pilularis	12.7	0.78	0.02	10	424	12	Shepherd *et al.* 2009 (unpub.)
E. urophylla	10.1	0.79	0.074	19	357	12	Payn *et al.* 2008
Mean of wide spread	13.9	0.83	0.07				
E. pellita	**18.01**	**0.811**	**0.17**	**12**	**85**	**13**	This study
E. resinifera	**19.70**	**0.876**	**0.13**	**11**	**77**	**13**	This study
Regional species							
E. cladocalyx	4.64	0.595	0.194	7		8	Byrne unpub. in Byrne 2008
E. sporadica	6.60	0.763		1		5	Byrne unpub. in Byrne 2008
E. lehmannii	5.60	0.745		1		5	Byrne unpub. in Byrne 2008
E. curtisii	4.96	0.540	0.30	12		4	Byrne unpub. in Byrne 2008
E. vernicosa/E. subcrenulta/E. johnstonii	22.5	0.86	0.12	11		4	Byrne unpub. in Byrne 2008
Mean of regional	5.45	0.661	0.205				
E. scias	**13.61**	**0.875**	**0.20**	**5**	**19**	**13**	This study

[A]: Number of populations
[B]: Number of individuals
unpub.: un publishment

Appendix 6 Inferred ancestry of 192 individuals for a K = 2 model determined using STRUCTURE

Index	Individual	Proportion of ancestry		Index	Individual	Proportion of ancestry	
		Resinfera group	Pellita group			Resinfera group	Pellita group
1	RES6002RAVQ	0.992	0.008	97	U1Timor	0.989	0.011
2	RES6003RAVQ	0.993	0.007	98	U2Timor	0.989	0.011
3	RES6004RAVQ	0.993	0.007	99	U64Wet	0.983	0.017
4	RES6005RAVQ	0.978	0.022	100	U89Wet	0.947	0.053
5	RES6006RAVQ	0.990	0.010	101	U99Wet	0.981	0.019
6	RES6090MARQ	0.973	0.027	102	U127Flo	0.899	0.101
7	RES6091MARQ	0.995	0.005	103	U129Flo	0.976	0.024
8	RES6092MARQ	0.981	0.019	104	U193Alo	0.903	0.097
9	RES6094MARQ	0.993	0.007	105	PEL401INDO	0.009	0.991
10	RES6096MARQ	0.987	0.013	106	PEL402INDO	0.010	0.990
11	RES6098MARQ	0.953	0.047	107	PEL405INDO	0.027	0.973
12	RES6100MARQ	0.994	0.006	108	PEL406INDO	0.005	0.995
13	RES6101MARQ	0.986	0.014	109	PEL407INDO	0.040	0.960
14	RES6032FRAQ	0.993	0.007	110	PEL410INDO	0.006	0.994
15	RES6035FRAQ	0.942	0.058	111	PEL411INDO	0.010	0.990
16	RES6070STRQ	0.988	0.012	112	PEL413INDO	0.037	0.963
17	RES6072BEEQ	0.979	0.021	113	PEL414SKIPN	0.006	0.994
18	RES6073BEEQ	0.993	0.007	114	PEL415SKIPN	0.005	0.995
19	RES6074BEEQ	0.982	0.018	115	PEL416SKIPN	0.016	0.984
20	RES6077BEEQ	0.970	0.030	116	PEL417SKIPN	0.007	0.993
21	RES6079BEEQ	0.987	0.013	117	PEL419SKIPN	0.009	0.991
22	RES6080BEEQ	0.992	0.008	118	PEL420SKIPN	0.013	0.987
23	RES6081BEEQ	0.987	0.013	119	PEL421SKIPN	0.014	0.986
24	RES6082POMQ	0.985	0.015	120	PEL422SKIPN	0.062	0.938
25	RES6083POMQ	0.962	0.038	121	PEL425SERPN	0.036	0.964
26	RES6084POMQ	0.979	0.021	122	PEL428SERPN	0.008	0.992
27	RES6085POMQ	0.996	0.004	123	PEL430SERPN	0.008	0.992
28	RES6087POMQ	0.990	0.010	124	PEL434SERPN	0.009	0.991
29	RES6088POMQ	0.973	0.027	125	PEL435SERPN	0.006	0.994
30	RES6089POMQ	0.995	0.005	126	PEL438SERPN	0.449	0.551
31	RES6007KENN	0.993	0.007	127	PEL439SERPN	0.143	0.857
32	RES6008KENN	0.985	0.015	128	PEL443SERPN	0.028	0.972
33	RES6009KENN	0.994	0.006	129	PEL444SERPN	0.005	0.995
34	RES6010KENN	0.992	0.008	130	PEL447SERPN	0.003	0.997
35	RES6011KENN	0.982	0.018	131	PEL448SERPN	0.004	0.996
36	RES6013KENN	0.996	0.004	132	PEL449SERPN	0.013	0.987
37	RES6014KENN	0.993	0.007	133	PEL450SERPN	0.010	0.990
38	RES6046KENN	0.995	0.005	134	PEL453SERPN	0.008	0.992
39	RES6048KENN	0.994	0.006	135	PEL454SERPN	0.007	0.993

Index	Individual	Proportion of ancestry		Index	Individual	Proportion of ancestry	
		Resinfera group	Pellita group			Resinfera group	Pellita group
40	RES6049KENN	0.994	0.006	136	PEL456SERPN	0.018	0.982
41	RES6050KENN	0.994	0.006	137	PEL457KIRPN	0.069	0.931
42	RES6052KENN	0.962	0.038	138	PEL458KIRPN	0.014	0.986
43	RES6054KENN	0.995	0.005	139	PEL459KIRPN	0.014	0.986
44	RES6066KENN	0.991	0.009	140	PEL461KIRPN	0.014	0.986
45	RES6016WOON	0.980	0.020	141	PEL463GOEPN	0.015	0.985
46	RES6017WOON	0.967	0.033	142	PEL464GOEPN	0.157	0.843
47	RES6018WOON	0.982	0.018	143	PEL465GOEPN	0.017	0.983
48	RES6020WOON	0.995	0.005	144	PEL466GOEPN	0.007	0.993
49	RES6021WOON	0.027	0.973	145	PEL468GOEPN	0.006	0.994
50	RES6022WOON	0.087	0.913	146	PEL469GOEPN	0.013	0.987
51	RES6023WOON	0.036	0.964	147	PEL501KURQL	0.319	0.681
52	RES6065WOON	0.989	0.011	148	PEL502KURQL	0.096	0.904
53	RES6066WOON	0.963	0.037	149	PEL503KURQL	0.022	0.978
54	RES6068WOON	0.993	0.007	150	PEL504KURQL	0.790	0.210
55	RES6069WOON	0.995	0.005	151	PEL505KURQL	0.425	0.575
56	RES6025COFN	0.109	0.891	152	PEL506KURQL	0.012	0.988
57	RES6026COFN	0.993	0.007	153	PEL507STAQL	0.745	0.255
58	RES6027COFN	0.916	0.084	154	PEL509STAQL	0.031	0.969
59	RES6028COFN	0.994	0.006	155	PEL510STAQL	0.043	0.957
60	RES6029COFN	0.927	0.073	156	PEL511STAQL	0.008	0.992
61	RES6030COFN	0.979	0.021	157	PEL512STAQL	0.818	0.182
62	RES6031COFN	0.992	0.008	158	PEL513ARIQL	0.068	0.932
63	RES6057COFN	0.993	0.007	159	PEL514ARIQL	0.231	0.769
64	RES6060COFN	0.995	0.005	160	PEL515ARIQL	0.011	0.989
65	RES6061COFN	0.984	0.016	161	PEL516ARIQL	0.005	0.995
66	RES6062COFN	0.993	0.007	162	PEL519ARIQL	0.018	0.982
67	RES6063COFN	0.987	0.013	163	PEL521ARIQL	0.006	0.994
68	RES6064COFN	0.996	0.004	164	PEL522ARIQL	0.007	0.993
69	RES6036NOWN	0.993	0.007	165	PEL523CARQL	0.009	0.991
70	RES6038NOWN	0.973	0.027	166	PEL524CARQL	0.011	0.989
71	RES6040NOWN	0.987	0.013	167	PEL527CARQL	0.006	0.994
72	RES6041NOWN	0.993	0.007	168	PEL529CARQL	0.012	0.988
73	RES6042NOWN	0.993	0.007	169	PEL533CARQL	0.012	0.988
74	RES6043NOWN	0.783	0.217	170	PEL535CARQL	0.011	0.989
75	RES6045NOWN	0.993	0.007	171	PEL539CARQL	0.007	0.993
76	RES6102EWIN	0.958	0.042	172	PEL542CARQL	0.035	0.965
77	RES6103EWIN	0.991	0.009	173	PEL543CARQL	0.007	0.993
78	SCIA301BOON	0.951	0.049	174	PEL546MOSQL	0.288	0.712
79	SCIA302BOON	0.977	0.023	175	PEL547MOSQL	0.011	0.989
80	SCIA303BOON	0.990	0.010	176	PEL548MOSQL	0.047	0.953
81	SCIA304BOON	0.993	0.007	177	PEL549KIRQL	0.120	0.880

Index	Individual	Proportion of ancestry		Index	Individual	Proportion of ancestry	
		Resinfera group	**Pellita group**			**Resinfera group**	**Pellita group**
82	SCIA305BOON	0.940	0.060	178	PEL550KIRQL	0.008	0.992
83	SCIA306MALN	0.987	0.013	179	PEL551KIRQL	0.008	0.992
84	SCIA307MALN	0.994	0.006	180	PEL552KIRQL	0.058	0.942
85	SCIA308BANN	0.992	0.008	181	PEL553KIRQL	0.014	0.986
86	SCIA309BANN	0.994	0.006	182	PEL554KIRQL	0.051	0.949
87	SCIC310BRON	0.965	0.035	183	PEL555KIRQL	0.013	0.987
88	SCIC311BRON	0.737	0.263	184	PEL558KIRQL	0.031	0.969
89	SCIC312BRON	0.981	0.019	185	PEL559HELQL	0.025	0.975
90	SCIC313BRON	0.991	0.009	186	PEL560HELQL	0.009	0.991
91	SCIC314BRON	0.943	0.057	187	PEL561HELQL	0.033	0.967
92	SCIC315BRON	0.984	0.016	188	PEL562HELQL	0.004	0.996
93	SCIS316BOUN	0.991	0.009	189	PEL563HELQL	0.099	0.901
94	SCIS318BOUN	0.981	0.019	190	PEL544SSO,N	0.099	0.901
95	SCIS319BOUN	0.994	0.006	191	PEL545SSO,N	0.009	0.991
96	SCIS320BOUN	0.982	0.018	192	PEL564SSO,N	0.004	0.996

Number preceding locality code (see table 1 for explanation of locality code) is family number and species (e.g. RES6002RAVQ: samples of *E. resinifera* family no. 6002, were collected from Ravenshoe, Queensland)