

**The invasion ecology of *Acacia pycnantha*:
A genetic approach**

Joice Ndlovu

Dissertation presented for the degree of

Doctor of Philosophy in Botany

at Stellenbosch University

(Department of Botany and Zoology)

Supervisors: Prof. D.M. Richardson, Dr J.J. Le Roux, Dr J.R.U. Wilson

December 2013

Declaration

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Abstract

Australian *Acacia* species are an important group of invaders and are known to form dense monospecific cultures in invaded habitats. Despite the ecological and economic importance of invasive acacias, little is known about their invasive biology both from an ecological and evolutionary perspective.

Molecular genetic methods have increasingly become important in identifying source populations for invasive species and determining the population genetic structure of these populations. This thesis applied molecular tools to understand the invasion ecology of *Acacia pycnantha* and its rhizobial symbionts as a model system of Australian *Acacia* introductions. Specific objectives were to: reconstruct the molecular phylogeny of invasive and native populations of populations of *Acacia pycnantha* and identify the native provenance of *A. pycnantha*; identify microsatellite markers for *Acacia pycnantha* and other invasive Australian acacias based on transferring microsatellite markers developed for *A. mangium*, *A. saligna*, *Paraserianthes lophantha* and universal chloroplast microsatellites developed from tobacco; assess the introduction dynamics of *Acacia pycnantha* in South Africa and identify the source populations in the species' native range; and determine which nitrogen fixing symbionts nodulate *A. pycnantha* and determine whether *A. pycnantha* brought its symbionts along from its native range or acquired them in the invasive range.

Nuclear and chloroplast DNA sequence data were used to reconstruct phylogeographic relationships between native and invasive *A. pycnantha* populations. The chloroplast phylogeny showed that Australian populations of *A. pycnantha* are geographically structured into two previously informally recognized lineages (representing wetland and dry land forms). Habitat fragmentation is probably the

result of cycles of aridity and abundant rainfall during the Pleistocene. The invasive population in Portugal was found to be the wetland form while South African populations were found to be predominantly wetland form although some dryland forms were identified.

Thirty microsatellites out of the forty nine tested microsatellites successfully amplified across all species tested (*A. implexa*, *A. longifolia*, *A. melanoxydon*, *A. pycnantha* and *A. podalyriifolia*). High Transfer rates varied between 85% for microsatellites developed for *A. mangium* to 50 % for those developed in *A. saligna*. Although transfer rates were high only twelve microsatellites (24%) out of the fifty tested were polymorphic while the chloroplast microsatellites showed no polymorphism. The low level of polymorphic loci calls for development of more microsatellites in this genus especially for species that have high commodity value.

Nuclear microsatellites revealed three genetic groupings with substantial admixture in the native range (1. wetland Victoria and South Australia populations; 2. dryland Victoria and Flinders Range population; and 3. New South Wales). Admixture in the native range may have occurred as a result of reforestation exercises. *Acacia pycnantha* has been widely used in reforestation projects in Australia because of its fast growth rate and ease of germination. Admixed populations were most-likely introduced to South Africa thus establishment of *A. pycnantha* may have been facilitated by already admixed propagules in the invasive range. Extensive admixture in the native range made it difficult to identify source populations of invasive *A. pycnantha* found in South Africa.

The rhizobial symbionts of *A. pycnantha* were identified, showing that this species utilizes a wider suite of symbionts in its invasive range than its native range and there is support for both the co-introduction and host jumping hypotheses. This creates

substantial opportunities for horizontal gene transfer between previously allopatric bacterial lineages, with as yet unknown consequences for plant and bacterial invasions.

Acknowledgements

I would like to thank the following people and organisations:

- Prof Dave Richardson (supervisor) who gave me a chance to do a PhD gave new ideas and insights into the project, for the financial support throughout the project and the constant encouragement.
- Dr J. J. Le Roux (co-supervisor) who guided me through the Molecular ecology field , bringing in new ideas into my work and spent a lot of time in the field and the lab with me and taught me how to write scientific articles.
- Dr J.R.U. Wilson for a lot of support and guidance in writing my thesis and bringing in new ideas into my work and the continuous encouragement throughout the thesis.
- Martin O'Leary from the State Herbarium and Bioknowledge South Australia, for helping with the collections in Australia, identifying *Acacia pycnantha* and his constant support throughout the work.
- Prof Andy Lowe for giving me an opportunity to work in his lab at the University of Adelaide in Australia.
- Prof Rob Reid for allowing me to use his lab at the University of Adelaide in Australia.
- Fiona Impson for helping with locality information for *Acacia pycnantha* in South Africa.
- Kate Le Roux for helping with *Acacia pycnantha* collections in Australia.
- Bruce Maslin for helping with locality information for *Acacia pycnantha* in Australia

- Christy Momberg, Mathilda van der Vyver, Sarah Davies, Anel Garthwaite
Karla Coombe-Davies for administrative assistance.
- My family for their constant support and encouragement.
- The National Research Foundation, the Working for Water Programme and
various other organisations for the funding.
- All my friends for the time well spent at Stellenbosch University.

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Chapter 1: General Introduction

1.1 Invasions

The acceleration in international trade has led to an increase in the movement of species globally, some of which have become naturalized and invasive (Campbell, 2001). Invasive plant species impact on the ecosystems they invade, affecting among other things, geomorphology, hydrology, nutrient cycling (Gordon, 1998), soil microbial structure (Gaertner *et al.*, 2009), and species composition (Simberloff & Von Holle, 1999). These impacts often have substantial economic implications (Pimentel *et al.*, 2005). In response to these impacts, a central applied research focus in invasion biology is to understand how, when and why species invade new ranges. Understanding these aspects can not only aid in the management of existing invaders but also help in determining the potential impacts of species before they are introduced into new environments. Furthermore, invasive species and especially invasive legumes offer an excellent opportunity to address basic research questions in plant invasion ecology e.g. interactions between plants and their associated symbionts.

1.2 How do plants invade?

1.2.1 Plant traits, invasiveness and invisibility

Baker (1965) postulated that weeds generally share common traits, including the ability to: reproduce both vegetatively and sexually; undergo rapid growth to sexual maturity; adapt to environmental stress; tolerate environmental heterogeneity; and have highly effective dispersal mechanisms. Many authors have argued that for

plants to become invasive, they must have all, or a subset of, the traits of Baker's "ideal weed" (Richardson & Pyšek, 2006). This has proved too simplistic (Williamson, 1993) and the quest to find robust correlates of invasiveness remains a key aim of much research in invasion ecology. Despite efforts to identify sets of traits associated with invasiveness a generalization regarding traits consistently associated with invasiveness is still lacking. However, more and more empirical evidence points to introduction dynamics and the potential for rapid evolution to be important determinants of invasive potential for many plant species (Lockwood *et al.*, 2005; Le Roux *et al.*, 2011; Taylor & Hastings, 2005; Thompson *et al.*, 2012). Attributes like high propagule pressure have been consistently linked to rapid geographical expansion while multiple introductions increases the likelihood of an introduction occurring in an invasion window (i.e. at a suitable time of year or co-inciding with a suitable disturbance event) (Taylor and Hastings, 2005). On the other hand, Allee effects can delay the onset of invasion, leading to a lag phase, and can lower the probability of successful establishment (Taylor & Hastings, 2005). In addition to aspects of the introduction dynamics, taxon-specific features are crucial for the detection and management of invasive species in different plant groups and species. For example, seed size and the length of the juvenile period are important for separating invasive from non-invasive pines (Richardson & Pyšek, 2006) and early establishment is an important correlate of invasiveness in *Cytisus scoparius* (Drenovsky *et al.*, 2012). Other abiotic characteristics like native geographical and latitudinal ranges are also considered important in influencing invasiveness (Richardson & Pyšek, 2006). Invasibility (the level or extent of susceptibility to invasion of recipient communities) is also a factor that affects the success of plant invasions (Richardson & Pyšek, 2006).

1.2.2 Evolutionary potential and genetic diversity

Although plant traits and invasibility coupled with propagule pressure are important for establishment success and subsequent invasion, evolutionary changes associated with an introduced population and standing genetic variation in the founder population may confer some opportunities for invasion success (Lavergne & Molofsky, 2007; Prentis *et al.* 2009; Le Roux & Wicczorek, 2009; Thompson *et al.*, 2012). The increase in molecular systematic and genetic studies in the past decade has presented opportunities for understanding the role of evolutionary dynamics in plant invasions.

The link between propagule pressure and multiple introductions and invasion success (Wilson *et al.*, 2009) may directly influence evolutionary potential by increasing gene diversity within introduced populations. High genetic diversity minimizes the effects of inbreeding depression and drift while increasing the likelihood of introducing pre-adapted genotypes (Simberloff, 2009). In addition, formation of new genotypes is more likely in the invaded ranges when the propagules introduced are from allopatric sources in the native range (Simberloff, 2009; Le Roux *et al.*, 2011), and in some instances such new genotypes have been shown to be highly invasive (e.g. *Phalaris arundinacea*; Lavergne & Molofsky, 2007).

Molecular ecological studies of plant invasions can complement conventional ecological studies in improving the management of invasive species. Molecular approaches have been essential to resolve taxonomic problems (e.g. *Tamarix* spp. in the US; Gaskin & Schaal, 2002), elucidate geographic origins of invasive populations (e.g. *Macfadyena unguis-cati* and *Jatropha gossypifolia*; Prentis *et al.*, 2009), detect introgression (e.g. *Parkinsonia aculeata*; Hawkins *et al.*, 2007), and track dispersal

and spread and the role of genetic diversity in invasion success (e.g. *Senecio madagascariensis*; Le Roux *et al.*, 2010).

1.2.3 Mutualistic interactions

Crucially, many introduced plant species are dependent on mutualistic interactions with pollinators, seed dispersal agents, mycorrhizal fungi, nitrogen fixing bacteria and plant-growth promoting endophytic bacteria (Richardson *et al.*, 2000a). These mutualists are important for overcoming barriers of plant establishment. It is therefore important that plants either form novel mutualistic interactions or retain the services of mutualists that exist in native ranges (by co-introduction) in the new range as these will confer direct establishment advantages (Rodríguez-Echeverría, 2010, Weir *et al.*, 2004). For example, nitrogen is a major limiting nutrient in many ecosystems globally and it is therefore conceivable that the ability to fix nitrogen by leguminous plants through their symbiosis with rhizobial bacteria is advantageous for their establishment and subsequent invasion potential (e.g. Vitousek & Walker, 1989).

1.3 Tree Invasions

Most tree invasions globally are the result of intentional introductions for ornamental horticultural, commercial forestry and agroforestry (Richardson & Rejmánek, 2011). Most are characterised by high propagule pressure, repeated introductions, concerted breeding efforts and introduction with compatible soil mutualists (Le Roux *et al.*, 2011). In many instances these species have escaped from areas set aside for their use to colonize neighbouring agricultural and natural areas (Richardson & Rejmánek, 2011). Despite their widespread occurrence in new

environments and the impacts that have been reported, invasive trees were not widely considered to be a major environmental problem until fairly recently (Richardson & Rejmánek, 2011). To date, 622 species of trees and shrubs are known to be “invasive” (sensu Pyšek *et al.*, 2004; Richardson & Rejmánek, 2011) with 21 woody plant species featuring in the world’s “top 100” invaders (Lowe *et al.*, 2000).

1.4 The genus *Acacia* as a model for studying invasive trees

Australian taxa in the genus *Acacia* (most of which were previously grouped in subgenus *Phyllodineae* in *Acacia*) offer exciting opportunities to study the molecular ecology of invasive woody plants (Richardson *et al.*, 2011). Australian acacias have been planted across the globe for agroforestry and ornamental purposes, and some species are now notorious invaders, especially in Mediterranean-type climates. For example, *Acacia mearnsii* and *A. longifolia* are the two most widespread woody invasive species in South Africa and Portugal respectively. Although some Australian species cause substantial negative impacts on the environment, some species are economically beneficial in different parts of the world and across different sectors of the economy (Kull *et al.*, 2011). These social, economic and conservation impacts contribute to the attractiveness of Australian acacias as a model system to study many aspects of invasion ecology (Richardson *et al.*, 2011).

1.5 *Acacia pycnantha*

In this thesis *Acacia pycnantha*, which is invasive in South Africa and Portugal and naturalised in the United States, was used to study aspects of the invasion

ecology of Australian acacias. *Acacia pycnantha* was chosen as a model system because: a) it has a well-documented introduction history in South Africa (Poynton, 2009); b) the species is found in different biogeographical regions in Australia (see Maslin & McDonald, 2004) and also invades different biogeographical regions in South Africa (see Henderson, 2001); c) nitrogen fixing mutualists are likely to be important determinant of the success of invasions in South Africa as *A. pycnantha* invades regions with nitrogen-poor soils; and d) management of the species (in terms of biocontrol) has been studied in South Africa (see Hoffmann *et al.*, 2002). These attributes used to select *A. pycnantha* as a model species for this study are a subset of attributes that make Australian acacias a good model system to study tree invasions (see Richardson *et al.*, 2011).

Acacia pycnantha, also known as the golden wattle, is an unarmed slender green tree which grows up to 8 m tall. It has pendulous leaflets that superficially resemble those of some species in the genus *Eucalyptus*. In Australia, *A. pycnantha* inhabits the understorey edges of forests dominated by *Eucalyptus* species (Maslin & McDonald, 2004). The tree has dull green leathery phyllodes which are up to 200 mm long, distinctly curved with a single prominent mid vein and have raised margins. The leaf tips are blunt or rounded and a large gland is situated at the base of each leaf. *Acacia pycnantha* has bright yellow flowers arranged in globular flower heads. Seeds are borne in brown pods which are almost straight and slightly constricted. In Australia *A. pycnantha* has variable leaf morphologies and exhibits different morphological variants which are allopatric (Fig. 1.1). Two distinct morphological variants (dryland form and the wetland form) which occur in different ecotypes are recognized (Le Roux *et al.*, 2011) (Fig. 1.1E). The dryland form occurs in the drier Flinders Range in South Australia and the drier areas of Victoria. The wetland form is found in South Australia, the wetter parts of Victoria and New South Wales. In South

Africa, *A. pycnantha* was introduced for dune reclamation, as an ornamental plant, and for tanbark production (Henderson, 2001). Introductions were made on at least two separate occasions, followed by extensive movement within the country.

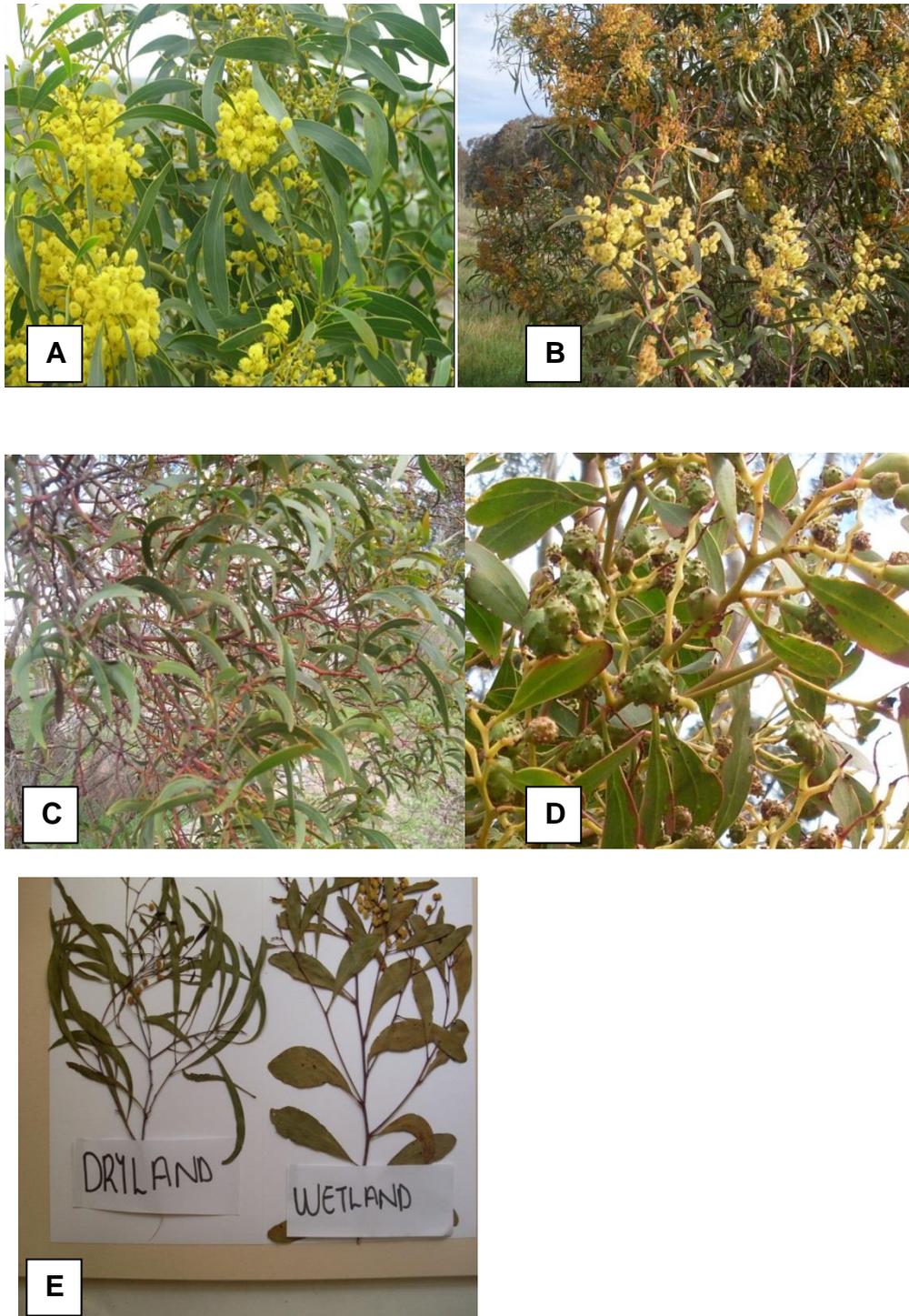


Fig. 1.1 Different variants of *Acacia pycnantha* occurring in the native range of the species in Australia: (A) South Australia, (B) New South Wales (Gundagai), (C) Victoria (Natimuk), (D) South Australia (Mt Compass) with *Trichilogaster signiventris* galls and (E) Herbarium specimens of the dryland and wetland forms of *A. Pycnantha* are housed at the Stellenbosch Herbarium. The specimens were identified by Martin O'Leary (State Herbarium of South Australia).

1.6 Distribution

Acacia pycnantha is widespread in Victoria and its range extends to the Flinders Range, York Peninsula, Southern Eyre Peninsula and Kangaroo Island in South Australia. It is also found in southern New South Wales, the Broken Hill area, and near Canberra in the Australian Capital Territory. The species also occurs in southern Western Australia where it is invasive (Maslin & McDonald, 2004). In South Africa *A. pycnantha* occurs in the Western Cape and Eastern Cape provinces (Henderson, 2001) (Fig. 1.2).

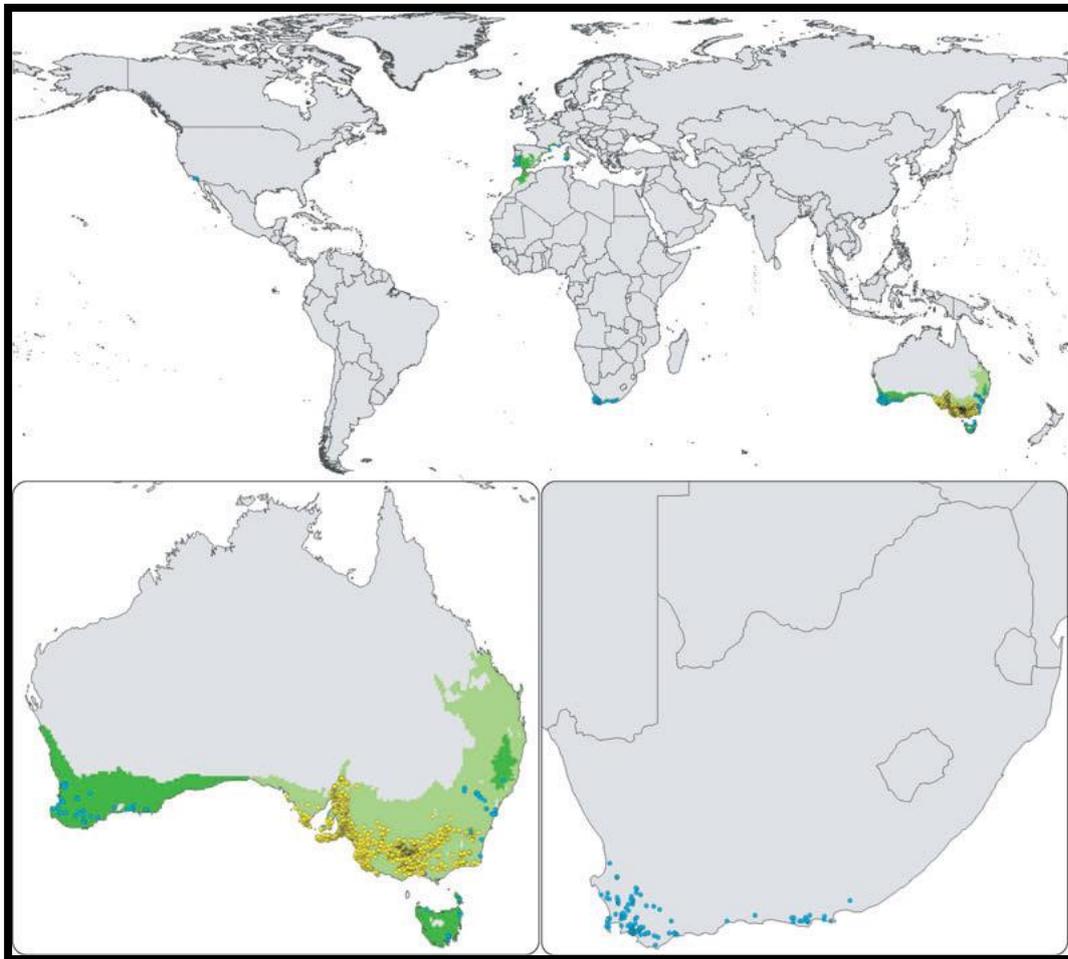


Fig.1.2 The global distribution of *Acacia pycnantha*. Native populations in Australia are indicated using yellow circles while blue circles show invasive and naturalised populations. Australian populations of *A. pycnantha* are indicated against restricted (light green shading) and full (dark green shading) correlative model backgrounds. Maps were extracted from Webber *et al.* (2011).

1.7 Aims and objectives

The main objective of this study was to use *Acacia pycnantha* as a study species to better understand how introduction history and mutualistic interactions affected South African invasions, using genetic tools. Specific objectives of the project, as addressed in the different chapters, are as follows:

- To reconstruct the phylogeography of *A. pycnantha* throughout its native (south eastern Australian) and invasive (South African, Portuguese, Western Australian) ranges (Chapter 2).
- To characterize a number of microsatellite markers previously developed for other taxa that are transferable and informative for *A. pycnantha* and related Australian acacias (Chapter 3).
- To assess the introduction dynamics of *A. pycnantha* in South Africa and identify the source populations in the species' native range using a population genetic approach (Chapter 4).
- To determine which nitrogen-fixing symbionts nodulate *A. pycnantha* and to determine whether these symbionts were co-introduced from the native range in Australia or whether *A. pycnantha* acquired novel symbionts in South Africa (Chapter 5).

Each chapter is presented as a stand-alone paper.

Chapter 1: General Introduction

This chapter provides a general overview of the entire study and sets a framework within which all the preceding research chapters will fall. It states the

aims, objectives and motivation of the study. A general literature review of tree invasions is provided and the use of genetic tools in studying plant invasions is justified.

Molecular genetics methods have increasingly become important in identifying source populations for invasive species and determining the population genetic structure of these populations. In this thesis, I used molecular tools to understand the invasion ecology of *Acacia pycnantha* and its rhizobial symbionts.

Chapter 2: Elucidating the native sources of an invasive tree species reveals unexpected native range diversity.

Knowledge of phylogeography of native and invasive range is important for designing effective biocontrol programmes for invasive alien plants. An extensive study of the genotypic diversity of *A. pycnantha* in different regions of the world (South Africa, Australia, and Portugal) was carried out. I used nuclear and chloroplast DNA sequence data to reconstruct phylogeographic relationships between native and invasive *A. pycnantha* populations. The aims were to: 1) reconstruct phylogenetic relationships among invasive (South Africa, Portugal, western Australia) and native (eastern Australia) populations of *A. pycnantha*; 2) compare genetic diversities within native and invasive populations; and 3) to infer the historical processes that may have shaped genetic structure in the natal ranges of the species.

Chapter 3: Transfer of orthologous microsatellite markers to *Acacia pycnantha* and other invasive Australian *Acacia* species.

To study the genetic diversity in *A. pycnantha* and other closely related invasive Australian acacias it is crucial to use molecular markers that are appropriate for the intended scales of investigation and the specific research questions. Population-level genetics that measure fine-scale genetic structure of recently diverged populations (such as introduced, invasive species) require fast-evolving markers such as microsatellite loci. Microsatellites have previously been developed for *A. mangium*, *A. saligna*, *Paraserianthes lophantha* and tobacco. These were transferred to *A. pycnantha* and other invasive Australian acacias. In this section the following questions were asked: 1) How many selected polymorphic microsatellite loci that were previously developed *A. mangium*, *A. saligna*, *Paraserianthes lophantha* and tobacco will cross-amplify in *A. pycnantha* and other invasive Australian acacias?; 2) How many universal chloroplast microsatellite markers will cross-amplify in *A. pycnantha* other Australian acacias?; 3) How many of the cross-amplified microsatellites are polymorphic? I also compared levels of gene diversity between invasive and native populations for five different acacia species with known and differential introduction histories (propagule pressure) in order to determine the native provenance of the invasive *Acacia* species and to compare the genetic diversities of the native and invasive populations.

Chapter 4: Native range plantings and admixture greatly alters genetic structure of invasive *Acacia pycnantha* (Benth) in South Africa.

Polymorphic microsatellites previously characterised for polymorphism in *A. pycnantha* were used to compare the genetic structure in the native (Australia) and invasive (South Africa) ranges of the species. The resulting data were used to determine whether the distribution of the species in its new range stemmed from a

single or multiple introduction events. The questions asked in this chapter were: 1) what genotypic diversity occurs in South African populations of *A. pycnantha* and what is their geographical distribution?; 2) What genotypic diversity occurs in native populations of *A. pycnantha* in south-eastern Australia and what is their geographic distribution?; 3) Can source populations of *A. pycnantha* invading South Africa be identified?; 4) Does admixture of native genotypes that are geographically isolated in Australia occur in South Africa?

Chapter 5: Co-invasion of South African ecosystems by an Australian legume and its rhizobial symbionts

South Africa's Cape Floristic Region, and the fynbos biome in particular, is home to a diversity of weedy herbaceous legumes, most notably Australian *Acacia* species. The ability of these *Acacia* species to establish in soils poor in nutrients (particularly nitrogen) may be due to their ability to enter effective symbiosis with compatible root-nodulating bacteria. In this chapter the rhizobial symbionts of *A. pycnantha* were identified associated with plants in their native and invasive ranges. The aims of this study were to: 1) determine the taxonomic diversity of rhizobia nodulating *A. pycnantha* in both native and invasive ranges; 2) determine whether rhizobial symbionts of invasive *A. pycnantha* in South Africa follow the co-introduction, host jumping hypothesis, or both; and 3) determine the level of promiscuity of *A. pycnantha*-rhizobial associations.

Chapter 6: Conclusions and recommendations

This section highlights the most important conclusions drawn from all facets of this study. Here I discuss the key findings from the thesis in the broader context of understanding and managing current invasions. I also discuss the potential future invasive threats and consequent research needs that my work has highlighted.

Chapter 2: Elucidating the native sources of an invasive tree species reveals unexpected native range diversity and structure.

This chapter was published in *Annals of Botany*.

Reference: Ndlovu, J., Richardson, D.M., Wilson, J.R.U., O'Leary, M. & Le Roux, J.J. (2013). Elucidating the native sources of an invasive tree species reveals unexpected native range diversity and structure. *Annals of Botany* doi10.1093/aob/mct057.

Abstract: Understanding the introduction history of invasive plant species is important for their management and identifying effective host-specific biological control agents. However, uncertain taxonomy, intra- and inter-specific hybridization, and cryptic speciation may obscure introduction histories, making it difficult to identify native regions to explore for host-specific agents. Here, our overall aim was to identify the native source populations of *Acacia pycnantha*, a tree native to south eastern Australia and invasive in South Africa, Western Australia and Portugal. Using a phylogeographic approach also allowed us to explore the historic processes that shaped genetic structure of *A. pycnantha* in the native range. We used nuclear (nDNA) and chloroplast DNA (cpDNA) sequence data in network and tree-building analyses to reconstruct phylogeographic relationships between native and invasive *A. pycnantha* populations. In addition, we used mismatch distributions, relative rates and Bayesian analyses to infer recent demographic processes and timing of events in Australia that led to population structure and diversification. The chloroplast network indicated that Australian populations of *A. pycnantha* are geographically structured into two informally recognized lineages, the wetland and dryland forms, while the nuclear phylogeny showed little geographic structure between these two

forms. Moreover, the dryland form of *A. pycnantha* showed close genetic similarity to the wetland form based on nDNA sequence data. Hybrid zones may explain these findings, supported here by incongruent phylogenetic placement of some of these taxa between nuclear and chloroplast genealogies. We hypothesize that habitat fragmentation due to cycles of aridity inter-dispersed with periods of abundant rainfall during the Pleistocene (ca. 100 KYA) probably gave rise to native dryland and wetland forms of *A. pycnantha*. Although the different lineages were confined to different ecological regions we also found evidence for intraspecific hybridization in Victoria. The invasive populations in Portugal and South Africa represent wetland forms, while some South African populations resemble the Victorian dryland form. The success of the biological control programme of *A. pycnantha* in South Africa may therefore be attributed to the fact that the gall forming wasp, *Trichilogaster signiventris*, was sourced from South Australian populations which closely match most of the invasive populations in South Africa.

Key words: *Acacia pycnantha*, biological control, biological invasions, genetic structure, hybridization, lineage divergence, native provenance, Pleistocene, systematics, taxonomy.

2.1 Introduction

Understanding the processes that shape species distributions and their evolutionary trajectories has long interested biogeographers, ecologists and phylogeographers. From a phylogeographic perspective, delineating genealogical relationships among taxa informs us about the historical processes that shaped

patterns of gene flow, local adaptation and, ultimately, speciation. There is growing interest in better understanding processes that underpin the evolutionary trajectories of contemporary species movements, especially those that involve the transfer of species that become established and invasive (Richardson *et al.*, 2011). Historical and contemporary biogeographies are both important, with the former influencing the latter in several ways (e.g., Hui *et al.*, 2011). Biological invasions are typically characterized by stochasticity, founder events and strong genetic drift, and introduced genotypes usually encompass only a small proportion of the total genetic diversity of the taxon (Bossdorf *et al.*, 2005; Kliber & Eckert, 2005). It is therefore important to understand the structure of natal populations when building a framework for testing hypotheses about the processes driving biological invasions, such as introduction histories, hybridization, gene diversity, and ultimately, evolutionary potential (Stepien *et al.*, 2002).

Relating historical biogeography with contemporary patterns of gene diversity associated with species introductions and invasions is not always straight forward, however (Le Roux *et al.*, 2011). Invasive plant populations can arise from individuals introduced from several previously allopatric parts of the native range. This creates opportunities for admixture, hybridization, and consequently genetic novelty (Prentis *et al.*, 2008). For example, *Acacia cyclops* sourced from different localities in Australia was introduced to a single location in South Africa that led to intra-specific hybridization (Le Roux *et al.*, 2011). On the other hand, a congener, *A. saligna*, shows high intraspecific diversification in its native range (Millar *et al.*, 2011), whereas invasive populations in South Africa comprise genetic entities not found in the native range (Thompson *et al.*, 2012). Similarly, the most invasive *Tamarix* genotype in the United States comprises a hybrid of two species, which are allopatric in the native Eurasian range (Gaskin & Schaal, 2002). Such complex introduction

scenarios are typical of species introduced for agroforestry in its widest sense, as species are often introduced from multiple sources on multiple occasions and normally are subjected to strong artificial selection following introduction.

Understanding introduction and invasion histories has important practical implications. The selection of effective host-specific biocontrol agents on invasive plants can depend on identifying which subspecific entities of the plant were introduced (Goolsby *et al.*, 2006; Harris, 1998; Wardill *et al.*, 2005). The identification of the native provenance of an invasive species can also improve the design of host specificity lists and subsequent host testing under the assumption that historical biogeographical processes similarly influenced hosts and agents, and thus co-evolution (Wardill *et al.*, 2005; McLeish *et al.*, 2007). However, it should be noted that the identification of the native provenance of invasive species can be complicated by long histories of plantings and cultivation within the species' native range (Thompson *et al.*, 2012).

Here we aim to place populations of *Acacia pycnantha* (Benth.) that are invasive in South Africa, Western Australia and in Portugal in the context of historical biogeographical patterns in the native range of the species in south eastern Australia. Specifically, we use chloroplast and nuclear DNA markers to: (a) reconstruct phylogenetic relationships among invasive (South Africa, Portugal, western Australia) and native (eastern Australia) populations of *A. pycnantha*; (b) compare genetic diversities within these invasive and native populations; and (c) to infer the historical processes that may have shaped genetic structure in the natal regions of the species.

2.2 Methods

2.2.1 Study system

Australia's national flower, *Acacia pycnantha* (Benth.), also known as the golden wattle, is native to New South Wales, Victoria, South Australia and is introduced and invasive in Western Australia. It is probably also naturalising in some areas of New South Wales and South Australia (Eyre Peninsula) from cultivated plantings in revegetation projects and along roadsides. The species, like many Australian acacias has visible phenotypic variation across its native range (Maslin, 2001; Maslin & McDonald, 2004). In particular informal morphological classification recognizes two very distinct ecotypes, the dryland and wetland forms (Fig. 2.1). The two forms have distinct phyllodes, flower colours and distributional ranges. Despite this variation, the species has not been formally separated into subspecific entities.

Seeds of *Acacia pycnantha* were introduced to South Africa on at least two occasions (1865 and 1890) for tannin production and for dune stabilization (Poynton, 2009), but the exact origin of the introduced seeds from the native range is unknown. Following introduction to South Africa, seeds of *A. pycnantha* were distributed extensively throughout the country (Poynton, 2009). Since then populations have become invasive in several locations with seven known localities of widespread invasions in the Eastern and Western Cape Provinces. *Acacia pycnantha* is now listed as a category-1 invasive plant meaning that all uses of the species are prohibited (Nel *et al.*, 2004; Henderson, 2001).

Following the success of other biological control agents against Australian acacias in South Africa, a gall-forming wasp, *Trichilogaster signiventris* was introduced in 1987 (Hoffmann *et al.*, 2002; Impson *et al.*, 2011), and a seed-feeding weevil *Melanterius maculatus* in 2003 (Impson *et al.*, 2011). While the wasp

dramatically reduced seed production, the impact of the weevil is still to be determined (Impson *et al.*, 2011).

Elsewhere in the world, *A. pycnantha* is invasive in Portugal (Richardson & Rejmánek, 2011) and naturalized in the United States (California) (<http://www.calflora.org>). An initial assessment of the potential for biocontrol of Australian acacias based on the experience in South Africa is underway in Portugal (Marchante *et al.*, 2011), with *T. signiventris* identified as a potential candidate for release.

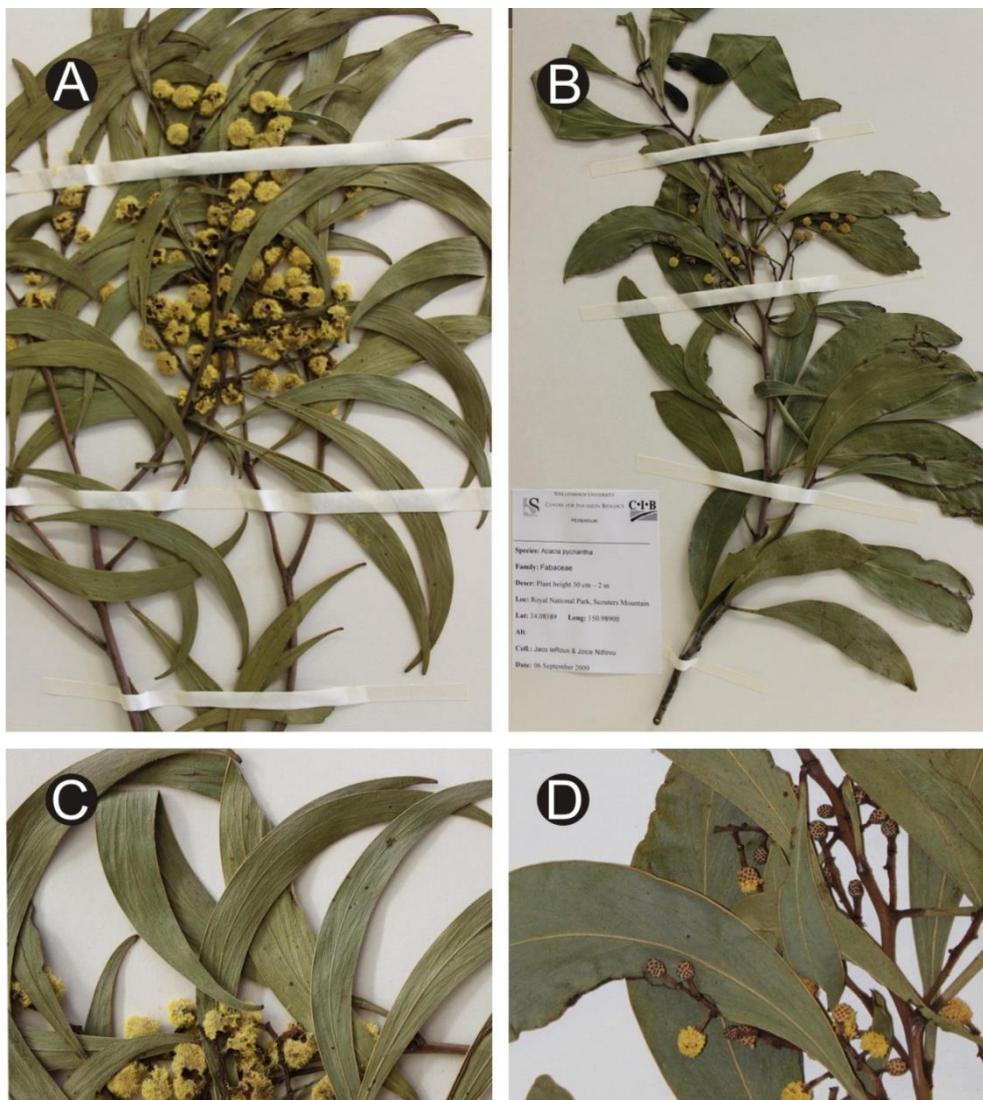


Fig. 2. 1 Herbarium specimens of the extreme ecotypes of *Acacia pycnantha* in Australia for the dryland form (A) characterized by thin phyllodes (C) and the wetland form (B) characterized by broad phyllodes (D).

2.2.2 Plant collection

Phyllodes of *A. pycnantha* were collected from throughout its native range (from what appeared to be natural populations) (south eastern Australia) and invasive (Western Australia and South Africa) ranges during 2009 (Table.2.1). In order to prevent sampling individuals from reforestation projects, most native populations were sampled away from the roadsides, e.g. in national parks. Between two and five individual trees were sampled from each population. A single population of *A.pycnantha* was also sampled in August 2010 in Portugal (see Table.2.1 for locality data). Phyllode material was dried in silica gel and kept at room temperature until DNA extraction. Duplicate voucher specimens were collected from each sampled population and deposited at the State Herbarium in Adelaide, South Australia and the Stellenbosch University Herbarium in Stellenbosch, South Africa.

2.2.3 DNA extraction, PCR amplification and sequencing

DNA was extracted from dried leaf samples using the CTAB method (Doyle & Doyle, 1987) modified by the addition of 5M NaCl. DNA concentrations were measured using a Nanodrop spectrophotometer (Infinite 200 PRO NanoQuant, Tecan Group Ltd, Switzerland) and diluted to 100 ng/μL and stored at -80 °C until further use. The chloroplast trnL region was amplified using the primers rpl32-F (5'CAGTTCCAAAAAACGTACTT-3') and rpl32-trnL(AUG) (5'CTGCTTCCTAAGAGCAGCGT-3') (Shaw *et al.*, 2005). In addition, two nuclear genes, the external transcribed spacer region (ETS) and internal transcribed spacer region (ITS) were amplified using primers described in Murphy *et al.* (2010). All PCR reactions were carried out in 50 μL reactions consisting of: 5 μL (5μM) of each primer, 5 μL of DNA template, 1 μL dNTP mix (20 mM), 2.0 mM MgCl₂, 5 μL of 10 X

buffer and 0.5 U Taq DNA polymerase (1 Super-Therm JMR-801; Southern Cross Biotechnologies, Cape Town, South Africa). For the chloroplast gene the following thermocycle was used: an initial denaturation of 95 °C for 2 min followed by 30 cycles of 95 °C for 30 sec, 60 °C for 30 sec and 72 °C for 60 sec, followed by a final extension at 72°C for 10 min. For both nuclear genes the following thermocycle was used: 95 °C for 3 min followed by 30 cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 2 min and a final extension of 72 °C for 10 min. Sequencing for all the three genes was carried out using Big dye Terminator cycle sequencing (Applied Biosystems, Foster City, CA). Given the relative short lengths of all genes include here, sequencing was done in one direction only for all gene regions. All DNA sequences have been deposited into GenBank (<http://www.ncbi.nlm.nih.gov/genbank>).

2.2.4 Phylogenetic analysis

DNA sequences were aligned using MAFFT (Kato *et al.*, 2005) with manual adjustments in BIOEDIT version 7.0.5.3 (Hall, 1999) for all gene regions separately. The nuclear ETS and ITS datasets were later combined into a single nDNA dataset. A haplotype network was constructed for the cpDNA dataset using statistical parsimony as implemented in TCS version 1.21 (Clement *et al.*, 2000). Phylogeny reconstruction was conducted separately on the nuclear dataset. Bayesian inference of phylogenetic relationships was done for the nDNA datasets using Mr Bayes 3.1.2 (Ronquist & Huelsenbeck, 2003). The best fit models were first estimated using jModelTest (Guindon & Gascuel, 2003; Posada, 2008). The nDNA dataset was run for two million generations and trees sampled every thousand generations. After

discarding 25 % of the burn-in trees a consensus tree was generated. Posterior probabilities were calculated using the 50 % majority rule consensus method.

Genetic distances among *A. pycnantha* populations were estimated using the library seqinR in the R statistical environment (Charif & Lobry, 2007). To obtain an indication of the expected intra-specific variation for the rpl32-trnL (UAG) gene, the genetic distances between subspecific entities of acacias and other species (*A. nilotica*, *Carex elata*, *Linaria viscosa*, *Linaria multicaulis*, *Limnanthes floccose* and *Centaurea aeolica*) were calculated using data downloaded from Genbank (<http://www.ncbi.nlm.nih.gov/genbank/>).

To test for neutrality in the chloroplast and nuclear datasets we used Tajima's D test, Fu and Li's D* test and Fu and Li's F* test in dnaSP version 5.0 (Librado & Rozas, 2009). Mismatch distributions and Harpending's ruggedness index for both datasets were also determined using dnaSP version 5.0 (Librado & Rozas, 2009).

An estimate of the divergence times of different native forms of *A. pycnantha* was based on previously published nucleotide substitution rates for acacias for the chloroplast genome of 0.1 % per million years (Byrne *et al.*, 2002). The value obtained from the nucleotide diversity estimate is very broad (Byrne *et al.*, 2002). Consequently, to validate this estimate, divergence times were also inferred using a relaxed molecular clock and a substitution rate of 0.1 per million years using a Bayesian Markov Chain Monte Carlo (MCMC) procedure as implemented in BEAST 1.4.7 (Drummond and Rambaut, 2007). We used a relaxed molecular clock as our data did not meet the assumptions of a strict molecular clock, as inferred from a likelihood ratio test (Verbruggen *et al.*, 2009). The MCMC model was run under a general time reversible model of nucleotide substitution with rate variation among sites modelled using a gamma-distribution implemented in jModelTest (Posada, 2008; Guindon & Gascuel, 2003). The Yule speciation process was selected as the

tree priori. Three independent MCMC analyses were each run for ten million steps and parameter values were sampled every ten thousand steps.

Table. 2.1 Localities in South Africa, Australia and Portugal where *Acacia pycnantha* was collected.

Sample ID	Latitude	Longitude	Country	Locality and abbreviation	Status	rpl32	ets	its
CAL11	-33.10701	19.29755	RSA	Caledon (CAL), WC	invasive	JF276987	KC261682	KC261745
GRT36	-33.46032	26.15991	RSA	Grahamstown (GRT), EC	invasive	JF276999	KC261683	KC261746
MTC70	-35.40585	145.95586	AUS	Mt Compass (MTC), SA	native	JF276989	KC261684	KC261747
MTC80	-35.40585	145.95586	AUS	Mt Compass (MTC), SA	native	JF276990	KC261685	KC261748
TOK162	-33.84179	18.66602	RSA	Tokai (TOK), WC	invasive	JF276991	KC261686	KC261749
MEL170	-32.78187	138.1973	AUS	Melrose (MEL), SEA	native	KC261785	KC261687	KC261750
MEL173	-32.78187	138.1973	AUS	Melrose (MEL), SEA	native	KC261786	KC261688	KC261751
MEL179	-32.78187	138.1973	AUS	Melrose (MEL), SEA	native	KC261787	KC261689	KC261752
MEL180	-32.78187	138.1973	AUS	Melrose (MEL), SEA	native	JF276992	KC261690	KC261753
MEL182	-32.78187	138.1973	AUS	Melrose (MEL), SEA	native	KC261788	KC261691	KC261754
HUM223	-34.03989	24.78687	RSA	Humansdorp (HUM), EC	invasive	JF276993	KC261692	KC261755
WOL255	-33.34012	19.16109	RSA	Wolseley(HUM), WC	invasive	JF276994	KC261693	KC261756
STE276	-34.06024	18.41480	RSA	Stellenrust (HUM), WC	invasive	JF276995	KC261694	KC261757
PIK302	-32.80084	18.71501	RSA	Piketberg (PIK), WC	invasive	JF276996	KC261695	KC261758
KIL357	-37.22176	145.021	AUS	Kilmore (PIK), VIC	native	JF276997	KC261696	KC261759
HNP374	-34.08589	150.989	AUS	Heathcote NP (HNP), NSW	native	JF276988	KC261697	KC261760
FRA419	-36.77054	141.18135	AUS	Frances (FRA), VIC	native	JF277005	KC261698	KC261762
FRA426	-36.77054	141.18135	AUS	Frances (FRA), VIC	native	JF277008	KC261699	KC261763
CB466	-35.99273	143.76538	AUS	Btwn Charlton &Boorte (CB)	native	JF277011	KC261700	KC261764
CB493	-35.99273	143.76538	AUS	Btwn Charlton &Boorte (CB)	native	JF277022	KC261701	KC261765
MTJ512	-35.36866	149.20332	AUS	Mt Jeramborera (MTJ), ACT	native	JF276998	KC261702	KC261767

MTJ520	-35.36866	149.20332	AUS	Mt Jeramborera (MTJ), ACT	native	JF277009	KC261703	KC261768
LOC541	-35.36866	146.64549	AUS	Lockheart (LOC), NSW	native	JF277012	KC261704	KC261769
LOC547	-35.36866	146.64549	AUS	Lockheart (LOC), NSW	native	JF277021	KC261705	KC261771
GUN579	-35.21065	147.76425	AUS	Gundagai (GUN), NSW	native	JF277023	KC261706	KC261772
RHSP608	-36.59888	145.95586	AUS	Reef Hills SP (RHSP), VIC	native	JF277004	KC261707	KC261773
RHSP610	-36.59888	145.95586	AUS	Reef Hills SP (RHSP), VIC	native	JF277007	KC261708	KC261774
ALB629	-34.31586	118.79919	AUS	Albany (ALB), WA	invasive	JF277016	KC261709	KC261775
ALB632	-34.31586	118.79919	AUS	Albany (ALB), WA	invasive	JF277013	KC261710	KC261776
ALB636	-34.31586	118.79919	AUS	Albany (ALB), WA	invasive	JF277019	KC261711	KC261777
RAV656	-33.59650	120.17688	AUS	Ravenssthorpe (RAV), WA	invasive	JF277016	KC261712	KC261778
NAT18	-36.00409	143.76041	AUS	Natimuk (NAT), VIC	native	KC261791	KC261814	KC261779
NAT22	-36.00409	143.76041	AUS	Natimuk (NAT), VIC	native	KC261792	KC261815	KC261780
NAT29	-36.00409	143.76041	AUS	Natimuk (NAT), VIC	native	KC261793	KC261816	KC261781
PORT15	FO	FO	PORT		invasive	KC261794	KC261817	KC261783
PORT31	FO	FO	PORT		invasive	KC261795	KC261818	KC261784
KIS823	-35.75669	137.89486	AUS	Kangaroo Isl (KIS), SA	native	KC261796	KC261713	KC261727
KIS825	-35.75669	137.89486	AUS	Kangaroo Isl (KIS), SA	native	KC261797	KC261714	KC261728
NCP832	-35.61298	138.47950	AUS	Newlands C. Park (NLCP), SA	native	KC261798	KC261715	KC261729
NCP833	-35.61298	138.47950	AUS	Newlands C. Park (NLCP), SA	native	KC261799	KC261716	KC261730
MTL841	-34.97175	138.6653	AUS	Mt Lofty (ML), SA	native	KC261800	KC261717	KC261731
MTL842	-34.97175	138.6653	AUS	Mt Lofty (ML), SA	native	KC261801	KC261718	KC261732
MB854	-35.31895	139.51193	AUS	Murray Bridge (ML), SA	native	KC261803	KC261719	KC261734
MB855	-35.31895	139.51193	AUS	Murray Bridge (ML), SA	native	KC261804	KC261720	KC261735
MB856	-35.31895	139.51193	AUS	Murray Bridge (ML), SA	native	KC261805	KC261721	KC261736

NEL867	-38.05003	141.01510	AUS	Nelson (NEL), VIC	native	KC261807	KC261722	KC261738
NEL869	-38.05003	141.01510	AUS	Nelson (NEL), VIC	native	KC261808	KC261723	KC261739
NEL872	-38.05003	141.01510	AUS	Nelson (NEL), VIC	native	KC261810	KC261724	KC261741
NEL873	-38.05003	141.01510	AUS	Nelson (NEL), VIC	native	KC261811	KC261725	KC261742
CAS877	-37.10758	144.09283	AUS	Castlemaine (CAS), VIC	native	KC261813	KC261726	KC261744

AUS- Australia, RSA-Republic of South Africa, Port- Portugal. WC-Western 1 Cape, EC-Eastern Cape, VIC-Victoria, WA- Western Australia, NSW-New South Wales, SA- South Australia, SEA, South East Australia, ACT-Australian Capital Territory

2.3 Results

The aligned cpDNA matrix was 608 bp long, requiring 12 gaps (indels) with an average length of 2 bp. Overall, twelve haplotypes were identified in *A. pycnantha* (Fig. 2.2). Five (A, D, F, H, I) unique haplotypes occurred in the distributional ranges of the wetland form in Australia, two haplotypes (B and C) encompassed both Victorian dryland and wetland taxa while two haplotypes (M and L) occurred in the drier Southern Flinders Ranges, where the dryland form occurs. Three haplotypes (E, G and J) were found in South Africa only (Fig. 2.2). The haplotype most commonly recorded in the natural range (A) also occurred in four invasive populations in South Africa (Fig. 2.2). Two of the invasive Australian populations (Western Australia) and the Portugal population also had haplotype A. The three remaining invasive populations from South Africa were closely related to haplotype A, B, G and haplotype H (Fig. 2.2). The Flinders Range haplotypes (L and M), corresponding to the dryland form of *A.pycnantha*, were not found in any of the invasive ranges.

Strong spatial clustering occurred throughout *A. pycnantha*'s range in Australia with most haplotypes having restricted distributions. Moreover, these groupings correspond to the morphologically recognized lineages within *A. pycnantha*: Flinders Range dryland (haplotypes M and L (slender trees with narrow phyllode at the species western natural range), wetland and Victoria dryland (haplotypes A, B, C, D, F, H, I) (Fig. 2.2). There was geographical overlap of haplotypes in Reef Hills State Park, Victoria (haplotype A and C). A distinct haplotype (haplotype I) was found in Mount Jeramborerra very close to the Australian Capital Territory (Fig. 2.2).

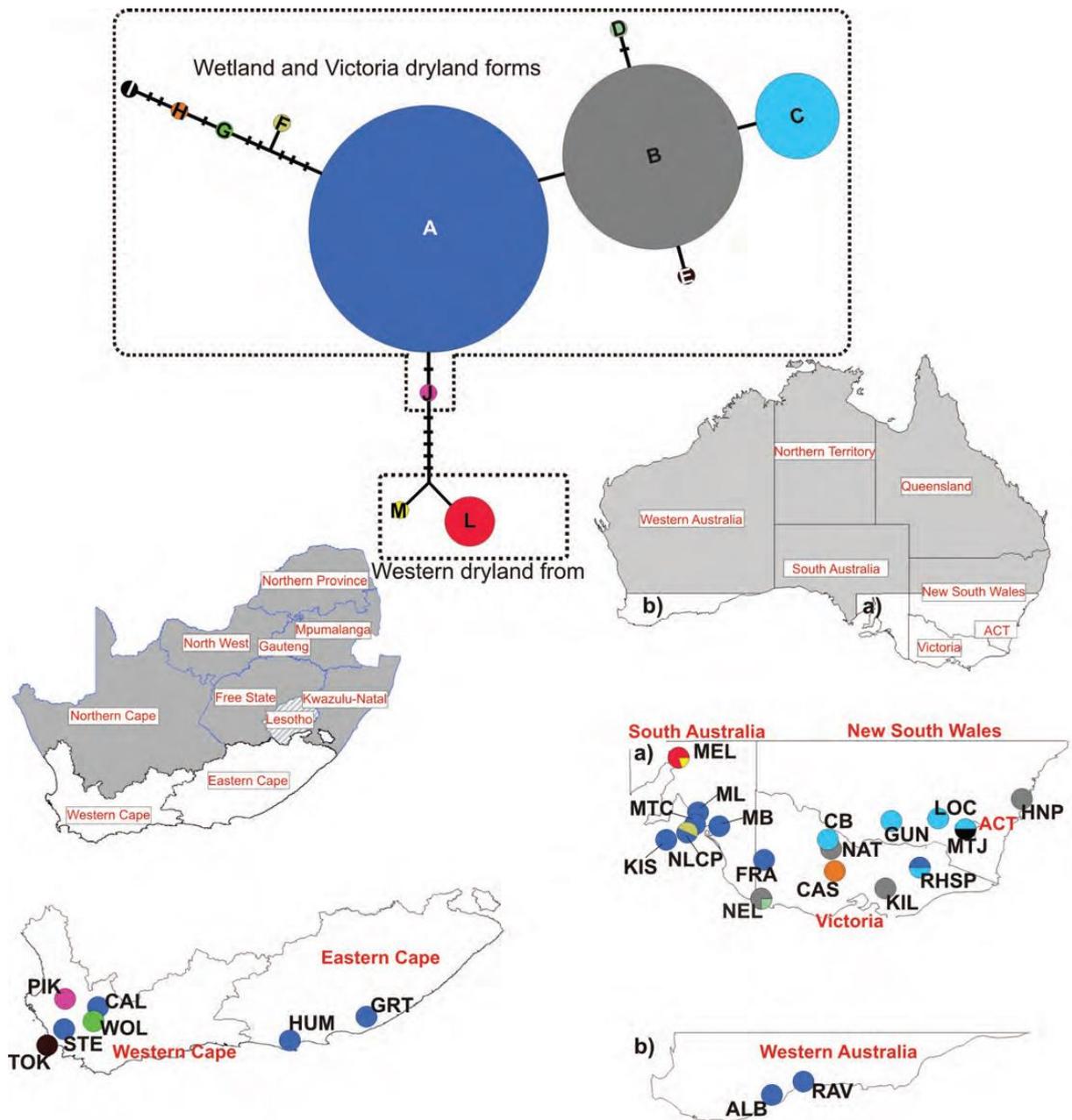


Fig. 2.2 Haplotype network of *Acacia pycnantha* (based on the rpl32-trnL(UAG) region) and geographical distribution of haplotypes in the native south-eastern Australia and invasive South Africa and western Australian ranges. Each unique haplotype is represented by a coloured circle and the size of the circle is proportional to the number of individuals possessing that haplotype. Pie charts represent the proportion and distribution of haplotypes across native (South Australia and New South Wales) and invasive (Western Australia and South Africa) ranges.

The best fit-model of evolution used in Bayesian inference, according to the Akaike information criterion (AIC) for the combined nuclear gene, was also the GTR model with a gamma-distribution. The nuclear gene tree retrieved from the Bayesian analysis was incongruent with the cpDNA haplotype network in the placement of certain taxa. Dryland taxa from Flinders Range clustered with the Natimuk taxa from Victoria. In addition, South Australian populations from Mt Compass were shown to be very closely related to the Flinders Range population. As in the chloroplast haplotype network, four South African populations clustered with South Australia populations. Two of the South African populations, (Humansdorp and Wolseley) were closely related to the dryland form of *A. pycnantha* (Fig. 2.3). The Nelson population formed a distinct cluster which was closely related to nearby Victorian populations. Overall, there was no clear geographical structuring of *A. pycnantha* for the nuclear analysis.

Genetic distances ranged from 0% to 0.018% between cpDNA haplotypes (Table 2.2). Haplotype L and M, which were collected from Melrose (MEL, Flinders Range), had larger distances of up to 0.018 % from the South Australia, Victoria and New South Wales haplotypes (mostly wetland haplotypes (Table 2.1).

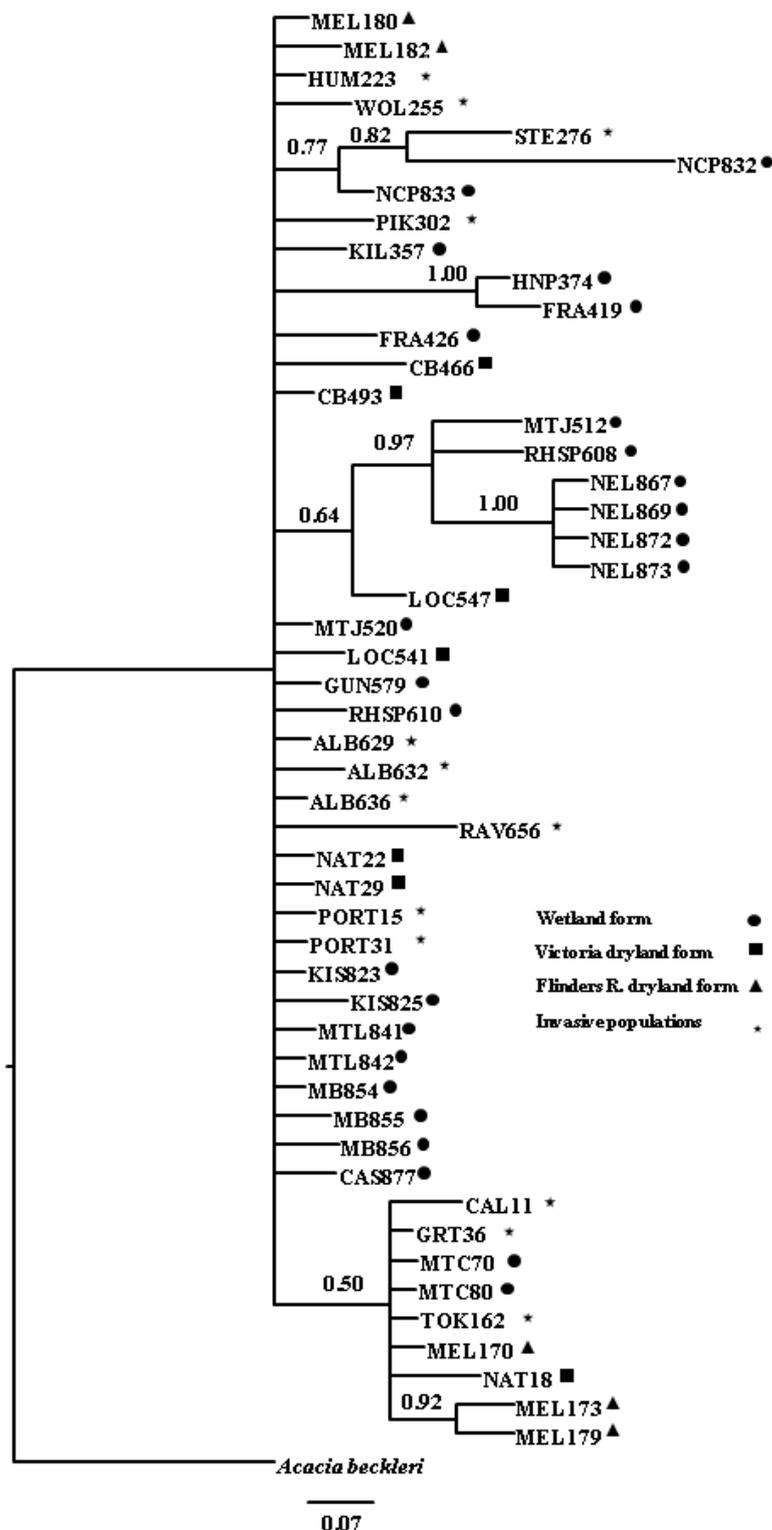


Fig. 2.3 Bayesian analysis tree for *Acacia pycnantha* using combined ITS and ETS regions. Numbers above the branches represent posterior probabilities derived from the analysis. Invasive taxa are indicated by asterisks for Australian, Portuguese and South African collections, as indicated in the key; also indicated are wetland, Victoria dryland and Flinders Range dryland forms of *A. pycnantha*.

Table. 2.2 Genetic distances between *rpl32-trnL^(UAG)* plastid gene haplotypes. A-H correspond to haplotypes as indicated in Fig. 2.2.

	A	B	C	D	E	F	G	H	I	J	L	M
B	0											
C	0	0										
D	0	0	0									
E	0	0	0.002	0								
F	0	0.004	0.007	0.004	0.004							
G	0.002	0.002	0.002	0.002	0.002	0.006						
H	0.004	0.004	0.002	0.004	0.004	0.004	0.002					
I	0.008	0.009	0.009	0.009	0.009	0.012	0.005	0.009				
J	0.002	0.002	0.002	0.002	0.002	0.007	0	0	0.005			
L	0.01	0.009	0.009	0.009	0.009	0.012	0.011	0.012	0.018	0.002		
M	0.01	0.011	0.012	0.011	0.011	0.015	0.011	0.015	0.016	0.01	0.002	

Tests of neutrality for the chloroplast gene using Tajima's D (-2.10088 $P < 0.05$), Fu and Li's D^* (-3.54960 $P < 0.02$) and Fu and Li's F^* (-3.62302 $P < 0.02$), all yielded negative values that were significantly different from zero. The population expansion hypothesis was investigated by computing the distribution of pairwise differences using dnaSP version 5. The mismatch distribution showed slight bimodality (Fig. 2.4A) with a Harpending's raggedness value resembling constant size population ($r=0.3667$, see Zink *et al.*, 2000). Using the rate of nucleotide divergence previously published for the same gene region

(rpl32-trnI), revealed that wetland and Flinders Range dryland forms of *A. pycnantha* diverged ca. 110 KYA. The upper limit of the Bayesian estimate of the age of the most recent common ancestor (TMRCA) was in agreement with the nucleotide diversity divergence times indicating that these lineages split ca. 66 KYA (95% CI of 58KYA to 87KYA).

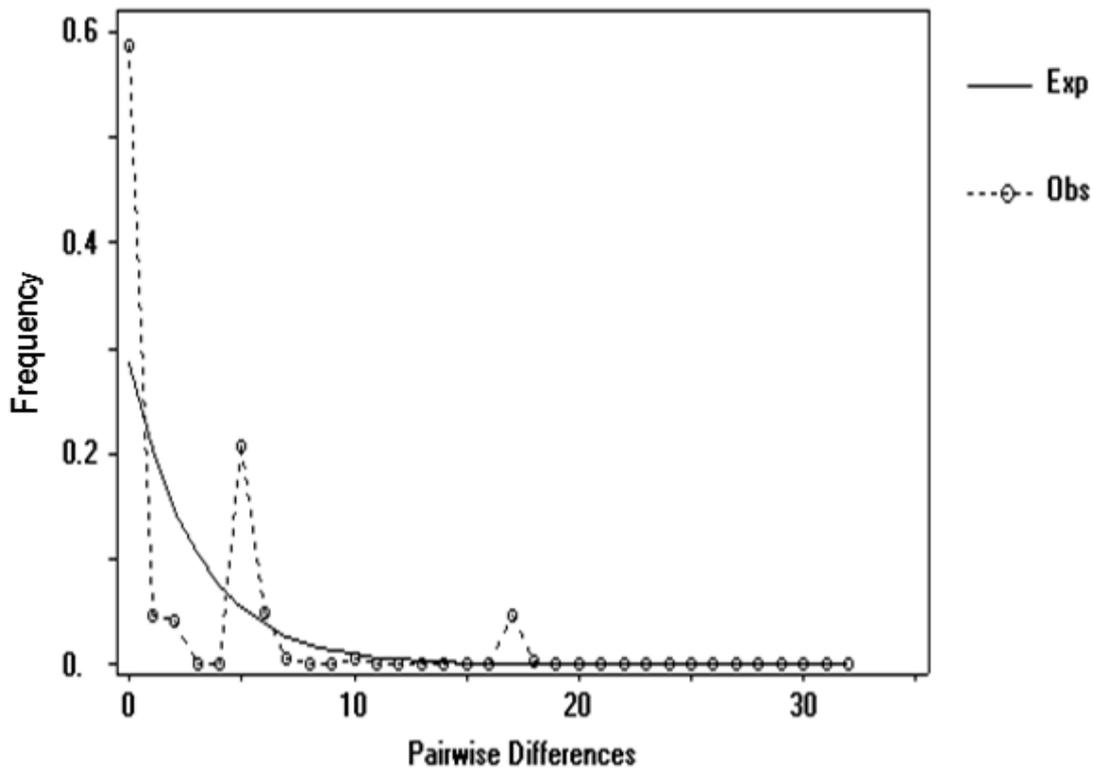


Fig. 2.4A. Mismatch distribution for DNA sequence data of the plastid genome for *Acacia pycnantha* in Australia (native range). The solid line represents the expected mismatch distribution of a constant-size population and the dotted line represents the observed mismatch distribution.

Similar to cpDNA, neutrality tests on the nDNA of Tajima's D (-2.84 $P < 0.001$), F_u and Li's D^* (-5.88 $P < 0.02$) and F_u and Li's F^* (-5.71 $P < 0.02$) yielded negatively significant values. However, pairwise mismatch distribution of the nuclear data yielded was unimodal with a raggedness index of 0.0265, indicative of recent population expansion (Fig. 2.4B).

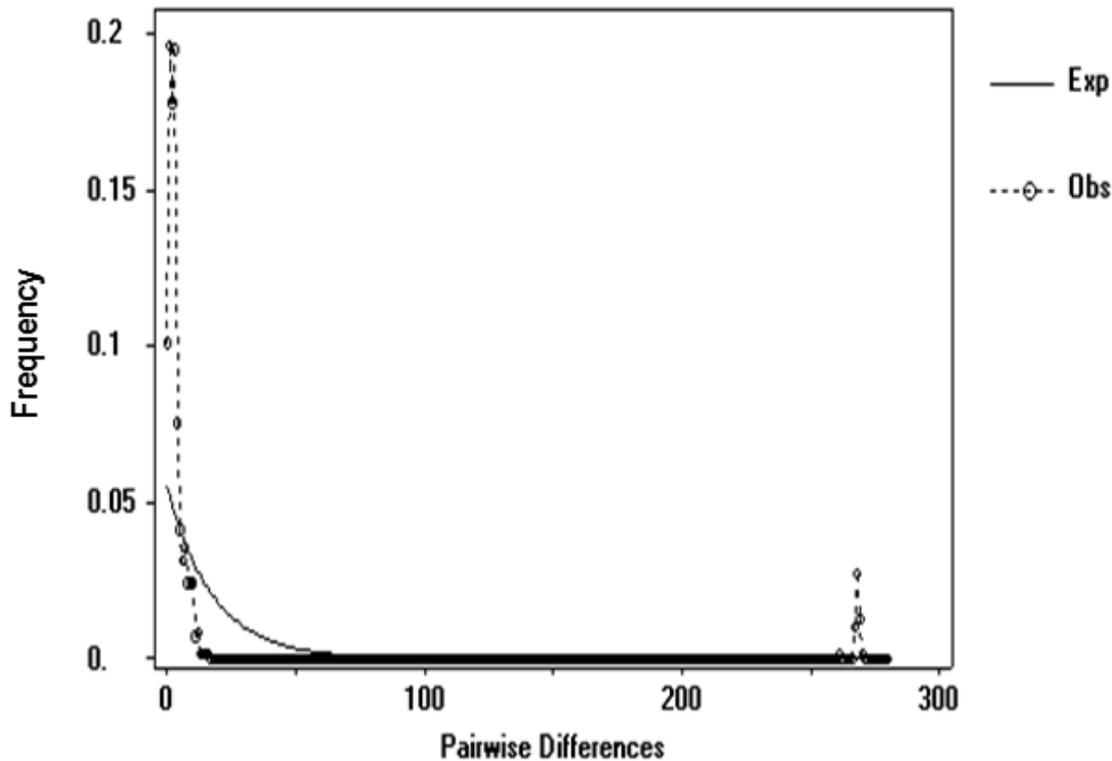


Fig. 2.4B Mismatch distribution for DNA sequence data of the nuclear genome for *Acacia pycnantha* in Australia (native range). The solid line represents the expected mismatch distribution of a constant-size population and the dotted line represents the observed mismatch distribution.

2.4 Discussion

Linking contemporary and historical biogeography remains a central theme in invasion biology (Stepien *et al.*, 2002). We were able to identify that invasive South African populations of *A. pycnantha* originated from the species' wetland form and Victorian dryland form distributions, while Portuguese and Western Australian invasive populations represent the species' wetland form only. Most invasive populations originated from the areas around Adelaide and the Mt Lofty ranges. This has important implications for the management of *A. pycnantha* in South Africa and Portugal as discussed below. But perhaps more interestingly, we found evidence to support previous informal morphological classifications of *A. pycnantha* as two distinct ecotypes (wetland and dryland forms) based

on chloroplast genealogies, providing a platform for guiding conservation efforts in the native range. However, the link between phylogenetic relatedness and morphological classification is not clear cut. The dryland forms of *A. pycnantha* found in Victoria, which had similar phyllode morphologies to the Flinders Range dryland variant, shared a close phylogenetic relationship with the wetland variants. However, the dryland variants found in the Flinders Range showed distinct genetic variation from the wetland/dryland Victoria group (Fig. 2.2).

2.4.1 Native range phylogeography

We suspect that the phylogeographic structure of *A. pycnantha* in eastern Australia is a result of relatively recent geological climatic shifts during the Pleistocene (Worth *et al.*, 2010, Worth *et al.*, 2009). During the Pleistocene (ca. 16KYA - 1.8 MYA) eastern Australia experienced a series of cycles of pluviality and aridity (Toon *et al.*, 2007). Numerous and isolated Pleistocene refugia during the extreme dry periods have been previously identified (Fig. 2.5, Ford, 1987), separated by arid barriers, climatic and edaphic factors (Ladiges, 2011). This fragmented landscape resulted in allopatric speciation in many Australian plant groups (Byrne *et al.*, 2002; Byrne *et al.*, 2003; Millar *et al.*, 2008). Using an estimate of 0.1 % DNA sequence divergence per million years for cpDNA as reported for other acacias (Byrne *et al.*, 2003), the divergence between the Flinders dryland and wetland/Victoria dryland form of *A. pycnantha* is estimated to have taken place around 100 KYA. This coincides with the mid Pleistocene, a period with high sedimentation rates and aridity in Eastern Australia (Prideaux *et al.*, 2007). This is supported by the *A. pycnantha* chloroplast structure which indicated the presence of two main haplotype groups that is indicative of the two distinct ecological zones where the different *A. pycnantha* variants occur. Kangaroo Island was connected to mainland Australia during late Pleistocene and is

estimated that the cut-off time of the island from mainland Australia is about 8800 to 13500 YBP (Hope *et al.*, 1977). This timeframe is in agreement with the *A. pycnantha* collections from Kangaroo Island that are genetically closely related to the wetland form (KIS 823 and KIS 825, Fig. 2.2).



Fig. 2.5 Refuge areas and geographical barriers in Australia during the Pleistocene. Stripped areas represent refugia in Australia and those with black dots represent refugia for *Acacia pycnantha* (Eyre Peninsula and Mt Lofty). The arrows show the Eyrean and Mallee geographical barriers (Ford *et al.*, 1987).

For the dryland and wetland lineages three putative refugia existed in the past: the Eyre Peninsula (Flinders Range dryland form), South East and Mt Lofty refugia (for the wetland and dryland Victorian lineage) (Fig. 2.5; Ford, 1987). These refugia were separated by the Eyrean and Mallee geographic barriers (Toon *et al.*, 2007; Ford, 1987)

and represent areas that had very low rainfall (Ford, 1987). Since this time, *A.pycnantha* has expanded its range eastwards and northwards. These expansions are supported by the presence of putative intra-specific hybrids identified by incongruent nDNA and cpDNA phylogenetic placements (e.g. NAT18 Victoria, Natimuk) and by the patterns of nDNA mismatch distribution which was unimodal, indicative of recent population expansion (Hwang *et al.*, 2003).

Morphologically, the wetland form of *A. pycnantha* has broader phyllodes and darker golden inflorescences than the dryland form (with narrower phyllodes and often pale yellow flowers, Fig. 2.1). However, slight morphological variants of *A. pycnantha* also exist within the wetland distributional range in South Australia, Victoria and New South Wales (Maslin & McDonald, 2004). The putative hybrid from Natimuk, Victoria, showed intermediate morphological characters between the wetland haplotypes and the Flinders Range haplotype (J Ndlovu & JJ Le Roux, pers. obs.). The presence of these intermediates in Victoria is likely the result of hybridization through pollen exchange between the wetland and the dryland forms.

To determine whether the genetic divergences between the extreme forms of *A.pycnantha* potentially represent different subspecies, we calculated genetic differences for known subspecies complexes and closely related species. At the inter-specific level genetic distances ranged from 0.04 % (*Acacia pulchella* and *A.koa*) to 0.15 % (*A. pulchella* and *A. longifolia*) and at the intra-specific level between 0% (recognized subspecies of *A. nilotica*) and 0.005% (recognized subspecies of *Linaria multicaulis*) while the level of genetic distances at the intraspecific level for *A. pycnantha* ranged between 0 % to 0.018%.

Overall, informal morphological classifications and our genetic results suggest that the *A. pycnantha* species complex needs a taxonomic revision. The genetic divergence

found between different forms, at least for the cpDNA, exceeds known divergences between other taxa consisting of subspecies (Table 2.2). However, the combination with our genetic and morphological data is not sufficient to separate the species into a subspecies complex. These attempts are hampered by many (other) characters showing geographic variation between the distributional ranges of the dry and wetland forms. For example, the Victorian dryland form, despite its geographic disparity, resembles the variant from the Flinders Range. These problems are also exacerbated by the history of cultivation of *A. pycnantha* in its natural range, possibly obscuring the natural distribution of taxa within the species. We recommend a finerscale population genetic study with a more extensive sampling scheme to resolve some of these issues.

2.4.2 Invasive range phylogeography and consequences for management

High frequency genotypes in the invasive range in South Africa, Western Australia and Portugal occur predominantly in South Australia, with some evidence of wetland variants from Victoria and New South Wales having also been introduced. Notably, however, *Acacia pycnantha* in South Africa harbours only a fraction of the genetic diversity found in its Australian range (Fig. 2.2). Clearly there has been a genetic bottleneck during either or both introduction and invasion (Fig. 2.2).

Assuming that historical genetic structure possibly determines co-evolutionary relationships between host plants and their antagonists and mutualists, our findings may explain why *Trichilogaster signiventris*, a gall-forming wasp initially released from Lake Natimuk in Victoria for biological control, did not establish successfully and did not achieve significant control of *A. pycnantha* in South Africa (Hoffmann *et al.*, 2002), as this region does not appear to be the source of invasive populations. However, the reintroduction of the same wasp species collected from Mt Compass in South Australia, a region identified

here as a potential source of the invasive populations in South Africa, has led to successful establishment and substantial impacts and control of invasive populations in South Africa (Hoffmann *et al.*, 2002). We therefore have reason to conclude that host-specificity and compatibility may be linked to natal phylogeographic structure in this system.

These results have important implications. First, we would expect populations in the native range to be able to survive drier conditions. If the dryland form was introduced there could potentially be a large expansion in the invasive range in South Africa. Second, the recommendations that biocontrol agents that have proved successful in South Africa should be considered for introduction to other countries where the species is invasive (e.g. Wilson *et al.*, 2011), might need to take subspecific identity and phylogenetic affinity into account. Initial results, however, are promising. *Acacia pycnantha* in Portugal appears to have a similar genetic origin to *A. pycnantha* in South Africa, suggesting the substantial reductions in seed-production caused by the *T. signiventris* in South Africa (Hoffmann *et al.*, 2002; Impson *et al.*, 2011) might be replicated in Portugal.

2.5 Concluding remarks

Identifying where invasive *Acacia* species originate from in their natal range is important for determining priorities for biological control. A comprehensive genetic diversity and phylogeographic study in the native range for all the known invasive acacias can potentially form a basis for recommendations of host-specific biological control organisms. In the case of *A. pycnantha*, the invasive genotype found in South Africa is similar to the invasive genotypes in Portugal and Western Australia thus introduction of the same variant of *T. signiventris* for biological control released in South Africa is recommended. Such studies for all the *Acacia* species should be based on chloroplast genetic diversity as the

chloroplast gene has shown to be more reliable for identifying source populations in this study and in other species in Australia. We therefore recommend that biological control efforts for Australian acacias must recognise the importance of genetic diversity by verifying the source of the invader before releasing biocontrol organisms.

2.6 Acknowledgements

This work was funded by the DST-NRF Centre of Excellence for Invasion Biology (C•I•B) and the Working for Water Programme through their collaborative research project on “Research for Integrated Management of Invasive Alien Species” and grants from South Africa’s National Research Foundation and the Hans Sigris Foundation. We thank Kate Le Roux for assistance during field collections.

Chapter 3: Cross-species amplification and characterization of microsatellite loci in invasive Australian *Acacia* species.

A version of this chapter will be submitted to *Molecular Ecology Resources*

Reference: Ndlovu, J., Richardson, D.M., Wilson, J.R.U. Le Roux J.J. (2013). Cross-species amplification of microsatellite loci for invasive Australian *Acacia* species. *Molecular Ecology Resources*: to be submitted.

Abstract: The introduction of Australian *Acacia* species around the world has caused the genus to be highly invasive. It has been proposed that the genus *Acacia* be used as a model system for invasion biology. The cross-species transfer of orthologous microsatellite markers to invasive Australian *Acacia* species could therefore provide a valuable tool to study the evolutionary aspects underlying invasion dynamics. Twenty nuclear microsatellites of *A. mangium*, ten of *A. saligna*, six of *Paraserianthes lophantha*, three of a *A. mangium* X *A. auriculiformis* hybrid, and ten of *Nicotiana tubacum* were assessed for cross-amplification and polymorphism in five invasive *Acacia* species: *A. implexa*, *A. longifolia*, *A. melanoxylon*, *A. podalyriifolia* and *A. pycnantha*. Thirty microsatellites successfully amplified across all species tested. Transfer rates varied between 85% for microsatellites developed for *A. mangium* to 50 % for those developed in *Acacia saligna*, with no successful transfers using the microsatellites developed for the hybrid. Although transfer rates were high, only twelve microsatellites were polymorphic. Observed heterozygosity ranged from 0.18 to 1.00 in the native range and 0 to 1.00 in the invasive range; number of alleles ranged from 3 to 12 in the native range and 2 to 12 in the

invasive range. The low level of polymorphic loci calls for development of more microsatellites in this genus especially for species that have high commodity value.

Keywords: *Acacia*, microsatellite transfer, invasion biology.

3.1 Introduction

Microsatellites, also known as simple sequence repeats (SSRs), are highly polymorphic genetic markers, valuable for the study of population genetic diversity and structure. (e.g. Genton *et al.*, 2005; González-Martínez *et al.*, 2004). Their co-dominant nature, high levels of polymorphism, and relative abundance throughout genomes make them markers of choice in many genetic studies (Ijaz, 2011). However, *de novo* development of microsatellite markers remains very expensive and time consuming (González-Martínez *et al.*, 2004). To offset the high costs of microsatellite development, cross-amplification of SSRs is often tested between closely related taxa, usually within the same genus (Liu *et al.*, 2011), but sometimes also between closely related genera (dos Santos *et al.*, 2007).

Australian acacias have been widely planted in different parts of the world for many reasons and have since become invasive and dominant in some ecosystems (Richardson *et al.*, 2011). To date, 23 species are recognised as invasive species, impacting on land use and ecosystem processes in different parts of the world (Richardson *et al.*, 2011), and 14 of these species are invasive in South Africa (van Wilgen *et al.*, 2011). The high invasion success and the wide range of occupied habitats across the world make Australian acacias an interesting model group for studying various aspects of plant invasions, including the evolutionary processes underlying invasions (Richardson *et al.*,

2011). Such an understanding is not only important from a theoretical perspective but also for the effective management and control of the invasive populations (Le Roux & Wiczorek, 2009). A number of intriguing questions such as the level of genetic diversity in the invading populations, the occurrence and extent of admixture, levels of gene flow, and the relationship between genetic diversity and propagule pressure remain unanswered for most *Acacia* taxa (but see Thompson *et al.*, 2012).

In this study I aim to transfer microsatellite loci previously developed for *Acacia mangium*, an *A. mangium* X *A. auriculiformis* hybrid, *A. saligna*, and *Paraserianthes lophantha* (a close relative of Australian acacias) to *A. implexa*, *A. longifolia*, *A. melanoxylon*, *A. podalyriifolia* and *A. pycnantha* that are invasive in South Africa. In addition, universal chloroplast loci developed for *Nicotiana tabacum* were also tested for transferability to these species. Specifically, the objectives were to determine: (a) how many selected polymorphic microsatellite loci that were previously developed will cross-amplify in *A. implexa*, *A. longifolia*, *A. melanoxylon*, *A. podalyriifolia* and *A. pycnantha* and (b) how many universal chloroplast microsatellite markers will cross-amplify in *A. pycnantha* and the other test species. Furthermore, I aim to compare levels of genetic diversity between invasive and native populations for five different *Acacia* species with known and differential introduction histories (propagule pressure) in order to determine the native provenance of the invasive *Acacia* species and to compare the genetic diversities of the native and invasive populations.

3.2 Methods

3.2.1 Sample collection and DNA isolation

Phyllode material was collected for 24 individuals per locality from *Acacia implexa*, *A. longifolia*, *A. melanoxyton*, and *A. pycnantha*, and 12 individuals per locality for *A. podalyriifolia*, from the native range (Australia) and the invaded range (South Africa) between February 2007 and February 2010 (Table 3.1). The samples were dried using silica gel and stored at room temperature. DNA was then isolated using the method of Doyle and Doyle (1987) modified by adding 5M sodium chloride following the method of Thompson *et al.*, (2012). DNA concentrations of all samples were measured using fluorimetric methods, diluted to 25ng/ μ L, and stored at -80°C until further use. In my analysis, however, some individuals were not considered because of lack of amplification.

Table 3.1: Australian and South African origins of all accessions used in this study

Species	South Africa			Australia					
	Latitude	Longitude	Locality	Latitude	Longitude	Locality			
<i>Acacia implexa</i>	-34.06098	18.42835	Papegaaiberg	-32.40088	149.39070	Wellington			
<i>Acacia longifolia</i>	-33.33221	26.57368	Grahamstown	-35.04203	138.75678	Mylor Parkways			
				-38.05878	141.01577	Sophora			
				-28.73358	153.60941	Lismore			
<i>Acacia melanoxylon</i>	-33.96969	23.43934	Knysna	-35.40585	138.59882	Mt Compass			
				-35.20750	138.70000	Kutipo Forest			
				-38.44788	145.91312	Gippsland			
<i>Acacia podalyriifolia</i>	-32.79895	18.71182	Piketberg	-34.64820	117.83483	O' Neil Road			
				-33.96585	23.45522	Western Cape	-28.01925	153.16867	Gold Coast
							-27.53294	152.85294	Brisbane
<i>Acacia pycnantha</i>	-33.34012	19.16109	Wolsely	-35.40585	138.59882	Mt Compass			
				-32.78187	138.1973	Melrose			

3.2.2 DNA amplification and fragment analysis

A total of 49 polymorphic microsatellite markers previously developed for *A. mangium* (20), *A. saligna* (10), *Paraserianthes lophantha* (6), *A. mangium* X *A. auriculiformis* hybrid (3) and 10 chloroplast microsatellite markers isolated from tobacco (*Nicotiana tabacum*) were tested for cross-amplification in all acacias sampled here. The PCR conditions described by Butcher *et al.* (2000), Millar & Byrne (2007), Ng *et al.* (2005) and Weising & Gardner (1999) were used to amplify *A. mangium*, *A. mangium* X *A. auriculiformis*, *A. saligna* and *Nicotiana tabacum* microsatellite markers respectively in 10 μ L reactions. Each reaction contained 1 μ L DNA, 1 μ L of each of the forward and reverse primers (5 μ M), 0.2 μ L dNTP mix (20 μ M), 1 μ L buffer (10X), 1.5mM-3mM magnesium chloride and 0.2 μ L of 250 U Supertherm Taq polymerase (Hoffman-La Roche). In addition 0,2 μ L of bovine serum albumen (10mg/ μ L) was added to reactions for amplifying microsatellites developed for *A. mangium* only. All PCR reactions were made up to 10 μ L final reaction volumes using distilled water. In addition, for problematic amplifications an annealing temperature gradient from 48°C to 60°C and a magnesium chloride gradient of between 1.5 mM to 3.0 mM (which was set at 0.5 mM intervals) were used for optimization (see Table. 3.2).

PCR products for all the microsatellites were visualized on 1.5% agarose gels containing ethidium bromide. Microsatellites that amplified and produced clear bands of expected sizes were amplified in all collected individuals using fluorescently-labelled primers and optimized PCR conditions (Table. 3.2). Each microsatellite locus was separately amplified, and resulting PCR products were combined into multiplexes (see Appendix. 3.1). Fragment analysis was conducted using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and the LIZ500 or HD400 internal size standards. Allele sizes were scored using GENEMARKER V1.95 (SoftGenetics LLC

Pennsylvania, USA). Arlequin version 3.5 (Excoffier & Lischer, 2010) was used to calculate the number of alleles (A), observed heterozygosity (H_O), expected heterozygosity (H_E) and to test for linkage disequilibrium. Levels of inbreeding (F_{IS}) were calculated in FSTAT version 2.9.3.2 (Goudet, 2002).

3.3 Results

Across the five *Acacia* species, 31 microsatellites with expected allele sizes were successfully amplified. The overall amplification success was high for microsatellite markers previously developed for *A. mangium* (17 out of 20), followed by *P. lophantha* (4 out of 6), *A. saligna* (5 out of 10), *N. tabacum* (7 out of 10), while all *A. mangium* X *A. auriculiformis* hybrid primers failed (0 out of 3). For all microsatellites tested there was also high variability in amplification success among test species; *A. implexa* (28%), *A. longifolia* (48%), *A. melanoxylon* (36%), *A. podalyriifolia* (38%), and *A. pycnantha* (46%).

Polymorphism was found in twelve nuclear microsatellite markers, of which nine were originally developed for *A. mangium*, two for *P. lophantha* and one for *A. saligna* (Table. 3.3). None of the *N. tabacum* chloroplast microsatellites were polymorphic. In the five *Acacia* species the number of alleles per locus in both native and invasive populations ranged from 2 to 12, and the observed heterozygosity (H_O) ranged from 0.00 to 1.00 while the expected heterozygosity (H_E) ranged from 0.18 to 0.92. In the native populations the number of alleles ranged from 3 to 12 per locus while the number of alleles in the invasive populations ranged from 2 to 12 per locus. Observed heterozygosity (H_O) in Australia ranged from 0.18 to 1.00 and 0.00 to 1.00 per locus in South Africa. Expected heterozygosity (H_E) ranged from 0.17 to 0.96 in Australia and 0.26 to 0.92 in South Africa. There was no significant linkage disequilibrium between the compared loci in all the five

study species. Generally, South African populations were more inbred than Australian populations (Table. 3.3) except for *A. implexa* and *A. melanoxyton* populations which showed high levels of inbreeding in the native range.

3.4 Discussion

While there was some cross-transferability of microsatellite markers to invasive Australian acacias (30 out of 49), only 12 of the tested SSRs were polymorphic, and so potentially informative.

The success of cross amplification of microsatellite markers has repeatedly been shown to be directly proportional to the genetic distance between species, The smaller the genetic distance, the higher the likelihood of transfer (Moreno *et al.*, 2011). For successful amplifications to occur, flanking regions must be conserved across taxa, thus more genetically similar species are likely to have conserved flanking regions and thus a higher chance of successful cross-species amplifications (Moreno *et al.*, 2011; Butcher *et al.*, 2005).

A similar result was evident here. The markers for *A. mangium* cross-amplified more often with the species in the same subsection Juliflora (*A. longifolia*: 12 out of 20 or 60 %), than with species from the section Plurinerves (*A. implexa*: 10 out of 20 or 50 %; *A. melanoxyton*: 11 out of 20 or 55%). Similarly, *A. saligna* (section Phyllodinae) microsatellites, successfully amplified in species in the same section (*A. podalyriifolia*: 2 out of 10 or 20%; and *A. pycnantha*: 1 out of 10 or 10%) while none of the *A. saligna* markers amplified for *A. implexa* or *A. melanoxyton*. Positive amplification for *A. saligna* microsatellites was also recorded for *A. longifolia* indicating that the subsections Juliflora

and Phyllodineae are quite closely related. Cross-amplification of *A. mangium* microsatellites to *A. podalyriifolia* (12 out of 20 or 60 %) and *A. pycnantha* (13 out of 20 or 65 %) was also indicative of the close phylogenetic relationship between subsections Juliflora and Phyllodineae.

Among the Australian acacias tested here, the degree of evolutionary divergence among subsections was probably a factor that led to low levels of microsatellite transferability, a phenomenon also recorded by Butcher *et al.* (2005). In addition, the transfer of *A. saligna* microsatellites to *A. pycnantha* and *A. podalyriifolia* also showed that transfer in the same subsection is preferred. The Australian acacias that invade South Africa do not all belong to the section Juliflora to which *A. mangium* which has a substantial number of developed microsatellites belongs; this accounts for the low levels of microsatellite transferability. It is therefore important to develop microsatellites from at least each of the subsections where an invasive species belongs (e.g. *A. melanoxylon* microsatellites in Plurinerves and *A. mearnsii* microsatellites in subsection Botrycephalae) to study *Acacia* invasions.

None of the inbreeding co-efficients were significantly different between the native and invasive ranges, though with more sampling important effects could become apparent. Inbreeding in the invasive range in South Africa might result from population bottlenecks during species introductions. In contrast in the native range in Australia, there is a known history of plantings and reforestation programmes for *Acacia* species which has caused random mixing of previously allopatric populations leading to intraspecific hybridisation (admixture). The microsatellites proposed here should be a useful tool to explore this and other hypotheses.

Table. 3.2: Microsatellite primers used for cross species amplification, Annealing temperatures, and results of positive amplification for *Acacia implexa*, *A. melanoxylon*, *A. longifolia*, *A. podalyriifolia* and *A. pycnantha*.

Locus	Species	Primer sequence 5'-3'	Exp size	MgCl ₂	T _m	FD	AMP
Am008	AM	CCACCCGTTACCCATTTATG CCGTGATTGACTCTCAGCG	88-106	1.5-3.0	48-60	na	na
Am012	AM	TGAGTCGATCGCTTAGCTTG TCCCGTTATTATGCCAAAGTG	150-156	1.5	55	6-FAM	L, PY
Am014	AM	GTACTAACGTTGCTATATGAGAAAGG CTGGTTGTTGCTTATATGG	150-154	1.5	55	PET	I, PO
Am018	AM	CACGGCTGTTATTTCTTCG GGAAAGAGGTGTGACAGAGGAC	129-144	1.5-3.0	48-60	na	na
Am030	AM	GAGGTAATATTTTGAATTCCTTGAAC GGTGTATACCTCTTTCCTGTGG	89-121	1.5	55	PET	I, M, L, PO, PY
Am041	AM	TAGGCTAATGGTCATATTCCTAG AGAGATAGGGGTACACACTAAAAAAC	114-147	1.5	55	NED	I, M, L, PO, PY
Am136	AM	CCCATTGCCGTTTCTTTG GCATTTCCCTTGGAACAGTC	111-125	1.5-3.0	48-60	na	na
Am326	AM	GGACCAAACCTTATGCAACACC GCATCAATGTACTAAACCATTTC	218-249	1.5	55	PET	I, L, PO, PY

Am352	AM	CCTCATGTCCTTGAATGTCAC GACTAACCCACAAGGAAGAGTTAC	127-129	1.5	55	6-FAM	I, M, L, PO, PY
Am384	AM	AGACTTCATAAATAAGATGGAAGAGG ATGCCAAATTTTCTTATTGGAG	195-197	1.5	55	VIC	M
Am387	AM	TGATACAAGGGAAGACAGAGTGG CCAAC TCAAACCTGACAACG	104-114	1.5-3.0	48-60	na	na
Am389	AM	AATCCTTCCGAAAGTTATACATGG GCACTTGTAAGTCGGAAC TGC	216-220	1.5	55	NED	I, M, L, PO, PY
Am424	AM	AATACATGGAAGAGGATGAGATG ATTGCATTTCA TTTGTTGCC	174-206	1.5	55	6-FAM	M, L
Am429	AM	CCTTCTTCTCTCATCTACCAAACC CCCACATCATCACTCACAAC T	170-180	1.5	55	PET	I, M, PY
Am435	AM	ACCCTTTATTTCTCACACGGA ACAGAAGAAGATGCAAAGAAGG	139-152	1.5	55	6-FAM	I, M, L, PO, PY
Am436	AM	ATGGATCTTGTCTTATCTTGA GGGCCAATTTGAGTTTGGAA	240-246	1.5	55	PET	L, PO, PY
Am460	AM	CACTAATTGCTCACACATTCCA ATTCATAGCCTCTCCCTTCAG	138-140	1.5	55	VIC	M, L, PO, PY

Am465	AM	TGGGTATCACTTCCACCATT AGGCTGCTTCTTTGTGCAGG	162-180	1.5	55	PET	I, M, PY
Am502	AM	CAAATGGCCAAGTTACGACTG TTCTGGTAATCCAACTTATGTGG	122-128	1.5	55	VIC	M, L, PO, PY
Am770	AM	CAGAGGTGGCAGATGATGTC AAGCCTTTAGTTGGGCGTTC	93-95	1.5	55	6-FAM	I, L, PO, PY
As2.04	AS	CAAATGAAAAGAATGCTTGGTG CATCTTGTTAAGGAATATTGGTTTCG	172-194	1.0-3.0	48-60	VIC	na
As2.13	AS	CGTACCAAATTGCTCCTTAACC TCCTGCCAAACATGAAAGC	125	1.5	56	PET	L
As2.17	AS	TCCTCGCTTCTCGACATTTT GCTCGAACCTTTCAAACGAA	119-134	1.0	56	VIC	PO, PY
As2.20	AS	TTAGTGAAATGCGACAGAGAGAC CATAGCCTGGCAAATCCTG	122-129	1.0-3.0	48-60	VIC	na
As2.34	AS	ACGGCCCTCGTTAGTCTG CTTGAACACCCCATGTGC	251-264	1.0-3.0	48-60	VIC	na
As2.46	AS	GTTCTCTTGCCCTGTTTGCT AGGCTGGAAATAAATGGAGGA	106	1.5	56	PET	L
As2.47	AS	CTCAGGTCCAAGAGGAACAAG	146-161	1.5	56	6-FAM	L, PO

		TTAGTGAATAGGTGGGAAATGG					
As2.57	AS	GGAAGAAGAGAATAGAGAAGAAAAAGA142–153		1.0-3.0	48-60	PET	na
		CACCCTACCCCTGCCAAT					
As2.61	AS	CTGAATGTGCTTCTTCTCTCTTGG	235–243	1.5	56	6-FAM	L
		GGGAATCTGCCTTTAGTTTGC					
As2.62	AS	GGATTTGCCATTTATTACCTACAAG	164–173	1.0-3.0	48-60	VIC	na
		GCTACACTCCCTCTTCCATT					
AH01	AMxAA	TTGAGGTTGAGGGTGATGAA	103–105	1.5-3.0	48-60	na	na
		GGCAAGCCTCTCTCTCTCT					
AH02	AMxAA	TGAACGGCTCTCTCTCTCT	78–80	1.5-3.0	48-60	na	na
		TTCATCACCCCTCAACCTCAA					
AH08	AMxAA	TTCAGGCCTCTCTCTCTCT	91–93	1.5-3.0	48-60	na	na
		TCGCCTAAATCCTTCCCAAC					
Plop4	PL	AAACCAAGGTCTTCTCTGCTTC	192-218	1.5	56	PET	L, M, PO, PY
		ACTCCCTCTCTTTCCATCTCT					
Plop6	PL	TGAAATGAGGGAGACGAGGA	122-128	1.5-3.0	48-60	na	na
		CACACATGTTCTCTCCTTACCTTG					
Plop8	PL	TTTGGCAGTCAGCAGAAGG	163-203	1.5-3.0	48-60	NED	PO

		TTCTTCAATTTCCGTTCCATC					
Plop11	PL	TGTCAAACACACCTATCCACA GACCGTCGGATCTGGAAGT	221-243	1.5-30	48-60	6-FAM	PO
Plop12	PL	GCATGTGACAATGGATGATTTTC CATTCTTCGCCATTCATTC	223-227	1.5-3.0	48-60	na	na
Plop18	PL	ATTGAAGCTGCCCTCACATT TGTTCCGGCCTCTTCTTTCTC	178-180	1.5-3.0	48-60	6-FAM	I, L, M, PY
ccmp1	NT	CAGGTAAACTTCTCAACGGA CCGAAGTCAAAGAGCGATT	139	1.5	50	na	na
ccmp2	NT	GATCCCGGACGTAATCCTG ATCGTACCGAGGGTTCGAAT	189	1.5	50	na	M
ccmp3	NT	CAGACCAAAGCTGACATAG GTTTCATTCCGGCTCCTTTAT	112	1.5	50	VIC	L, PY
ccmp4	NT	AATGCTGAATCGAYGACCTA CCAAAATATTBGGAGGACTCT	126	1.5	50	PET	PY
ccmp5	NT	TGTTCCAATATCTTCTTGTCATTT AGGTTCCATCGGAACAATTAT	121	15	50	VIC	M, L, PO, PY
ccmp6	NT	CGATGCATATGTAGAAAGCC	103	1.5	50	VIC	PY

		CATTACGTGCGACTATCTCC					
ccmp7	NT	CAACATATAACCACTGTCAAG	133	1.5	50	6-FAM	I, M, L, PO, PY
		ACATCATTATTGTATACTCTTTC					
ccmp8	NT	TTGGCTACTCTAACCTTCCC	77	1.5	50	na	na
		TTCTTTCTTATTTTCGCAGDGAA					
ccmp9	NT	GGATTTGTACATATAGGACA	98	1.5	50	na	na
		CTCAACTCTAAGAAATACTTG					
ccmp10	NT	TTTTTTTTTAGTGAACGTGTCA	103	1.5	50	NED	I, M, L, PO, PY
		TTCGTCGDCGTAGTAAATAG					

Exp size: Expected size

AM: *Acacia mangium*

I: *Acacia implexa*

Tm: Annealing temperature

AS: *Acacia saligna*

M: *Acacia melanoxylon*

FL: Flouriscent label

AA: *Acacia auriculiformis*

L: *Acacia longifolia*

AMP: Amplified product

PL: *Paraserianthes lophantha*

PO: *Acacia podalyriifolia*

na: Not applicable

NT: *Nicotiana tabacum*

P: *Acacia pycnantha*

Table 3.3: Results of primer screening for polymorphisms for the 5 tested species. Number of PCR successes (N), Number of alleles (N_A), observe heterozygosity (H_O), Expected heterozygosity (H_E), Inbreeding coefficient (F_{IS}). None of the inbreeding coefficients (F_{IS}) differed significantly between native and invasive populations.

Australian Population							South African population					
<i>Acacia implexa</i>												
Locus	N	N_A	Allele range	H_O	H_E	F_{IS}	N	N_A	Allele range	H_O	H_E	F_{IS}
AM352	24	11	(113-133) 20	0.33	0.84	0.61	24	9	(113-133)20	0.76	0.80	0.05
AM460	24	10	(111-117) 6	0.79	0.76	-0.04	24	5	(107-117) 10	0.67	0.53	-0.26
AM770	24	5	(89-94) 5	0.92	0.75	-0.23	24	2	(89-94)5	1.00	0.51	-1.00
Plop18	24	5	(168-170) 2	0.22	0.20	-0.08	24	4	(162-70) 8	0.67	0.50	-0.34
AVERAGE				0.57	0.64	0.065				0.775	0.585	-0.3875
<i>Acacia longifolia</i>												
Locus	N	N_A	Allele range	H_O	H_E	F_{IS}	N	N_A	Allele range	H_O	H_E	F_{IS}
AM352	29	7	(111-125) 14	0.83	0.68	-0.22	17	11	(105-127) 22	0.81	0.85	0.05

AM387	29	9	(103-121) 18	0.55	0.73	0.25	17	5	(107-121) 14	0.69	0.62	-0.06
AM429	29	9	(169-189) 20	0.96	0.85	-0.14	15	12	(167-189) 22	0.76	0.92	0.15
AM502	29	7	(108-129) 21	0.63	0.73	0.14	17	7	(111-123) 12	0.86	0.77	-0.13
plop4	29	4	(168-180)12	0.83	0.56	-0.49	17	2	(168-176) 8	0.73	0.48	-0.56
Plop18	24	3	(160-170)10	0.75	0.49	-0.53	14	4	(168-170) 2	0.57	0.42	-0.37
AVERAGE				0.76	0.67	-0.17				0.74	0.68	-0.15

Acacia melanoxylon

Locus	N	NA	Allele range	Ho	He	Fis	N	NA	Allele range	Ho	He	Fis
AM352	24	5	(107-117)10	0.75	0.69	-0.10	23	5	(107-117) 10	0.91	0.73	-0.26
AM435	21	12	(121-141) 20	0.52	0.88	0.41	21	8	(125-141) 16	0.48	0.83	0.44
AM502	18	6	(122-140) 18	0.56	0.81	0.32	23	5	(119-140) 21	0.83	0.72	-0.16
Plop18	17	3	(160-170) 10	0.76	0.52	-0.48	22	2	(164-170) 6	0.91	0.51	-0.83
AVERAGE				0.65	0.73	0.04				0.78	0.70	-0.20

Acacia podalyriifolia

Locus	N	NA	Allele range	Ho	He	Fis	N	NA	Allele range	Ho	He	Fis
AM041	12	3	(125-134)9	0.38	0.68	0.46	12	6	(125-134)9	0.56	0.68	0.19
AM352	12	6	(108-117)9	0.92	0.85	-0.08	12	7	(103-117)14	1.00	0.74	-0.38
AM435	12	3	(122-128)6	0.82	0.54	-0.55	12	3	(122-128)6	0.83	0.57	-0.51
AM436	12	4	(230-238)8	1.00	0.70	-0.46	12	4	(224-238)14	0.10	0.59	0.83
AM502	12	5	(125-140)15	0.91	0.75	-0.22	12	6	(116-134)18	0.75	0.81	0.07
AM770	12	5	(85-95)10	0.83	0.69	-0.22	12	5	(85-95)10	0.75	0.77	0.04
Plop4	12	4	8 (176-184)	1.00	0.65	-0.58	12	8	(162-184)22	0.83	0.69	-0.21
Plop18	12	3	(160-170)10	0.18	0.18	-0.03	12	2	(168-170)2	0.00	0.26	1.00
AVERAGE				0.76	0.63	-0.21				0.60	0.64	0.13

Acacia pycnantha

Locus	N	NA	Allele range	Ho	He	Fis	N	NA	Allele range	Ho	He	Fis
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AM352	24	6	(107-120)	13	0.96	0.61	-0.66	24	3	(111-117)	6	0.95	0.53	-0.83
AM429	24	9	(170-190)	20	0.76	0.81	0.059	24	8	(172-188)	16	0.84	0.83	-0.018
AM435	24	5	(123-131)	8	0.79	0.61	-0.30	24	4	(125-135)	10	0.59	0.57	-0.03
AM436	24	6	(252-272)	22	0.59	0.71	0.17	24	6	(250-264)	14	0.52	0.63	0.17
AM502	24	5	(125-135)	10	0.50	0.56	0.11	24	3	(127-135)	8	0.63	0.46	-0.36
AS2.17	24	4	(105-113)	8	0.64	0.67	0.05	24	4	(105-113)	8	0.86	0.66	-0.31
Plop4	24	7	(170-192)	22	0.55	0.68	0.21	24	8	(170-192)	22	0.64	0.71	0.10
Plop18	24	4	(154-173)	19	0.18	0.17	-0.01	24	3	(158-170)	12	0.45	0.37	-0.23
AVERAGE					0.62	0.60	-0.05					0.69	0.60	-0.19

Chapter 4: Native range plantings and admixture greatly alters genetic structure of invasive *Acacia pycnantha* (Benth) in South Africa.

A version of this chapter will be submitted to *BMC Evolutionary Biology*.

Reference: Ndlovu, J., Richardson, D.M., Wilson, J.R.U. Le Roux J.J. (2013). Native range plantings and admixture influence the spread of *Acacia pycnantha* (Benth) in South Africa. *BMC Evolutionary Biology*: to be submitted.

Abstract: Evolutionary studies in invasion biology have tended to focus on the influences of introduction or post-introduction dynamics in the invaded range. However, human influences on the native range of species prior to a species being selected for introduction can also affect invasion dynamics like genetic diversity and genetic structure. In this study we examined *Acacia pycnantha* populations in their native range (south-eastern Australia) and invasive range in South Africa. Nuclear microsatellite loci were used to compare genetic diversity and structure in the native range (Victoria and New South Wales and South Australia) and invasive range (South Africa) and to trace the landscape-scale native provenance of invasive *A. pycnantha* populations. Using assignment tests, three genetic groupings with substantial admixture were found in the native range (wetland Victoria and South Australia populations; dryland Victoria and Flinders Range population; and New South Wales). Bayesian assignment of invasive populations in South Africa indicated a similar genetic structure of highly admixed individuals. We argue that the genetic structure of *A. pycnantha* in Australia has been greatly altered through intentional human movements and plantings which have led to highly admixed populations. These

already-admixed propagules reached South Africa before becoming established and invasive.

Key words: *Acacia pycnantha*, admixture, genetic structure, native range, restoration

4.1 Introduction

The link between genetic diversity and invasion history has been documented for numerous species (Genton *et al.*, 2005; Kelly *et al.*, 2006; Kolbe *et al.*, 2007). It is clear that invasions are often founded by genetically bottle necked populations which harbor only a small proportion of the total genetic diversity found in their native ranges and therefore may display reduced evolutionary potential (Dlugosch & Parker, 2008). Various factors can result in invasion success despite low genetic diversity; these include release from natural enemies (De Walt *et al.*, 2004) and competitors (Funk & Vitousek, 2007), broad environmental tolerance (Shea & Chesson, 2002), and pre-adaptations (Schlaepfer *et al.*, 2009). On the other hand, many successful invasions are characterized by high genetic diversity as mediated through multiple introductions (Dlugosch & Parker, 2008). Intuitively, high genetic diversity is likely to be beneficial to any species introduced into a new environment. Multiple introductions can cause an immediate breakdown of natural gene flow barriers in the native range, often leading to admixture (e.g. Lavergne & Molofsky, 2007), increased population genetic diversity (Lavergne & Molofsky, 2007), hybridization (Gaskin & Schaal, 2002) and even genetic novelty (e.g. Thompson *et al.*, 2012) in the invaded range. High propagule pressure may also simply enhance the likelihood of introducing suitable genotypes to the new environment (Richardson & Pyšek, 2006). However, the native range population structure can also influence this process. For example for highly structured populations, multiple introduction events from a single

population may lead to lower overall diversity than a single introduction event sourced from numerous native range populations (Le Roux *et al.*, 2011).

Tree species introduced for forestry (in the broad sense) are a particularly interesting case. Human-mediated introductions of plants for use in forestry, agroforestry and horticulture have substantially accelerated the rate of plant invasions globally (Richardson *et al.*, 2011). Plantations are often abandoned when demand declines or when they cease being economically viable, creating opportunities for plants to escape, establish, and invade (Richardson *et al.*, 2011), while other species may accidentally escape active cultivation to become problematic weeds (Richardson *et al.*, 1994, Rosmann, 2001). Forestry species are also typically sampled over large native range distributions and in large numbers prior to introduction to maximize genetic diversity, environmental sampling, and thus evolutionary/breeding potential (Coggeshall & Woester, 2010). Extensive breeding programmes in both the native and introduced range might also 'pre-adapt' introductions to local environmental conditions (Le Roux *et al.*, 2011). Consequently, the introduction and establishment of forestry species are characterised by a suite of characteristics often associated with invasion success: high propagule pressure, short generation times, high growth rates (see Richardson & Pyšek P, 2006), as well as high adaptability as mediated through high genetic diversity (Okada *et al.*, 2007; Thompson *et al.*, 2012; Dlugosch & Parker, 2008; Williams *et al.*, 2007, Lavergne & Molofsky 2007).

Disentangling the deterministic and stochastic processes that underlie the genetic diversity and structure of successful invaders remains problematic (Keller & Taylor, 2008), but has important implications for the effectiveness of management interventions, particularly biological control (e.g. Goolsby *et al.*, 2006). Given the complex introduction histories often associated with forestry species, elucidating the processes that shape genetic diversity in the invasive ranges requires accurate and detailed introduction records

(Le Roux *et al.*, 2011). Here we took advantage of the detailed records of introduction of *Acacia pycnantha* (Benth.) to South Africa (Poynton, 2009). *Acacia pycnantha*, commonly known as the golden wattle, is native to south eastern Australia and was introduced to South Africa on two separate occasions in 1865 and 1893 as a potential source of tanbark and for dune reclamation purposes (Poynton, 2009). Experimental plantings of *A. pycnantha* showed the species to be a promising candidate for tanbark production. While the exact size of both introduction events is unknown, the redistribution of ca. 22 - 29 million seeds sourced within South Africa throughout the coastal regions of the country was documented (Poynton, 2009, Webber *et al.*, 2011). Like many other Australian acacias, *A. pycnantha* is now invasive in parts of South Africa (Henderson, 2001). Previous phylogeographic work has shown that *A. pycnantha* is structured into two distinct ecotypes in its native range distribution (the dryland and wetland forms) and that hybridization appears to occur between these two forms (Ndlovu *et al.*, 2013). This study also showed that South African populations are genetically less diverse than Australian populations and most closely resemble the wetland form from southern Australia (Ndlovu *et al.*, 2013). However, revegetation and roadside plantings from cultivated plants have led to established populations of *A. pycnantha* in southern Australia and this may complicate phylogeographic signatures in the native range. Given the known impacts of cultivation on the genetic makeup of invasive species, including Australian acacias (Thompson *et al.*, 2012), our overall aim was to use comparative landscape genetics to better understand the provenance, genetic diversity, structure, and dynamics of invasive *A. pycnantha* populations in South Africa. Specifically, the following questions were addressed: 1) What is the genetic structure in the native range of *A. pycnantha*? 2) How much of *A. pycnantha*'s native range genetic diversity has been introduced to South Africa? 3) Can

source populations of *A. pycnantha* invading South Africa be identified? 4) Does admixture of geographically isolated genotypes from Australia occur in South Africa?

4.2 Materials and methods

4.2.1 Sample collection

Phyllode material of *A. pycnantha* was collected at seventeen sites throughout the native range (southeastern Australia) and at seven sites from the invaded ranges in South Africa (Table. 4.1). Material was also collected from Mt Compass in South Australia from where the biocontrol agent *Trichilogaster signiventris* which has successfully established in South Africa was collected (Hoffman *et al.*, 2002). For each site, material was collected from between five and 30 trees and preserved in silica gel until DNA extraction. Collection sites were geo-referenced using a handheld GPS.

4.2.2 DNA extraction and PCR conditions

DNA was extracted from phyllode material using the CTAB extraction protocol (Doyle and Doyle, 1987) as modified by Ndlovu *et al.* (2013). DNA concentrations were measured using spectrophotometry (Infinite 200 PRO NanoQuant, Tecan Group Ltd, Switzerland) and good quality DNA diluted to 20 ng/μL and stored at -80°C until further use. Eight nuclear microsatellite markers that were previously developed for *Acacia mangium* (Butcher *et al.*, 2000), *A. saligna* (Millar & Byrne, 2007) and *Paraserianthes lophantha* (Brown & Gardner, 2011) were amplified for each individual in the study (see Appendix. 4.1). PCR was conducted in two separate multiplexes which were performed

using the Qiagen multiplex PCR kit following the manufacturer's instructions: 5 μ L of 2X Qiagen mix, 2 μ L of (2 pmols per reaction) mix, 2 μ L of Rnase free water and 1 μ L of DNA template to make up a final volume of 10 μ L (Table. 4.2). The following thermocycle was used: an initial denaturation step of 95°C for 15 min followed by 35 cycles of 94°C for 30s, an annealing temperature of 57°C for 90s and 72°C for 60s. A final elongation step of 60°C for 30 min was performed. Separation of PCR fragments was done on an ABI Prism 3100 Genetic analyzer (Applied Biosystems, Foster City, USA) using GENSCAN TM- 500 (-250) as an internal size standard. Allele scoring was done using GENEMARKER version 1.95 (SoftGenetics LLC, Pennsylvania, USA).

4.2.3 Data analysis

4.2.3.1 Genetic diversity

Microchecker version 2.2.3 (van Oosterhout *et al.*, 2004) was used to check for null alleles, problems with large allele dropouts and allele stutter. In addition, FreeNA (Chapius & Estoup 2007) was also used to examine the presence of null allele frequencies for each locus and population following the expectation maximisation algorithm (Franck *et al.*, 2007). All microsatellite loci were tested for departures from the Hardy-Weinberg equilibrium and linkage disequilibrium using Adegnet (Jombart, 2008). ARLEQUIN version 3.5.1.2 (Excoffier, 2010) was used to calculate the number of alleles (N_A), allelic richness (R), observed and expected heterozygosities (H_O and H_E), the fixation indices (F_{ST}) and the inbreeding coefficients (F_{IS}) for Australian (native range) and South African (invasive range) ranges. Statistical comparisons to evaluate differences in the genetic diversity indices were calculated using FSTAT version 2.9.3.2 (Goudet, 1995). To obtain a more detailed representation of genetic diversities, the following within population diversity

indices: number of alleles (N_A), observed and expected heterozygosities (H_O and H_E), inbreeding coefficients (F_{IS}) were computed in ARLEQUIN version 3.5.1.2 (Excoffier, 2010). The mean number of private alleles per population was computed in GenAlex (Peakal & Smouse, 2006).

4.2.3.2 Genetic structure

A Bayesian clustering method implemented in the programme STRUCTURE version 2.3.4 (Pritchard *et al.*, 2010) was used to detect the number of genetic clusters present in the native range dataset and to assign individuals probabilistic to these clusters. The admixture model with correlated allele frequencies was chosen and 10 replicates of each run were done. Each run consisted of a burnin of 10000 MCMC steps, followed by 100000 iterations. The method of Evanno *et al.* (2005) was used to determine the approximate number of genetic clusters.

To validate the number of native range genetic clusters identified in the STRUCTURE analysis, a second Bayesian method as implemented in Geneland package version 3.0 (Gulliot *et al.*, 2009) was used. Ten independent Monte Carlo Markov chains were performed using the following settings: 100 000 iterations with 100 thinning intervals using a correlated allele frequency model. The maximum number of populations/clusters was set to ten. A map of population membership was obtained by post process chain and tessellation functions into Geneland by tesseling the landscape at a resolution of 5 m in R (R Core Development).

To assign the invasive populations (South Africa) to a genetic cluster in the native range (Australia), the clusters obtained from the Geneland analysis were used. We used the

Geneland clusters since those identified by the STRUCTURE analysis were highly admixed and not clearly differentiated. The pop-flag setting was used as an additional a priori in STRUCTURE version 2.3.4 (Pritchard *et al.*, 2010), in other words, allele frequencies were updated using only pop-flagged native range populations (Geneland clusters).

To assess the distribution of genotypes in the native and invasive ranges a covariance standardised Principal Co-ordinate analysis (PCoA) implemented in GenAlex (Peakall & Smouse, 2006) was used. An analysis of molecular variance (AMOVA) (Excoffier *et al.*, 2010) was performed with genetic variation being partitioned into two hierarchical levels between regions and among regions.

4.3 Results

4.3.1 Genetic diversity

There was evidence of null alleles at one locus (Plop 18) in eight of the populations and so this loci was not used in further analyses. The Hardy Weinberg probability tests carried out on the remaining seven microsatellites showed that the 153 out of 168 of the locus-by-site comparisons did not deviate from the Hardy Weinberg equilibrium. Fifteen loci-by-site comparisons deviated from expectation and showed an excess of homozygotes. Of the 15 deviations only three populations out of the 24 sampled populations showed more than one locus deviating from the Hardy Weinberg equilibrium. Three loci (As 2.17, Am435 and Plop 4) were out of Hardy-Weinberg equilibrium for at least three sampled populations. However, the homozygote excess was not attributed to the presence of null alleles in the dataset.

Overall, the number of alleles (N_A), unbiased genetic diversity (H_S), and allelic richness (R_S) were slightly higher in the native region (Australia), compared to the invasive range (South Africa). Furthermore, the native range populations were slightly more structured ($F_{ST} = 0.084$) compared to invasive populations in South Africa ($F_{ST} = 0.052$) (Table 4.1). Native range populations were less inbred than the introduced populations (see Table. 4.2).

Table. 4.1: Microsatellite genetic diversity indices for native and invasive populations of *Acacia pycnantha*. R_S = allelic richness, H_S = unbiased gene diversity, F_{IS} = Inbreeding coefficient, F_{ST} = Among-population differentiation.

Region	R_S	H_S	H_O	F_{IS}	F_{ST}
Native (Australia)	79	0.631	0.673	-0.078	0.084
Invasive (South Africa)	68	0.611	0.651	-0.067	0.052

For the intra-population genetic diversities, the mean number of alleles was slightly lower in the introduced populations (3.86 to 6.43) than in the native range (3.33 to 7.29). Similarly, genetic diversity (measured as expected heterozygosity, H_E) was slightly higher in the native range (0.53 to 0.73) than in the invaded ranges (0.53 to 0.66) (Table 4.2). There were fewer private alleles (N_P) found in the invaded ranges in South Africa (6) than in the native range (11) (see table 4.2).

Table 4.2: Genetic diversity indices at 7 microsatellite loci and 24 populations (17 invasive and 7 native) of *Acacia pycnantha*. N = Number of individuals per population, N_a = Number of alleles, H_O = Observed heterozygosity, H_E = Expected heterozygosity, F_{IS} = Inbreeding coefficient and N_{PA} = Number of private alleles.

Sample ID	Latitude	Longitude	N	N _a	H _O	H _E	F _{IS}	N _{PA}
South Africa (invasive)								
Caledon (CAL)	-33.10701	19.29755	27	3.86	0.59	0.53	0.09	0
Grahamstown (GRT)	-33.46032	26.15991	25	5.86	0.58	0.57	0.8	2
Tokai (TOK)	-33.84179	18.66602	28	4.86	0.55	0.60	0.24	0
Humansdorp (HUM)	-34.03989	-24.78687	18	6.00	0.70	0.64	-0.13	1
Wolsely (WOL)	-33.34012	19.16109	26	6.43	0.72	0.67	-0.02	1
Stellenrust (STE)	-34.06024	18.41480	27	6.00	0.69	0.62	-0.12	2
Piketberg (PIK)	-32.80084	18.71501	21	4.86	0.72	0.66	-0.15	0
AVERAGE				5.41	0.65	0.61	0.10	
Australia (native)								
Mt Compass (MTC)	-35.40585	145.95586	19	6.43	0.73	0.69	-0.12	0
Melrose (MEL)	-32.78187	138.1973	28	6.43	0.63	0.70	-0.04	3
Kilmore (KIL)	-37.22176	145.021	26	5.57	0.57	0.59	0.14	0
Natimuk (NAT)	-36.00409	143.76041	26	6.29	0.68	0.66	-0.09	3

Frances (FRA)	-36.77054	141.18135	25	5.42	0.70	0.66	0.08	0
Border NSW &VIC (NSW)	-35.83107	147.22716	29	7.29	0.78	0.69	-0.11	1
Charlton & Boorte (CB)	-35.99273	143.76538	28	6.14	0.81	0.67	-0.19	4
Mt Jeramborera (MTJ)	-35.36866	149.20332	21	4.86	0.63	0.53	-0.05	0
Lockheart (LOC)	-35.36866	146.64549	21	4.86	0.72	0.66	-0.08	1
Gundagai (GUN)	-35.21065	147.76425	22	4.71	0.60	0.54	-0.01	0
Reef Hills (RHSP)	-36.59888	145.95586	22	5.57	0.61	0.62	0.03	0
Kangaroo Isl (KI)	-35.75669	137.89486	5	4.29	0.74	0.75	0.33	0
Newlands C. P(NLHCP)	-35.61298	138.47950	5	3.33	0.74	0.62	-0.10	0
Nelson (NEL)	-38.05003	141.01510	8	3.71	0.63	0.60	0.04	0
Castlemaine (CAS)	-37.10758	144.09283	5	3.43	0.61	0.57	-0.08	0
Border Town (BT)	-35.32020	139.51302	5	3.57	0.67	0.55	-0.39	0
Hall's Gap	-37.11027	142.57697	6	3.71	0.70	0.73	0.01	0
AVERAGE				5.04	0.68	0.63	-0.04	

4.3.3 Genetic structure

Structure analysis of the Australian data set revealed three different genetic clusters (Fig. 4.1B and Fig. 4.1D). The genetic clusters roughly corresponded to three distinct regions within *A. pycnantha*'s natural range (see Fig 4.1A). These included New South Wales and some wetter parts of Victoria (cluster 1, indicated in red in Fig. 4.1A); the drier parts of Victoria (cluster 2, indicated in green in Fig. 4.1A); and South Australia and Flinders Range (cluster 3, indicated in blue in Fig. 4.1A). However, the majority of the native range populations could not be confidently assigned to a single cluster ($q > 0.8$), indicative of extensive admixture. The Geneland analysis clustered the Australian populations into two distinct clusters: the dryland Flinders Range population and the rest of the populations (also see Ndlovu *et al.* 2013). A further STRUCTURE analysis to assign South Africa invasive populations to native range genetic clusters did not accurately place South African populations in a particular cluster (Fig. 4.1C and Fig.4.2). These results showed that South African invasive populations probably originated from seed collected from anywhere in Australia or from a single population in the native range distribution in Australia. Although the invasive *A. pycnantha* populations clustered with the Flinders Range (dryland form of *A. pycnantha*) it is highly unlikely that this region is a putative source region because of the marked differences in leaf morphologies. Furthermore, chloroplast sequence data obtained from the Flinders Range population showed no similarity to South African invasive populations (see Ndlovu *et al.*, 2013).

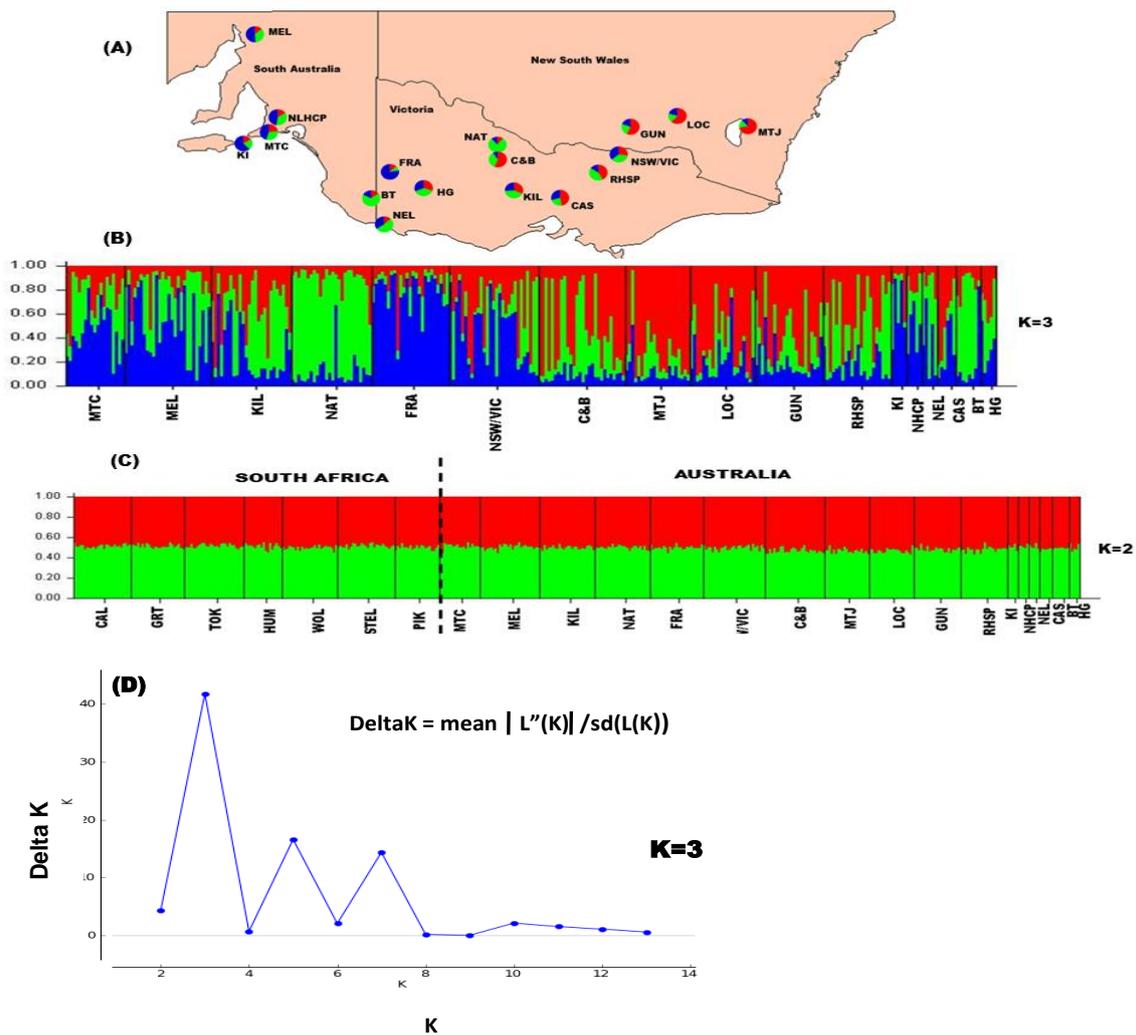


Fig. 4.1 Results of the STRUCTURE analysis showing Phylogeographic structure of *A. pycnantha* populations in its native range (SE Australia) and an invasive range (South Africa). (A) Genetic groups as obtained in the analysis are overlaid on the native range geographic map. (B) Bayesian clustering of Australian genetic groups in *Acacia pycnantha* based on STRUCTURE analysis. Each individual included in the analysis is represented by a vertical bar. (C) Bayesian clustering of Australian and South African groups of *Acacia pycnantha*. (D) Results for Delta K for the native Australian populations using the method of Evanno *et al.*, 2005

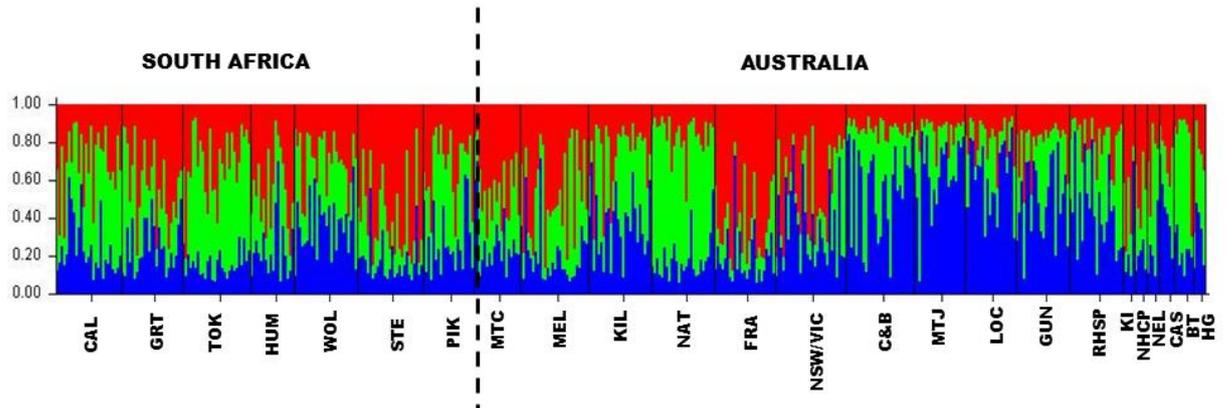


Fig. 4.2 Bayesian clustering of Australian and South African groups of *Acacia pycnantha*.

Hierarchical AMOVA comprising all samples (Table. 4.4) showed low differentiation between the native and invasion ranges (0.30%) but considerable differentiation among populations (6.76%) whereas the majority of genetic variation resided within individuals (95.9%). PCA results (Fig. 4.3 indicated a close relationship between invasive *A. pycnantha* from South Africa and native populations from southern Australian populations, particularly Mt Compass and some Victorian populations. A non-significant pattern of isolation by distance was detected among native Australian populations ($r=0.20$, $P=0.075$)

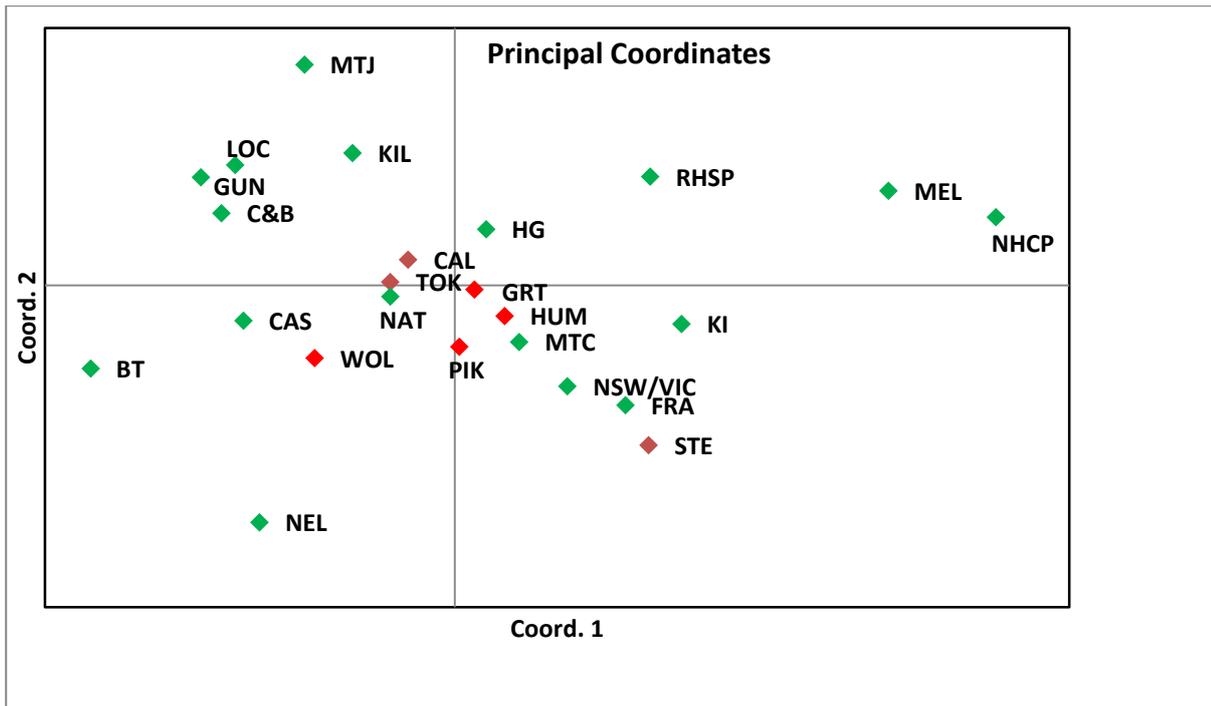


Fig.4.3 Principal co-ordinate analysis of Australian (native populations represented by green triangles) and invasive South African populations (represented by red triangles) of *Acacia pycnantha*.

Table. 4.3: Hierical analysis of molecular variance (AMOVA) in *Acacia pycnantha* among native and invasive populations, among populations, within populations, within populations and within individuals.

Source of variation	df	Sum of squares	Variance	Percent variation (%)
Among native and invasive populations	1	4.701	0.00258	0.30
Among populations	22	66.297	0.05794	6.75
Within populations	449	347.016	-0.02530	-2.95
Within individuals	473	389.500	0.82347	95.90

4.4 Discussion

We found very low fine-scale population genetic structure and high levels of gene flow throughout *A. pycnantha*'s native distribution in south eastern Australia (see Fig. 4.1A). While this observation supports a previous phylogeographic analysis indicating that admixture frequently occurs in Australian populations it is also in contrast to the genetic structure previously identified between different ecotypes (dry and wetland forms) of *A. pycnantha* (Ndlovu *et al.* 2013). These results most likely reflect the extensive movement and plantings of *A. pycnantha* in Australia.

Acacia pycnantha has a long history of usage in Australia. Recently *Acacia pycnantha* has been identified as a promising forestry tree because of its hardiness, drought tolerance and good performance under a range of soil conditions and trial plantations have been established at numerous sites in Australia (Maslin & McDonald, 2004). The species has also been used for soil stabilisation as it shows high natural colonising ability and fast growth under field conditions (<http://www.treesforlife.org.au>). However, as early as the 1840s *A. pycnantha* was harvested in the wild like other wattles (Maria Hitchcock, personal notes). The unsustainable harvesting practices of wattle bark in the wild prompted the parliament of Victoria to appoint a Wattle Board of enquiry which recommended wattle plantations as a sustainable source of tannin bark (Frawley, 2010). Wattle plantations were therefore set up in Victoria, New South Wales, Tasmania and South Australia during the last two decades of the nineteenth century (1880-1900) (Frawley, 2010). In consequence, there has been substantial history of seeds being moved throughout *A. pycnantha*'s native range, providing many opportunities for interbreeding to occur between previously allopatric populations. The genetic effects of such breakdowns of reproductive barriers will be profound. Therefore, admixture of *A. pycnantha* could have

occurred before the species was brought to South Africa in the second introduction in 1893 (Poynton, 2009).

The role of genetic admixture in successful establishment and invasion has been documented for numerous species (Rosenthal *et al.*, 2008, Kang *et al.*, 2007, Kolbe *et al.*, 2007). This study is however, as far as we know, the first to show how anthropogenic actions (revegetation and restoration in this case) can lead to admixture in the native range prior to a species' introduction into its new range, thus changing important aspects of the invasion/dispersal pathway. Admixture may aid in the invasion success of species by allowing species to pre-adapt to more bioclimatic regions before they are introduced to new regions, increasing the overall likelihood of invasive success. Furthermore, because admixture in the native range may increase overall genetic diversity, the likelihood of introducing highly diverse genotypes is enhanced, also contributing to an enhanced likelihood of invasion success. Other benefits of admixture to invasion success include masking of deleterious alleles and the creation of novel genotypes (Verhoeven *et al.*, 2010). What makes our finding particularly interesting is the fact that extensive admixture have occurred in the native range as this means the likelihood of introducing highly diverse genotypes from a fraction of native range populations is enhanced.

Since the Australian landscape is characterised by many revegetated forests, it is difficult to identify putative sources of invasive *A. pycnantha* because admixed propagules of *A. pycnantha* might have been introduced to South Africa. The origins of invasive populations of *A. pycnantha* might explain why the introductions of *Trichilogaster signiventris* from both the dryland (Natimuk, Victoria) and the wetland regions of *A. pycnantha* (Mt Compass) were successful. The dryland populations found in South Africa could have been controlled by the Natimuk genotype of *T. signiventris* (Hoffmann *et al.*, 2002) while the remaining wetland populations could have been controlled by the Mt

Compass genotype. This will be an interesting hypothesis to test, in line with other work on the influence of phylogeographic structure on the efficacy of biological control (e.g. Goolsby *et al.*, 2005).

4.5 Conclusions

Introduction events are known to often affect genetic diversity such as causing losses in allelic richness and heterozygosity (Dlugosch & Parker, 2008). Founder effects caused by small populations often lead to reduced genetic diversity. However, we found only slightly lower genetic diversity (measured as expected heterozygosity; H_E) and a similar mean number of alleles (N_a) in South Africa compared to Australia (Table. 4.1). These results are consistent with the STRUCTURE results which suggest that invasive populations of *A. pycnantha* were either sourced from across the whole native range distribution in Australia or from a small number of already admixed populations. Given the long history of cultivation of *A. pycnantha* in its native range and the likely resulting admixture, we suspect the latter. In summary, we hypothesise that human influences on native range phylogeographic structure prior to a species being even considered for introduction, can ultimately influence invasion success.

Chapter 5: Co-invasion of South African ecosystems by an Australian legume and its rhizobial symbionts.

This chapter was published in *Journal of Biogeography*.

Reference: Ndlovu, J., Richardson, D.M., Wilson, J.R.U., & Le Roux, J.J. (2013). Co-invasion of South African ecosystems by an Australian legume and its rhizobial symbionts

Abstract: The role of nitrogen fixing bacteria (rhizobia) has been widely recognised in invasive legumes for the purposes of plant establishment and subsequent invasion. Here, the aim was to determine and compare the taxonomic identity and diversity of root nodule and rhizospheric microbial symbionts associated with *Acacia pycnantha* Benth. in its native (Australian) and invasive (South African) ranges, and to establish whether these associations are general or host specific. Bacteria were isolated from root nodules collected from 18 populations of *A. pycnantha*. Repetitive element polymerase chain reaction (REP-PCR) fingerprinting was used to assess overall bacterial diversity and clustering. Molecular phylogenies for a subset of isolates representing major REP-PCR clades were reconstructed using maximum parsimony and Bayesian phylogenetic analyses of the nuclear 16S–23S rRNA intergenic spacer (IGS), 16S rRNA, and the symbiotic *nodA* genes. Twelve clusters were identified from the REP-PCR analysis; 11 included isolates from both the native range in Australia and invasive range in South Africa, while one cluster comprised only Australian isolates. Six rhizobial species were found in association with *A. pycnantha*: *Bradyrhizobium japonicum*, *Rhizobium gallicum*, *R. lusitanum*, *R. miluonense*, *R. multihospitium* and *R. tropici*. We also identified three plant-growth promoting bacteria isolated from root nodules of *A. pycnantha*: *Burkholderia caledonica*, *B. graminis* and *B. phytofirmans*. Phylogenetic analysis of the IGS gene

retrieved clades containing symbionts from both Australia and South Africa while others comprised only South African taxa, suggesting the introduction of bacterial lineages from Australia to South Africa. Our phylogeographic analysis of the *nodA* gene confirmed that *A. pycnantha* was co-introduced with its symbionts to South Africa. *Acacia pycnantha* is a promiscuous legume, associated with at least six different rhizobial symbionts, and forms associations with plant growth promoting rhizosphere bacteria from the genus *Burkholderia*. In the invasive range of *A. pycnantha* in South Africa, nodules contained some symbionts of South African origin while other symbionts appear to have been co-introduced from Australia. *Acacia pycnantha* is associated with a wider suite of symbionts in its invasive than native range.

Keywords

Acacia pycnantha, Australia, biological invasions, co-introduction hypothesis, generalist, host-jumping hypothesis, mutualisms, plant growth promoters, rhizobia, South Africa.

5.1 Introduction

The establishment of introduced plants is dependent on a variety of factors, including direct interactions with mutualists, such as pollinators and soil microbiota (Richardson *et al.*, 2000). A growing body of evidence suggests that mutualisms between introduced plants and soil microbiota could serve as major drivers for plant invasions by improving the host's nutrient status, e.g. mycorrhizal fungi and nitrogen-fixing bacteria (rhizobia) (Reinhart & Callaway, 2006). Similarly, other beneficial microbial associations can promote plant growth by inducing metabolic processes that counter negative feedbacks brought about by biotic and abiotic stress (Compant *et al.*, 2008).

In general, mutualisms between invasive plants and rhizobia have been shown to increase plant biomass and to improve establishment success (Weir *et al.*, 2004). For example, invasive *Acacia longifolia* in Portugal, grown in soils that were collected from sites with established legume populations (*Acacia longifolia*, *Cytisus grandiflorus* and *Ulex europaeus*), produced higher above-ground biomass and nodule densities than plants grown in soils collected from established *Pinus pinaster* stands (Rodríguez-Echeverría *et al.*, 2009). High levels of nodulation in the invasive range are indicative of the functionality and the importance of mutualisms during spread and invasion processes (Parker, 2001). For example, in the USA, inoculation with *Bradyrhizobium* associated with invasive *Cytisus scoparius* more than doubled the average plant biomass while non-inoculated plants without root nodules had significantly lower biomass (Parker *et al.*, 2006).

The origin(s) of nitrogen-fixing symbionts utilized by invasive legumes in their new ranges has been a subject of discussion in the invasion literature (Weir *et al.*, 2004; Chen *et al.*, 2005; Parker *et al.*, 2007; Wei *et al.*, 2009; Rodríguez-Echeverría, 2010; Porter *et al.*, 2011; Rodríguez-Echeverría *et al.*, 2011; Crisóstomo *et al.*, 2013). Many successful invasive legumes form new mutualisms with bacteria found in the introduced environment (host-jumping hypothesis). However, symbionts can also be co-introduced with host plants, either directly as inoculants for agroforestry species (Marques *et al.*, 2001) or indirectly by hitchhiking on introduced plant material (co-introduction hypothesis) (Weir *et al.*, 2004; Porter *et al.*, 2011). Some invasive legumes form a variety of different mutualisms, and can conform to both hypotheses. For example, invasive populations of *Acacia longifolia* and *A. saligna* from Portugal, *Medicago polymorpha* from California, USA, and *Acacia decurrens* from New Zealand, all recruit co-introduced bacterial symbionts (Weir *et al.*, 2004; Rodríguez-Echeverría, 2010; Porter *et al.*, 2011;

Crisòstomoet *al.*, 2013) and some Australian acacias are able to recruit novel symbionts when grown in non-native soils (Birnbaum *et al.*, 2012).

This mixing of symbionts from different origins can have several consequences. In some instances, subsequent conjugation between different bacterial strains of different origins can lead to novel genetic combinations and may even enhance invasiveness (Menna & Hungria, 2011). Alternatively, co-introduced bacteria may represent a preferred symbiotic lineage or be a different species, which can outcompete local microbiota, resulting in multiple invasions, both above and below ground (Rodríguez-Echeverría, 2010). However, different rhizobial strains usually vary in effectiveness, with co-evolved associations being the most effective. Therefore, while the ability of an invader to form mutualisms with a larger variety of rhizobia (symbiotic promiscuity) significantly improves invasion potential and success (Weir *et al.*, 2004; Rodríguez-Echeverría *et al.*, 2011) through host-jumping, these may not be the most effective associations. Generally, promiscuous host plants also tend to be more effective in fixing nitrogen, implying that in new environments where host plants have a choice of symbiotic partners, mutualistic interactions are likely to favour generalists and exclude specialists (Wilkinson & Parker, 1996).

In addition to housing nitrogen-fixing bacteria, some legumes can also interact with other forms of plant-growth promoting microbes in their root nodules. For example, co-inoculation of soya bean with *Bradyrhizobium japonicum* and the plant-growth promoting bacterium *Serratia proteamaculans* increases the onset of nitrogen fixation, percentage plant nitrogen produced, and plant protein content (Dashti *et al.*, 1998). Similarly, endophytic plant-growth promoting bacteria and nitrogen-fixing *Rhizobium* species were found to work in synergy to promote nitrogen fixation efficiency in *Lens culinaris* (Veena & Poonam, 2011).

Not surprisingly, legumes are often over-represented among invasive plant taxa (Daehler, 1998) with some taxa emerging as model systems within invasion biology, e.g. Australian acacias (Richardson *et al.*, 2011). Many species of acacias have been moved around the world for various purposes, with many records of invasiveness. In particular, in South Africa dense monospecific stands of acacias cover tens of thousands of hectares, with substantial impacts, including changes in the soil microbial structure as a result of increased soil nitrogen (Gaertner *et al.*, 2009; Le Maitre *et al.*, 2011). The success of *Acacia* invasions globally has been attributed to a number of factors such as repeated introductions, introductions of large seed volumes, and concerted breeding efforts by scientists (Le Roux *et al.*, 2011). However, it is also likely that mutualisms, such as the legume–rhizobial symbioses, play an important role in the establishment and invasion success of acacias (Rodríguez-Echeverría *et al.*, 2011). Despite the huge introduction efforts and successful invasions by Australian acacias in South Africa, little is known of the diversity and origin of rhizobia nodulating these species and their role in the invasion process (but see Rodríguez-Echeverría *et al.*, 2011). Here, we use *Acacia pycnantha* Benth., a tree native to eastern and southeastern Australia and invasive in the Eastern and Western Cape Provinces of South Africa, to understand the diversity and role of rhizobial symbionts in its invasion success. Specifically we aimed to: (1) determine the taxonomic diversity of rhizobia associated with root nodules of *A. pycnantha* in both native and invasive ranges; (2) determine whether rhizobial associates of invasive *A. pycnantha* in South Africa follow the co-introduction, host-jumping hypothesis, or both; and (3) determine the level of promiscuity of *A. pycnantha*–rhizobial associations.

5.2 Methods

5.2.1 Root nodule collection

During 2009 root nodules were collected from *A. pycnantha* plants from 10 sites from throughout its native range in Australia. We also collected plants from seven sites in South Africa and one site in Western Australian (Table 5.1), representing its introduced ranges. At each site, five root nodules were sampled from 10 individual plants. Root nodules from a single host plant were treated as one unit and preserved in the same vial containing silica gel. For phylogenetic analyses we sequenced only one bacterial isolate/host plant because most bacteria retrieved represented *Bradyrhizobium* spp. (see Results) and all bacteria collected from single hosts appeared to be the same species based on morphology and growth rates. We therefore focused our phylogenetic analyses to include more sites over wider geographic regions rather than more nodules/individuals.

5.2.2 Isolation and culturing of rhizobia

Dried root nodules were washed thoroughly using tap water to remove all soil particles. Nodules were transferred to sterile water and stored in a refrigerator at 4 °C overnight to rehydrate (Burdon *et al.*, 1999). Rehydrated nodules were surface-sterilized in 70% ethanol for 30 s followed by 3.5% sodium hypochlorite solution for 5 min. Sterilized nodules were washed at least five separate times in sterile distilled water. The surface sterilized root nodules were then crushed in 300 µL sterile distilled water using a sterilized toothpick. 50 µL of the turbid suspension was diluted and streaked on yeast manitol agar (YMA) medium (Vincent, 1970) containing Congo Red. Plates were incubated at 28 °C and examined for bacterial growth after 3–10 days. Well-separated single colonies were

restreaked on to fresh plates until pure cultures were obtained. Colony purity was ascertained using Gram staining and light microscopy.

5.2.4 Genomic DNA isolation

Genomic DNA was extracted by heating bacterial cells in a lysis buffer (1 X TE: TrisHCl and EDTA) for 5 min at 95 °C followed by centrifuging cell lysates for 2 min. The DNA-containing supernatant was collected and washed with chloroform (Parker *et al.*, 2007). All DNA concentrations were quantified by spectrofluorimetry and high quality extractions stored at -20 °C until further use.

Table 5.1 Rhizobial strains used in the phylogenetic analyses of root nodule and rhizospheric microbial symbionts associated with *Acacia pycnantha* in its native (Australian) and invasive (South African and Western Australian) ranges.

Sample ID	Country	Locality	Latitude	Longitude	GenBank accession numbers		
					16S–23S IGS	16SrRNA	<i>nodA</i>
JNR1	SA	Caledon	-33.10701	19.29755	NA	KC207926	NA
JNR2	SA	Caledon	-33.10701	19.29755	HQ895988	NA	NA
JNR5	SA	Caledon	-33.10701	19.29755	HQ895989	NA	NA
JNR6	SA	Caledon	-33.10701	19.29755	HQ895990	NA	KC297661
JNR8	SA	Caledon	-33.10701	19.29755	HQ895991	NA	NA
JNR20	SA	Humansdorp	-34.03989	24.78687	NA	KC207924	NA
JNR22	SA	Humansdorp	-34.03989	24.78687	NA	KC207922	NA
JNR24	SA	Humansdorp	-34.03989	24.78687	NA	KC207913	NA
JNR25	SA	Humansdorp	-34.03989	24.78687	NA	KC207925	NA
JNR26	SA	Humansdorp	-34.03989	24.78687	NA	KC207920	NA
JNR27	SA	Humansdorp	-34.03989	24.78687	NA	KC207923	NA
JNR28	SA	Humansdorp	-34.03989	24.78687	NA	KC207911	NA
JNR30	SA	Humansdorp	-34.03989	24.78687	HQ895993	NA	KC297663
JNR31	SA	Wolseley	-33.34012	19.16109	HQ895994	NA	KC297664
JNR32	SA	Wolseley	-33.34012	19.16109	KC207904	NA	KC297665
JNR35	SA	Wolsely	-33.34012	19.16109	HQ895995	NA	KC297666
JNR37	SA	Wolsely	-33.34012	19.16109	HQ895996	NA	KC297667
JNR42	SA	Tokai	-33.84179	18.66602	NA	KC207921	NA
JNR44	SA	Tokai	-33.84179	18.66602	HQ895997	NA	KC297668
JNR53	SA	Stellenbosch	-34.06024	18.41480	KC207906	NA	KC297669

Table 1 (continued)

Sample ID	Country	Locality	Latitude	Longitude	GenBank accession numbers		
					16S–23S IGS	16SrRNA	<i>nodA</i>
JNR54	SA	Stellenbosch	-34.06024	18.41480	HQ895998	NA	KC297662
JNR56	SA	Stellenbosch	-34.06024	18.41480	NA	KC207917	NA
JNR57	SA	Stellenbosch	-34.06024	18.41480	KC207905	NA	KC297670
JNR138	SA	Piketberg	-32.80084	18.71501	HQ896012	NA	NA
JNR62	AUS	Esperance	-34.31586	118.79919	NA	NA	KC297671
JNR63	AUS	Esperance	-34.31586	118.79919	KC207903	NA	NA
JNR65	AUS	Esperance	-34.31586	118.79919	NA	KC207930	NA
JNR67	AUS	Esperance	-34.31586	118.79919	NA	KC207933	NA
JNR69	AUS	Esperance	-34.31586	118.79919	HQ896000	NA	NA
JNR71	AUS	Esperance	-34.31586	118.79919	KC297672	NA	KC297672
JNR78	AUS	Melrose	-32.78187	138.1973	KC207902	NA	NA
JNR80	AUS	Melrose	-32.78187	138.1973	HQ896003	NA	KC297674
JNR83	AUS	Melrose	-32.78187	138.1973	HQ896004	NA	KC297675
JNR85	AUS	Melrose	-32.78187	138.1973	KC207899	NA	KC297676
JNR86	AUS	Frances	-36.77054	141.18135	NA	KC207912	NA
JNR89	AUS	Frances	-36.77054	141.18135	HQ896005	NA	KC297680
JNR93	AUS	NSW	-35.99273	143.76538	KC207900	NA	KC297677
JNR94	AUS	NSW	-35.99273	143.76538	NA	KC207932	NA
JNR98	AUS	MTJ	-35.36866	149.20332	NA	NA	KC297678
JNR100	AUS	MTJ	-35.36866	149.20332	NA	KC207910	NA

Table 1 (continued)

Sample ID	Country	Locality	Latitude	Longitude	GenBank accession numbers		
					16S–23S IGS	16SrRNA	<i>nodA</i>
JNR101	AUS	MTJ	-35.36866	149.20332	KC20790	NA	KC297679
JNR104	AUS	Natimuk	-36.00409	143.76041	NA	KC207916	NA
JNR109	AUS	Natimuk	-36.00409	143.76041	NA	KC207919	NA
JNR112	AUS	Natimuk	-36.00409	143.76041	NA	KC207931	NA
JNR117	AUS	Mt Compass	-35.40585	145.95586	NA	KC207914	NA
JNR118	AUS	Mt Compass	-35.40585	145.95586	NA	KC207928	NA
JNR120	AUS	Wagawaga	-35.21065	147.76425	NA	KC207918	NA
JNR125	AUS	Wagawaga	-35.21065	147.76425	NA	KC207909	NA
JNR126	AUS	Wagawaga	-35.21065	147.76425	NA	KC207908	NA
JNR129	AUS	Lockhart	-35.36866	146.64549	NA	KC207907	NA
JNR131	AUS	Lockhart	-35.36866	146.64549	NA	KC207915	NA
JNR132	AUS	Lockhart	-35.36866	146.64549	NA	KC207927	NA
JNR135	AUS	Lockhart	-35.36866	146.64549	NA	KC207929	NA

5.2.5 DNA fingerprinting

To obtain an overall idea of bacterial diversity among isolates a preliminary repetitive element polymerase chain reaction (REP-PCR) fingerprinting was performed using a fluorescently labelled enterobacterial repetitive intergenic consensus primer (ERIC1) (5'-ATG TAA GCT CCT GGG GAT TCA-3') and a BOX primer (5'-CTA CGG CAA GGC GAC GCT GAC G-3') (Versalovic *et al.*, 1994). The REP-PCR was carried out in 20 μ L reactions containing 5 pmol of each primer, 25 ng template bacterial DNA, 0.4 μ L of 10mM dNTPs, 5 U taq (Kapa Biosystems, Boston, MA, USA), 10 mg mL⁻¹ bovine serum albumen (BSA) and 4 μ L of 5 \times 9 buffer. PCR amplifications were performed in an automated thermocycler with initial denaturation of 95 °C for 2 min, followed by 40 cycles of denaturation (94 °C for 60 s, 53 °C for 60 s, 65 °C for 8 min) and a final extension temperature of 65 °C for 8 min. PCR products were separated on 2% agarose gels to confirm successful amplification prior to genotyping (Versalovic *et al.*, 1994). PCR fragments were separated on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), using GeneScantm-500 (-250) as an internal size standard (Applied Biosystems). Allele sizes were visualized and scored as presence or absence of amplicons using GeneMarker 1.95 (SoftGenetics LLC, State College, PA, USA). A binary matrix of presence and absence data was generated for all amplified fragments. These data were used to reconstruct a dendrogram using a neighbour-joining distance algorithm as implemented in paup* 4 (Swofford, 2002).

5.2.6 PCR conditions and DNA sequencing

Distinct clusters from the ERIC and BOX fingerprinting tree were visually identified. At least two representative isolates were chosen from each cluster and sequenced using

the 16S–23S rRNA intergenic spacer region (IGS). IGS amplification was carried out using the primers FGPS 1490-72 (5'-TGC GGC TGG ATC CCC TCC TT-3') and FGPL 132'-38 (5'-CCG GGT TTC CCC ATT CGG-3') (Romdhane *et al.*, 2005). Each 50 µL reaction contained 5 pmol of each primer, 0.5 µL of 20mM dNTPs, 10X buffer, 1.5 mM magnesium chloride and 0.5 µL Super-Therm polymerase JMR-801 (5 U µL) (Roche, Mannheim, Germany). The following PCR cycle was used: 94 °C for 5 min denaturation followed by 30 cycles of (94 °C for 30 s, 58 °C for 30 s annealing and 72 °C for 60 s) followed by a final extension step at 72 °C for 7 min and a final holding temperature of 15 °C. A preliminary BLAST search of the 16S–23S IGS sequences was performed to compare sequences obtained against the NCBI database. Sequences that had a similarity index below 96% could not be used to reliably identify isolates to species level were reamplified with the 16S rRNA primers, 16SA (5'-AGA GTT TGA TCC TGG CTC AG-3') and 16SB (5'-AAG GAG GTC ATC CAG CC-3') (Weisburg *et al.*, 1991). For the latter, PCR reactions were carried out in 50 µL reactions containing 5 pmol of the forward and reverse primers, 1 µL of 20 Mm dNTP mix, 5 µL of 10 X buffer, 1.5 mM magnesium chloride, 1 µL BSA and 24.5 µL water. The following cycle was used: an initial denaturation of 95 °C for 2 min followed by 30 cycles of (95 °C for 30 s, 54 °C for 30 s annealing and 72 °C for 4 min) followed by a final extension step of 72 °C for 20 min and a holding temperature of 15 °C.

The *nodA* gene, which is commonly used as a biogeographic marker in rhizobial phylogenetics (Rodríguez-Echeverría, 2010), was amplified for isolates that were identified as *Bradyrhizobium* spp. using the primers TSnodD1-1C (5'- CAG ATC NAG DCC BTT GAA RCG CA-3') and TSnodB1 (5'- AGG ATA YCC GTC GTG CAG GAG CA-3') /TSnodA2 (5'-GCT GAT TCC AAG BCC YTC VAG ATC-3') (Moulin *et al.*, 2004). The following cycle was used: 94 °C for 2 min, followed by 30 cycles of (95 °C for 30 s, 56 °C/ 55 °C for 30 s, 72 °C for 45 s) and a final extension temperature of 72 °C for 7 min

(Rodríguez-Echeverría, 2010). The *nodA* gene (350 bp fragment) was sequenced for all isolates included in the 16S rRNA analysis and also to check whether taxa identified as *Burkholderia* (see Results) were actually nodulating bacteria. *Burkholderia terricola* was used as a positive control for the latter. For *Burkholderia* accessions, the *nodA* gene was amplified using the primers *nodA* A (5'-TGG ARV BTN YSY TGG GAA A-3') and *nodB* (5'-CCR AAV SCR AAY GGV AC-3') and the following thermocycle: 94 °C for 2 min followed by 35 cycles of (94 °C for 45 s, 55 °C for 30 s, 72 °C for 45 s) and a final extension temperature of 72 °C for 7 min (Chen *et al.*, 2005).

5.2.7 Molecular characterization of isolates and phylogenetic analysis

A BLAST search of the 16S–23S IGS, 16S rRNA and the *nodA* gene sequences was performed to compare sequences obtained in this study against existing data available in Gen-Bank (<http://blast.ncbi.nlm.nih.gov>). Australian and South African sequences available from GenBank that revealed high similarities to those obtained here (96–100% similarity) were also included in the reconstruction of phylogenies. Datasets for all gene regions were aligned using Clustal W (Thompson *et al.*, 1994) and manually edited in BioEdit 7.0.5.3 (Hall, 1999). All three gene phylogenies were reconstructed using maximum parsimony as implemented in paup* 4 with the heuristic search options (Swofford, 2002) and Bayesian inference in MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003). MrBayes was run for two million generations and trees sampled every 100 generations for all the three genes. Confidence in tree topologies for maximum parsimony was determined using 1000 bootstrap replicates while Bayesian posterior probabilities were determined with the Bayesian Markov chain Monte Carlo method implemented in MrBayes 3.1.2. Prior to analyses, the best fit model for evolution for each of the datasets

was determined using the Akaike information criterion (AIC) as implemented in Modeltest 3.7 (Posada & Crandall, 1998). The model of evolution used to reconstruct the *Bradyrhizobium* phylogeny was the HKY + I + G model, while the TrN + I + G and the TVM + G models were used for the 16S rRNA and *nodA* phylogenies, respectively. Because the latter two models are not implemented in MrBayes, we used the GTR + G model.

5.3 Results

From 180 collected root nodules, 125 produced colonies resembling rhizobia (see Appendix 5.1). Among these there was a mixture of fast-growing and slow-growing bacteria. Most colonies from native range regions were fast growing (c. 63%) while most colonies from invasive ranges were slow growing (c. 80%). Neighbour joining analysis of the REP-PCR and ERIC-PCR fingerprints revealed 12 distinct genetic clusters of bacterial symbionts. Overall, Australian (native) and South African (invasive) isolates were found in most clusters retrieved, with the exception of one cluster that contained only Australian isolates.

5.3.1 16S–23S rRNA – *Bradyrhizobium*

All sequences of slow-growing bacteria showed high similarity (> 96%) to accessions of *B. japonicum* lodged in Gen-Bank. All the trees obtained from the two different analyses (Bayesian and maximum parsimony) retrieved similar topologies so only the Bayesian tree is shown here (Fig. 5.1). All analyses separated the *Bradyrhizobium* isolates into nine well supported and distinct clades (Fig. 5.1). Clade 6 consisted of isolates with Australian origins, while clade 2 and clade 9 consisted of accessions collected from *A. pycnantha*

from both native and invasive ranges. Clade 1 and Clade 5 consisted of isolates that were unique to the invasive range in South Africa. Most of the Portuguese isolates that had an Australian origin (based on the *nodA* and *nifD* genes, (see Rodríguez-Echeverría, 2010) were found in cluster seven.

5.3.2 *nodA*

Partial *nodA* DNA sequences obtained in this study, and additional data for *Bradyrhizobium* spp. isolated from root nodules of different species in Australia and southern Africa (Botswana, South Africa and Zimbabwe) obtained from Gen-Bank, were used for phylogenetic reconstruction. Two geographically distinct clades were resolved in the analysis (Fig. 2). Clade 1 consisted of exclusively southern African accessions (isolated from root nodules of *Arachis hypogaea* and *Vigna unguiculata*), while Clade 2 included mostly Australian accessions and isolates from South Africa (invasive *A. pycnantha* and *A. decurrens*). All invasive *A. pycnantha* isolates from South Africa grouped within the Australian clade.



Figure 5.1 Bayesian tree of *Bradyrhizobium japonicum* symbionts associated with *Acacia pycnantha* based on the 16S–23S rRNA intergenic spacer (IGS) gene. The tree also includes reference bacterial strains isolated from various legume species, as indicated. Nodal support is given as bootstrap values. The scale bar represents the number of substitutions per site. Geographical origins are abbreviated: AUS, Australia; PORT, Portugal; SA, South Africa. Please note that, despite being collected in Portugal, accessions labelled with asterisks are thought to be of Australian origin based on a previous phylogeographic study (Rodríguez-Echeverría, 2010).

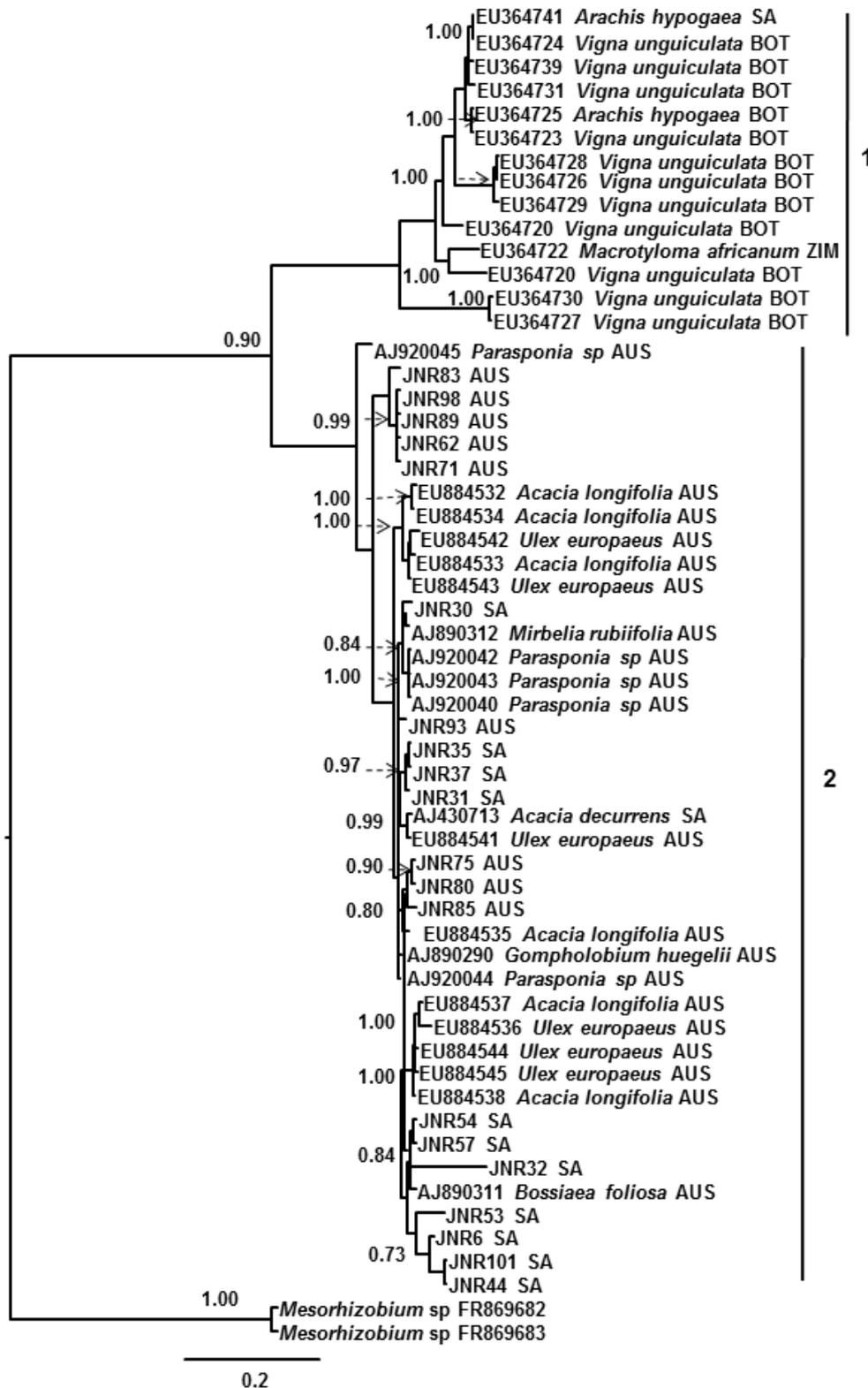


Figure 5.2 Bayesian tree based on the *nodA* gene of *Bradyrhizobium japonicum* symbionts associated with *Acacia pycnantha*. The tree also includes reference bacterial strains isolated from various legume species, as indicated. Posterior probability values > 80% are indicated. The scale bar represents the number of substitutions per site. Geographical origins are abbreviated: AUS, Australia; BOT, Botswana; SA, South Africa; ZIM, Zimbabwe.

5.3.3 16S rRNA

All of the fast-growing bacteria sequenced using the 16S–23S rRNA IGS region showed low overall similarity (< 96%) to any accessions deposited in Gen-Bank. Re-sequencing these fast-growing isolates with the 16S rRNA primers yielded sequences with a high similarity index to sequences contained in Gen-Bank and identified fast-growing bacteria as similar to *Burkholderia*, *Rhizobium* and *Mesorhizobium* species. The 16S rRNA phylogeny retrieved two major clades with one belonging to the alpha proteobacteria (*Mesorhizobium* and *Rhizobium*) and the other belonging to the beta proteobacteria (*Burkholderia*) (Fig. 5.3). In South Africa, isolates resembling *Mesorhizobium* spp., *Rhizobium miluonense*, *R. multihospitium* and *R. tropici* were retrieved, while in Australia *R. gallicum* and *R. lusitanum* were most closely related to fast-growing bacteria isolated from *A. pycnantha*. In addition, rhizobia closely related to *R. cellulolyticum* and *R. yanglingense* (which are known to form ineffective nodules in *Phaseolus vulgaris*; Tan *et al.*, 2001; García-Fraile *et al.*, 2007) were found in the root nodules of *A. pycnantha* from Australia. The 16S rRNA phylogenetic tree placed the *Burkholderia* sequences from Australia and South Africa into a single well-supported clade that included *Burkholderia caledonica*, *B. graminis* and *B. phytotfirmans* (Fig. 5.3). Both South African and Australian bacteria included *B. phytotfirmans* and *B. caledonica*, while a strain similar to *B. graminis* (JNR104) was found in Australia only. All three species identified here are not known to nodulate legumes or fix nitrogen and therefore amplification of the *nodA* gene (which facilitates nodulation) failed for all the isolates. To confirm this, we successfully amplified *nodA* in *B. terricola*, a known nodulating symbiont of *Virgilia oroboides* in South Africa (A. Magadlela, Stellenbosch University, pers. comm.), using the same PCR conditions and primers.

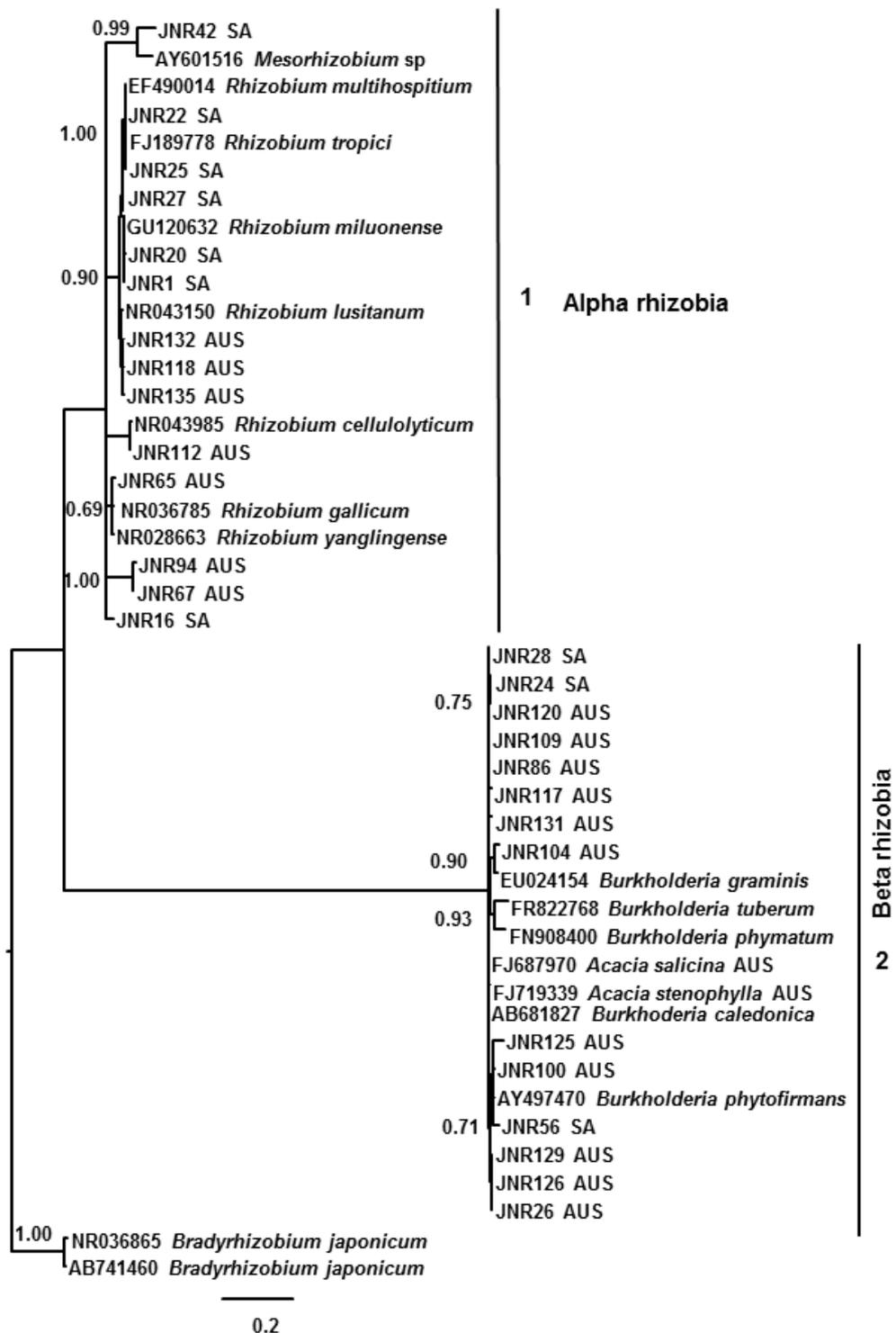


Figure 5.3 Bayesian tree based on the 16S rRNA gene for *Burkholderia*, *Rhizobium* and *Mesorhizobium* symbionts found associated with *Acacia pycnantha* in South Africa and Australia. GenBank accession numbers are given for reference taxa included from GenBank. Nodal support is given as Bayesian posterior probability values. The scale bar represents the number of substitutions per site. Geographical origins of *A. pycnantha* symbionts are abbreviated: AUS, Australia; SA, South Africa.

5.4 Discussion

Acacia pycnantha in its invasive range in South Africa was co-introduced with at least some of its native symbionts from Australia (co-introduction hypothesis). *Acacia pycnantha* is also a highly promiscuous nitrogen fixer capable of forming mutualistic associations with a wide range of symbionts in its introduced range. It forms associations with members of the genus *Burkholderia*, although these associations probably do not result in the fixation of atmospheric nitrogen. Such promiscuity is not surprising because other closely related species such as *Acacia cyclops*, *A. saligna*, *A. melanoxylon* and *Paraserianthes lophantha* also form new rhizobial associations when cultivated in soils from their non-native ranges (Birnbaum *et al.*, 2012). *Acacia* therefore appears to consist of generalist species, which do not require specific rhizobial species to fix nitrogen outside their native range (Rodríguez-Echeverría *et al.*, 2011; Birnbaum *et al.*, 2012).

Given the pivotal role of mutualisms in invasion success (Richardson *et al.*, 2000), species with highly specialized obligate mutualistic associations are often under-represented in invasive floras, e.g. members of the Orchidaceae (Daehler, 1998). The ability of Australian acacias to nodulate and fix nitrogen must have been a substantial factor contributing to their success in South Africa's fynbos biome, which is characterized by soils that are generally poor in nutrients, especially nitrogen (Slabbert *et al.*, 2010). While it is an advantage to form these mutualisms, the ability to form mutualistic partners with a wider range of rhizobial symbionts (generalist legumes) must confer advantages to introduced legumes in their new environments. Invasive *A. pycnantha* in South Africa is a generalist species associated with a range of different and distantly related rhizobia, including *Bradyrhizobium japonicum*, *Rhizobium gallicum*, *R. miluonense*, *R. multihospitium* and *R. tropici*. To the best of our knowledge this is the first record of both *R. miluonense* and *R. multihospitium* associated with an Australian *Acacia*. *Rhizobium*

multihospitium was isolated from several legumes in China (Han *et al.*, 2008). Similarly, *R. miluonense* was isolated from the root nodules of *Lespedeza* species in China (Gu *et al.*, 2008). Highly promiscuous legumes do not require co-introduction with compatible rhizobia from their native regions and may easily recruit novel microsymbionts in their new environments (Rodríguez-Echeverría *et al.*, 2011).

Our phylogenetic results indicate that *B. japonicum* symbionts were most likely co-introduced with *A. pycnantha* from Australia (Figs 5.1 & 5.2). Without knowledge of native legume– rhizobia associations, novel mutualisms between *A. pycnantha* and South African rhizobia remain speculative. However, the association between *A. pycnantha* and bacteria known only from Asia (*R. miluonense* and *R. multihospitium*) supports the host-jumping hypothesis. While the phylogeographic utility of the 16S–23S rRNA IGS gene region (Fig. 5.1) has been questioned (Rodríguez-Echeverría, 2010), the co-introduction of symbionts from Australia is further supported by our nodulation gene (*nodA*) phylogeny. Nodulation genes have been widely used and have proven to be reliable phylogeographic markers for rhizobial lineages (Weir *et al.*, 2004). Our results confirmed that *A. pycnantha* brought most, if not all, of its *Bradyrhizobium* symbionts along from Australia (Fig. 5.3). All the *A. pycnantha* isolates from South Africa grouped within the Australian clade (Clade 2; Fig. 5.2). Overall, invasive *A. pycnantha* in South Africa appears to conform to both the co-introduction and the host-jumping hypothesis. Consequently, *A. pycnantha* utilizes a wider suite of symbionts in its invasive range than its native range. This finding contrasts with previous work that showed Australian acacias to generally utilize a wider variety of mutualists in their native than invasive ranges (e.g. Weir *et al.*, 2004; Rodríguez-Echeverría, 2010; Rodríguez-Echeverría *et al.*, 2011).

In addition to the conventional rhizobial taxa found associated with *A. pycnantha* in Australia and South Africa, we also identified various *Burkholderia* taxa. The association of some beta-proteobacterial genera capable of fixing atmospheric nitrogen, including *Burkholderia*, has been known for some time (Moulin *et al.*, 2001). In particular, we isolated and identified *B. caledonica*, *B. graminis* and *B. phytofirmans* from root nodules of *A. pycnantha*. While nitrogen-fixing *Burkholderia* endosymbionts have been previously described from numerous South African legumes (Elliot *et al.*, 2007), and it is hypothesized that the group has a primarily Neotropical origin (Bontemps *et al.*, 2010). As far as we know, no indigenous nodulating *Burkholderia* strains are known from Australia. Numerous attempts to amplify nodulation genes (*nodA*) in these isolates failed, whereas we could successfully amplify nodulations genes from *B. terricola*, which nodulates South African legumes and fixes nitrogen (A. Magadlela, pers. comm.). Nevertheless, the frequency of *Burkholderia* strains was overall very high, and therefore not considered to be the result of contamination but is likely to reflect true symbionts. Whilst these taxa appear incapable of fixing nitrogen, the data raise the fundamental question of whether these bacteria have benefits to their hosts. Two of these species, *B. caledonica* and *B. graminis*, have been previously recorded as common rhizosphere inhabitants (Compant *et al.*, 2008), while others have been found to be specifically associated with the rhizospheres of Australian acacias (Hoque *et al.*, 2011). These rhizosphere bacteria can form beneficial associations with their hosts, although the nature of these associations remains uncertain. Plant-growth promoting rhizobacteria can colonize the plant anterior and thrive as endophytes in various plant organs without causing harm to their hosts (Compant *et al.*, 2008). These bacteria initially colonize the root surface followed by the subsequent entrance into the endorhiza mainly through the root tip, lateral root cracks or rhizodermal cells via cell wall degrading enzyme secretions (Compant *et al.*, 2008). Plant growth

promoters can directly exert positive effects to plants through the synthesis of phytohormones and solubilization of inorganic phosphates and mineralization of organic phosphates (Rodríguez & Fraga, 1999).

The isolation of *B. phytofirmans* from root nodules of *A. pycnantha* from both native and invasive ranges is a significant finding. *Burkholderia phytofirmans* has been largely recognized as a plant-growth promoting bacterium. For example, associations with *B. phytofirmans* enlarge root systems with enhanced secondary roots and more root hairs and thus the opportunity to form root nodules by rhizobia. Association with *B. phytofirmans* also frequently leads to the development of more and larger leaf hairs, steadier stems, higher lignin deposits around the vascular system, larger amounts of chlorophyll, increased levels of cytokinins and phenylalanine ammonia, and pathogen resistance (Compant *et al.*, 2008). Assuming that the endophytic bacteria confer some or all of these advantages to legumes that already have the ability to overcome the negative effects caused by nitrogen stress, plant establishment and subsequent invasions will therefore benefit. However, more research is needed to understand the role of the endophytic bacteria not only in the establishment success of the genus *Acacia*, but for introduced legumes in general. This information will not only advance our understanding of the invasion dynamics of Australian acacias but may also increase the value of these species for agroforestry. To date, *Burkholderia* has been isolated from root nodules or the rhizosphere of *A. pycnantha*, *A. salicina*, *A. stenophylla* and *A. decurrens* (Menna *et al.*, 2006; Hoque *et al.*, 2011; Rodríguez-Echeverría *et al.*, 2011). We recommend that future research should focus on assessing the beneficial properties that these endophytic *Burkholderia* species may render invasive legumes.

Finally, in the South African fynbos, where *A. pycnantha* is invasive, indigenous legumes show distinct, and some even peculiar, rhizobial associations. For example, the genus *Cyclopia* is nodulated primarily by *Burkholderia* species, while some *Lotononis* species are nodulated by *Methylobacterium nodulans* (Sy *et al.*, 2001; Ardley *et al.*, 2009); both groups are very distantly related to conventional rhizobia. The diversity of rhizobial symbionts in the fynbos offers an exciting opportunity to study how the interactions between indigenous and introduced bacteria affect both native and introduced legume species, a largely unexplored topic. A comprehensive study of symbiotic genes will allow for a detailed framework on how lateral gene transfer (if and when it occurs) between different species of bacteria allows for new genetic combinations that can form novel genotypes, which may enhance invasiveness.

5.5 Acknowledgements

This work was funded primarily by DST-NRF Centre of Excellence for Invasion Biology (C•I•B) and the Working for Water Programme through their collaborative research project on 'Research for Integrated Management of Invasive Alien Species'. Additional funding was supplied by grants to D.M.R. from the National Research Foundation and the Hans Sigrist Foundation. J.J.LeR. also acknowledges the National Research Foundation for funding. The authors thank Pauline Ladiges and two anonymous referees for helpful comments on previous drafts on the manuscript.

Chapter 6: Conclusion

Alien tree invasions have increased in recent decades, especially in regions with large plantings of non-native trees for forestry such as South Africa (Richardson & Rejmanek 2011). Fourteen species in the Australian genus *Acacia* (within the subgenus *Phyllodineae*) are invasive in South Africa (van Wilgen *et al.* 2011); most of these species have invaded large areas and cause major impacts in invaded ecosystems. Many traits have been consistently associated with invasion success of plants, including propagule pressure (Taylor and Hastings, 2005), hybridization (Gaskin & Schaal, (2002), and the role of mutualists (Richardson *et al.*, 2000a). In this thesis I focussed on the biogeography of the bacterial symbionts associated with woody legumes and how this and introduction history affects the likelihood of invasion success in new environments. *Acacia pycnantha* (Benth) (golden wattle) was used as a model system for studying how introduction histories impact on standing genetic diversity and mutualist associations in particular with nitrogen-fixing symbiotic bacteria. This chapter summarises the findings reported in the thesis, discusses some challenges encountered during the course of the work, and explores the priorities for further work to elucidate remaining issues.

6.1 Summary

This thesis showed that in its native range *A. pycnantha* is phylogeographically structured into two genetic lineages, the wetland form (found in South Australia, some parts of Victoria and New South Wales) and the dry land form (found in the drier regions of Victoria and the dry Flinders Range in South Australia). These two forms have distinct leaf morphologies and were probably separated during the Pleistocene epoch. The chloroplast gene (rpl32-trnI^(AUG)) and two nuclear genes (ETS and ITS) that were used to reconstruct

the separate *A. pycnantha* phylogenies which give insights into the native provenance of *A. pycnantha* found in South Africa. These results indicated that populations of *A. pycnantha* in South Africa originated from South Australia and Victoria. However, the chloroplast and nuclear phylogenies obtained from this study were not congruent, suggesting the possibility of intraspecific hybridisation in the native range. I therefore used nuclear microsatellite markers that were developed for *Acacia mangium*, *A. saligna* and *Paraserianthes lophantha* to further examine the causes of incongruence between the nuclear and the chloroplast phylogenies. For these markers, low genetic structure was observed and high levels of intraspecific hybridisation throughout the species' native ranges in south eastern Australia. I concluded that intraspecific hybridisation (admixture) occurred in the native range during the 18th century when *A. pycnantha* and other native acacias were widely moved and planted for revegetation in many parts of Australia. It was therefore from the native admixed populations that *A. pycnantha* populations in South Africa were founded. This breakdown in allopatry in the native range provided opportunities to bring in genotypes that could have been already pre-adapted for different biogeographical and bioclimatic regions of South Africa. In summary, a study motivated by understanding invasion dynamics provided useful insights into native range dynamics.

The second major finding was that *A. pycnantha* is a generalist species that is nodulated by a suite of different rhizobial symbionts. At least six different rhizobial symbionts were found to be associated with *A. pycnantha* in the native and invasive ranges. *Acacia pycnantha* also formed associations with new symbionts in South Africa, indicating that the species can fix nitrogen in new environments even in the absence of its co-evolved symbionts. More interestingly, *A. pycnantha* was found to form associations with a wide range of bacteria from the genus *Burkholderia*, which are plant-growth

promoters (although I did not explore the nature, if any, of the benefit of these bacteria on *A. pycnantha* and their role in plant invasions).

These results shed new light on the invasion dynamics of one of the most widely distributed genera of woody plants. Firstly, the work has shown the importance of human activities in the native range in altering the genetic structure of the species. This has important implications for understanding the dimensions of the introduction pathway and its role in determining invasive success in novel environments (Wilson *et al.*, 2009). The results presented in the thesis has also provided major new insights on the ecology of mutualisms in determining the performance of introduced legumes (Richardson *et al.*, 2000), thus substantially improving our ability to predict invasive success of the many species that are widely planted in many parts of the world.

6.2 Future work/opportunities

The ability to identify putative sources of invasive *A. pycnantha* in the native range using cpDNA, nDNA sequences and nuclear microsatellites opens up exciting opportunities to study how the genetic makeup of *A. pycnantha* may change when introduced to new habitats. The study also offers excellent opportunities to study co-evolutionary relationships between an invasive species and biocontrol organisms.

Further investigations into the role of genetic diversity and structure are important to improve our understanding of the invasion dynamics of alien trees used for different purposes, such as commercial forestry, agroforestry and horticulture. The effects of admixture in the native range on different aspects of performance in novel ranges need to be further explored. Further studies will help to understand whether creating new

genotypes in the native range reduces the lag phase of invasions thereby facilitating quicker invasions. It is also important to understand the extent to which admixture in the native range “prepares” species for invasion by, for example, allowing them deal with a wider range of climatic conditions in the new range.

Although I identified some polymorphic microsatellites using microsatellite transfer techniques, designing new microsatellites would improve the study of these species since the rate of transferability is low. Design of microsatellites for species like *A. longifolia* and *A. melanoxylon* which are global invaders (Richardson & Rejmanek 2011) would greatly improve the utility of this genus as a model group for the study of many aspects of invasion ecology. Since some *Acacia* species are still widely used in different forestry, characterisation of microsatellites should focus on species that are both invasive and commercially important, such as *A. mangium* and *A. mearnsii*. This will serve a dual function for improvement and invasion biology studies. Finally, the diversity of rhizobial symbionts in the fynbos as shown by the high number of nitrogen-fixing bacteria associated with *A. pycnantha* offers excellent opportunities to study how the interactions between indigenous and introduced bacteria affect both native and introduced legume species, a largely unexplored topic. A comprehensive study of symbiotic genes will allow for a detailed framework on how lateral gene transfer (if and when it occurs) between different species of bacteria allows for new genetic combinations that can form novel genotypes, which may enhance invasiveness. In addition, studying the role of *Burkholderia* species in *Acacia* invasions will provide a platform that will show how plant endophytic bacteria may aid plant invasions.

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Appendices

Appendix 3.1: Multiplexes of all amplified loci for the five *Acacia* species to which microsatellites were transferred. M= Multiplex.

Species	Multiplex
<i>Acacia pycnantha</i>	M1: Am 012 (6- FAM) Am030 (PET), Am 041 (NED), AM429 (PET), AM502 (VIC), Am 770 (6-FAM). M2: Am326 (VIC), ccmp3 (VIC), ccmp4 (PET), ccmp7 (6-FAM), ccmp10 (NED) M3: AM465 (PET), ccmp6 (VIC) M4: Plop 18 (6-FAM), AS2.17 (HEX) M5: Am352, Am2.17, Am436 M6: Plop4, Plop 18 M7: Am435
<i>Acacia implexa</i>	M1: Am030 (PET), Am041 (NED), Am 465 (PET), Am770 (6-FAM), Plop18 (6- FAM) M2: ccmp7 (6-FAM), ccmp10 (NED) M3: Am014 (PET), Am389 (NED), Am352 (6-FAM), Am460 (VIC), Am326 (PET).
<i>Acacia podalyriifolia</i>	M1: ccmp6 (VIC), ccmp7 (6-FAM), ccmp10 (NED) M2: Am770 (6-FAM), Am502 (VIC), Am041(NED), Am030 (PET), Plop18 (6-FAM) M3: AS2.17 (HEX), AS2.47 (6-FAM). M4: Am389 (NED), Am460 (VIC), Plop4 (PET), Plop 8 (NED), Plop11 (6- FAM), Am352 (6-FAM), Am436 (PET). M5: Am424 (6-FAM), Am435 (6-FAM), Am 014 (PET), Am326 (PET).
<i>Acacia melanoxylon</i>	M1: Am465 (PET), Am424 (6-FAM), Am326 (VIC), ccmp7 (6-FAM), ccmp10 (NED), ccmp5 (VIC). M2: Am030 (PET), Am041 (NED), Am502 (VIC), Am429 (PET), Plop 18 (6-FAM) M3: Am460 (VIC), AM522 (PET), Am341 (NED), Am352 (6-FAM)

M4: Am384 (VIC), Am435 (6-FAM).

Acacia longifolia

M1: Am326 (VIC), Am429 (PET), Am424 (6-FAM), Am387 (VIC),
ccmp10 (NED)

M2: Am012 (6-FAM), Am502 (VIC), Am041 (NED), Am030 (PET).

M3: ccmp7 (6-FAM), ccmp3 (VIC)

M4: ccmp5 (VIC), Plop 18 (6-FAM)

M5: As2.13 (PET), As2.46 (PET), As2.47 (6-FAM), As2.61 (6-FAM)

M6: Am326 (PET), Am341 (NED), Am352 (6-FAM), Am389 (NED)

M7: Plop4 (PET), Am435 (6-FAM), Am522 (PET)

Appendix 4.1: Polymorphic microsatellites and multiplexes used in this studying the genetic structure and diversity of *Acacia pycnantha*.

Primer name	Microsatellite sequence	Flourescent label	Size	Multiplex
Am352	F: CCTCATGTCCTTGAATGTCAC R: GACTAACCCACAAGGAAGAGTTAC	6 – FAM	127-129	1
Am429	F: CCTTCTTCTCTCATCTACCAAACC R: CCCACATCATCACTCACAACCT	PET	170-180	1
Am435	F: ACCCTTTATTTCTCACACGGA R: ACAGAAGAAGATGCAAAGAAGG	6 – FAM	139-152	2
Am436	F: ATGGATCTTGTCTTATCTTGA R: GGGCCAATTTGAGTTTGGAA	VIC	240-246	1
Am502	F: CAAATGGCCAAGTTACGACTG R: TTCTGGTAATCCAACTTATGTGG	VIC	122-128	1
As2.17	F: TCCTCGCTTCTCGACATTTT R: GCTCGAACCTTTCAAACGAA	VIC	119–134	2
Plop4	F: AAACCAAGGTCTTCTCTGCTTC R: ACTCCCTCTCTTTCCATCTCT	PET	192-218	2
Plop18	F: ATTGAAGCTGCCCTCACATT R: TGTTTCGGCCTCTTCTTTCTC	NED	178-180	1

Appendix 5.1: Number of *Acacia pycnantha* rhizobial isolates obtained per site in South Africa and Australia. Isolates that were sequenced in this study were chosen from the localities presented here.

Country	Locality	Latitude	Longitude	No of isolates
South Africa	Caledon	-33.10701	19.29755	8
South Africa	Grahamstown	-33.46032	26.15991	9
South Africa	Humansdorp	-34.03989	24.78687	10
South Africa	Wolseley	-33.34012	19.16109	8
South Africa	Tokai	-33.84179	18.66602	9
South Africa	Piketberg	-32.80084	18.71501	9
South Africa	Stellenbosch	-34.06024	18.41480	9
Australia	Charlton and Boorte	-35.99273	143.76538	4
Australia	Albany	-34.31586	118.79919	7
Australia	Reef Hills State Park	-36.59888	145.95586	6
Australia	Melrose	-32.78187	138.1973	8
Australia	Frances	-36.77054	141.18135	5
Australia	Border NSW & VIC	-35.99273	143.76538	7
Australia	Mt Jeramborerra	-35.36866	149.20332	3
Australia	Natimuk	-36.00409	143.76041	6
Australia	Mt Compass	-35.40585	145.95586	5
Australia	Gundagai	-35.21065	147.76425	5
Australia	Lockhart	-35.36866	146.64549	7
Total				125