# *Chapter 1* **Introduction**

In order to understand current distribution patterns in the Australian flora it is important to consider genetic diversity as a hierarchy, with evolutionary processes occurring at a range of scales: landscapes, ecosystems, communities, species, populations and individuals. In recently diverged taxa this consideration is crucial for understanding speciation processes since discerning boundaries between populations and species can be difficult (Drummond and Hamilton 2007). Distribution patterns of plant taxa are the result of complex historical processes including climatic change, vicariance, long-distance dispersal, competition and selection. However, contemporary factors such as tolerance or adaptation to habitats and human activity can be equally important. Determining the long- and short-term processes that have led to isolated and, consequently, vulnerable populations of plant species, is crucial for the conservation of the Australian flora.

The genera *Tremandra* R.Br. ex DC., *Platytheca* Steetz and *Tetratheca* Sm. have traditionally comprised the Australian endemic family Tremandraceae (Thompson 1976). They are small perennial shrubs distributed mostly across the temperate zone in relatively open, dry habitats ranging from heath and sclerophyll forest to rock outcrops in the arid zone. Recent molecular phylogenetic research (e.g. Savolainen *et al.* 2000, Bradford and Barnes 2001, Crayn *et al.* 2006) confirms that these three genera form a clade within Elaeocarpaceae, a family of mostly rainforest trees and shrubs that are widespread in tropical and subtropical regions and extend into temperate areas. Placement of these genera within Elaeocarpaceae has been recently formalised (APG 2003, Coode 2004). This unexpected relationship provides an ideal opportunity for studying processes contributing to speciation in plant groups characteristic of a broad range of Australian vegetation types.

The main aim of the present study is to investigate long and short term processes affecting and/or driving speciation in *Tetratheca* using a hierarchical approach. Evolutionary dynamics will be investigated at three levels: a phylogenetic study of the genus *Tetratheca*, a phylogeographic study, and a study of population dynamics within and among populations.

## 1.1 Chapter overview

The purpose of the present chapter is to provide a brief introduction to the genus *Tetratheca* in the context of the Australian flora, along with a discussion of the influences that have shaped current distribution patterns and plant assemblages. The three molecular approaches applied in the present study are outlined here, followed by the specific aims of each. Subsequent chapters have been written as stand-alone studies based on each of the three approaches and comprehensive introductions to the study species, evolutionary concepts and molecular techniques are therefore presented within the relevant chapters.

Chapter 2 presents the first detailed molecular phylogeny for *Tetratheca* providing the basis for biogeographic interpretation and phylogeographic studies in the genus. In Chapter 3, taxonomic concepts in two species complexes and the phylogeography of *Tetratheca* (investigated using chloroplast microsatellites) are discussed. Chapter 4 describes the development and application of novel nuclear microsatellites for a study of population structure and gene flow within and among two closely related species of *Tetratheca*. A summary and conclusions are presented in Chapter 5.

## 1.2 Background to the Australian flora

Distribution patterns in the Australian flora today are the result of combined evolutionary and environmental histories (Coode 2004, Crisp *et al.* 2004). Major palaeoclimatic changes, a long history of weathering, changes in fire frequency, glaciations and human impacts have all shaped the flora of present day Australia. Approximately 55 million years ago (mya) Australia began to break away from the great southern landmass Gondwana comprising Australia, Africa, Antarctica, New Zealand and South America. By 35 mya Australia had separated completely from Antarctica: its final connection with Gondwana. The isolation of Antarctica led to the formation of the circumpolar Antarctic current and with it came a much cooler, drier and more seasonal climate for Australia, including periods of glaciation (Hill 2004). By 25 mya a flora dominated by rainforest and wet heath (defined as the aseasonal-wet biome by Crisp *et al.* 2004) gave way to more sclerophyllous

vegetation dominated by eucalypts, acacias and casuarinas (SW and SE temperate biomes; Crisp *et al.* 2004).

After separating from Gondwana, Australia began to raft northward at approximately seven centimetres per year, moving a total distance equivalent to approximately 20 degrees latitude to its present position (Hill 2004). Expansion of the Antarctic ice sheet resulted in three major chilling events around 34, 14 and 3 mya (Crisp and Cook 2007). Between these events, warmer wetter periods resulted in higher sea levels and inundation of the Eucla basin (in the Nullarbor region of central, southern Australia). Deposition of limestone sediments in the Nullarbor, from marine incursions, possibly formed an edaphic barrier to the migration of many southern Australian taxa (Mast and Givnish 2002, Crisp and Cook 2007).

Rapidly increased aridification (approximately 5 mya) led to a large arid zone in the centre of the continent, effectively isolating the western and eastern floras. Rainforest habitats were severely contracted to small refugia in coastal areas during the last ice age, and sclerophylly and fire frequency became increasingly important features of the landscape (Hill 2004). Many Gondwanan lineages have persisted, however those in aseasonal-wet biomes are in general species poor compared with sclerophyllous sister taxa (Crisp *et al.* 2004). Many Gondwanan genera are present in both SW and SE biomes (either side of the Nullarbor Plain) indicating past contact. At the species level, however, the south-western and south-eastern floras are highly distinctive: relatively few species span both sides of the continent. Despite evidence of contact between the two biomes in the past, the disjunction of floras at the species level indicates subsequent isolation or barriers to contact (Mast and Givnish 2002, Crisp *et al.* 2004) and independent diversification of the two floras.

## 1.3 Elaeocarpaceae

Elaeocarpaceae is one of six families comprising the order Oxalidales (APG 2003), the others are: Brunelliaceae, Cephalotaceae, Connaraceae, Cunoniaceae and Oxalidaceae. Most members of the family are large rainforest trees, or less often shrubs, that are widely distributed throughout the tropical, sub-tropical and temperate regions of South America, Australasia, Southeast Asia and Madagascar. A southern origin for the family has been

hypothesised due to an absence of taxa from continental Africa, Europe and North America (Raven and Axelrod 1972). There are over 500 species within 12 genera, with the highest taxonomic diversity (nine genera) found in Australia. Seven genera are endemic to Australia: *Ceratium* DC., *Dubouzetia* Pancher ex Brongn & Gris, *Peripentadenia* L.S.Sm., *Platytheca, Sericolea* Schltr., *Tetratheca* and *Tremandra* and two small genera (*Crinodendron* Molina and *Vallea* Mutis ex L.f.) are endemic to South America (Crayn *et al.* 2006). There is an extensive fossil record for Elaeocarpaceae preserved as pollen, leaves and fruit (Baker *et al.* 1998). Rozefelds and Christophel (1996, p. 229) describe the genus *Elaeocarpus* L. as "a ubiquitous element of Tertiary floras of eastern Australia" on the basis of *Elaeocarpus*-type endocarps.

Molecular dating analyses by Crayn *et al.* (2006) estimate Elaeocarpaceae to be approximately 120 million years old, with the tremand lineage arising 37-64 mya. The origin of *Tetratheca* is estimated at 17-19 mya (crown age) and 37-39 mya (stem age) with major diversification occurring during the Miocene 6-7 mya, with rates of evolution much faster than their rainforest relatives (Crayn *et al.* 2006). This corresponds in timing with the rapid radiation of other sclerophyllous groups such as *Banksia* L.f., *Eucalyptus* L.Hér., two Fabaceae tribes Mirbeliae and Bossieae, and *Allocasuarina* L.A.Johnson (Crisp *et al.* 2004).

## 1.4 *Tetratheca* and allies

The placement of the former Tremandraceae family (*Tremandra, Platytheca* and *Tetratheca*) has long been controversial and it has been included in several different orders and suborders, e.g. Pittosporales, Polygalales, Lasiopetalae, and Geraniales. A detailed literature review of past familial and ordinal relationships is presented in Downing (2005). Affinity with Elaeocarpaceae has been hypothesised a number of times in the past (e.g. Nandi *et al.* 1998, Boesewinkel 1999, Bradford and Barnes 2001). Floral morphology indicative of a buzz pollination syndrome occurs across the tremand lineage supporting its placement within Elaeocarpaceae (Matthews and Endress 2002). The tremand lineage of Elaeocarpaceae is endemic to temperate regions of Australia, and species occur in all states but not in the Northern Territory. There are 52 currently recognised species in the three genera, *Tremandra, Platytheca* and *Tetratheca*, however this figure is likely to be an

underestimate: several newly discovered entities are yet to be described (R. Butcher, pers. comm. 2008).

In contrast to most other genera within the Elaeocarpaceae, the tremand lineage is represented by small perennial sub-shrubs, ranging from tiny procumbent plants to small shrubs, sometimes lax or trailing. *Tetratheca* is the most species rich genus consisting of 48 currently recognised species. *Platytheca* and *Tremandra* (each comprising two species) are endemic to south-western Western Australia while *Tetratheca* occurs widely across south-western and south-eastern Australia. There is an absence of species from the Nullarbor Plain, and no species occur on both sides of the continent.

Thompson's (1976) treatment of *Tetratheca* described the majority of the species and offered the first hypotheses of species relationships based on an intuitive assessment of morphology. Her proposed species relationships were based primarily on ovule number, unusual floral characters and hair types (particularly stem indumentum). Thompson (1976) proposed nine groups of closely related species based on shared morphological traits and suggested that affinities within these groups warrant further investigation. Downing (2005) tested Thompson's species concepts with a phenetic analysis and provided the first cladistic analysis of the genus based on morphology.

Until recently little was known about the ecology of *Tetratheca.* A study of a newly discovered population of *Tetratheca juncea* (Payne 1993) was the first to assess the habitat and conservation requirements of this species, listed as vulnerable under the Threatened Species Conservation Act, 1995. Studies of floral structure and the breeding system of *T. juncea* (Bartier *et al.* 2001, Gross *et al.* 2003) indicate that autogamy is rare and pollinators probably play an important role in the reproductive success of the species. The flowers of *Tetratheca* are consistent with a buzz pollination syndrome, however for a long time pollinators of many species eluded observers. In 2003, two species of native bee were observed visiting the flowers of *T. juncea* and buzz pollination was confirmed (Driscoll 2003). The fruits of *Tetratheca* have small locules that dehisce as they dessicate and release one to four seeds (occasionally five). The seeds have a chalazal appendage that functions as a food source for ants which collect, disperse and bury the seeds (Boesewinkel 1999, Bartier *et al.* 2001). Examples of *Tetratheca* flowers and growth habit are shown in Figure 1.1 and Figure 1.2.

**Figure 1.1 Examples of *Tetratheca* flowers – *Tetratheca rupicola* (left) and *Tetratheca ericifolia* (right)**

**Figure 1.2 Examples of *Tetratheca* habit, showing different substrates – *Tetratheca pilosa* (left) and *Tetratheca bauerifolia* (right)**

## 1.5 Biogeography and endemism in *Tetratheca*

A key characteristic of the Australian flora is the high levels of narrow endemism of many plant taxa. Distribution patterns of *Tetratheca* are similar in both western and eastern Australia: few species are widespread (albeit sometimes in disjunct populations) but the majority are localised endemics and many are rare or endangered. There are very few widespread taxa in the genus, with only seven species occurring in more than one state of Australia (Thompson 1976, Gardner and Murray 1992, Jeanes 1999, Downing 2005, Australia's Virtual Herbarium). Crisp *et al.* (2001) described eleven centres of high richness and endemism in the Australian flora (an area of endemism is defined as the smallest area to which at least two taxa are restricted in distribution). Areas of species richness and endemism for *Tetratheca* correspond with four of their centres: South West Western Australia; Adelaide-Kangaroo Island (South Australia); Sydney Sandstone (New South Wales); and Tasmania. Two species are also found in the Australian Alps (Victoria), however neither is endemic to the area. Approximately 32% of the species of *Tetratheca* were listed as rare or threatened by Walter and Gillett in 1998. Nine new species have been described since then (Butcher and Sage 2005, Bull 2007, Butcher 2007a, Butcher 2007b, Butcher 2007c), and all of them have been listed as Priority or Declared Rare Flora for Western Australia (Butcher 2007b).

Thompson (1976) proposed a biogeographical scenario for *Tetratheca* based on an assessment of morphological characters. She suggested that due to a higher number of genera and a greater variety of morphological characters present in Western Australia, a western origin for *Tetratheca* is likely. Thompson's (1976) hypothesis requires the assumption that the three genera are monophyletic and that long distance dispersal from west to east has occurred in *Tetratheca*, followed by diversification of the two floras in isolation. She also suggested that Kangaroo Island was colonised from both sides at a later date since the two endemic species seem to have separate affinities: *Tetratheca halmaturina* more closely related to leafless Western Australian species such as *T. harperi*; and *T. insularis* more closely related to *T. pilosa* in eastern Australia.

An alternative explanation is that the separation of western and eastern species is the result of a vicariant event. This scenario assumes that a formerly widespread ancestral lineage

has been split into two as a result of climatic or environmental change, and each has diversified independently (Ladiges 1998). Crisp and Cook (2007) studied the divergence times of south-western and south-eastern Australian floras across multiple plant lineages with disjunct distributions. They found that divergence times for several lineages correspond with the formation of the Nullarbor Plain, which continues to be a substantial barrier to migration of species between the south-west and south-east sides of the continent. Downing *et al.* (2008) propose that vicariant events have had a profound influence on the distribution of *Tetratheca.* A molecular phylogeny is required for the genus to test these biogeographical scenarios, particularly since a cladistic analysis of morphological characters (Downing 2005) was poorly resolved.

## 1.6 Approaches and molecular tools

The present study utilises three molecular approaches to investigate the processes driving speciation in *Tetratheca* at a variety of taxonomic and temporal scales. The first approach is to infer molecular phylogenies using plastid and nuclear sequence data to reconstruct relationships at the genus level. The second approach uses chloroplast microsatellites to investigate relationships at the species and population levels. The third is a nuclear microsatellite study at the population level. Comparative studies across a range of closely related taxa using each of these approaches will determine points of congruence and conflict among data retrieved by the various molecular tools. This will highlight scenarios in which reliance on a single species or a single molecular approach could lead to incorrect interpretations of evolutionary hypotheses and conversely allow greater confidence in the patterns and relationships proposed.

### 1.6.1 Phylogeny

Phylogenetic analyses use morphological or molecular data to construct patterns of ancestor-descendent relationships. Phylogenetic inferences based on morphological datasets often contain high levels of homoplasy or convergent evolution of many traits. Resolution can be poor, and clade support is often weak. Molecular sequence datasets generally contain a large number of characters (many more than morphological datasets)

and therefore resolution and clade support tend to be stronger and homoplasy can be less influential over-all. Either, phylogenies can be reconstructed using both types of data, or morphological traits can be mapped onto molecular phylogenies to test which traits are most informative.

There are many markers available for molecular phylogenetic inference. Choosing those that vary at the appropriate taxonomic level requires finding a balance between rates of mutation that are high enough to detect variation between species but low enough that unambiguous alignment can be achieved. Phylogenetic studies of plants most often use chloroplast DNA (cpDNA) genes and non-coding regions for studies at various taxonomic levels. Single copy genes and non-coding regions of nuclear DNA (nrDNA) or mitochondrial DNA (mtDNA) are also used.

There are many factors that can cause conflict between independent data sources, and congruence is frequently not achieved. Low resolution or conflict between uniparentally and biparentally inherited data sources can indicate incomplete lineage sorting (ILS) or hybridisation events. ILS is described as the persistence of ancestral polymorphisms through multiple speciation events (Avise 1994, Avise 2000). Hybridisation, or interspecific geneflow, results in shared chloroplast haplotypes among the maternal parent and hybrid offspring (McKinnon *et al.* 2001, McKinnon *et al.* 2004a, McKinnon *et al.* 2004b, Heuertz *et al.* 2006), and therefore patterns differ between chloroplast and nuclear data. Both evolutionary processes often occur in recently diverged lineages and can affect the accuracy of phylogenetic inferences. At the phylogenetic level it is often impossible to distinguish between them. An understanding of the distribution of molecular variation within and between species can help to elucidate which of these factors is affecting phylogeny inferences.

### 1.6.2 Phylogeography

Phylogeography brings together phylogenetics, biogeography and population genetics to investigate influences of historical and evolutionary processes on the distribution of populations and species (Avise 2000, Byrne 2007). Chloroplast DNA has been widely used to investigate phylogeographic patterns in plants. It is uniparentally (usually maternally)

inherited, has restricted gene flow and does not recombine. It can therefore be used to construct lineage genealogies which can then be analysed in a spatial context (Schaal *et al.* 1998, Byrne 2007). These kinds of studies have revealed the postglacial recolonisation routes of many European plants including, for example, *Quercus* (Petit et al. 2002, Magri et al. 2007), *Fraxinus* (Heuertz et al. 2004) and *Pinus* (Afzal-Rafii and Dodd 2007). Phylogeographic studies of Australian plants have appeared in the literature over the past decade (Steane *et al.* 1998, Jackson *et al.* 1999, McKinnon *et al.* 2001, Byrne *et al.* 2002, McKinnon *et al.* 2004a, McKinnon *et al.* 2004b) however most have focussed on large, long-lived trees. This study represents the first comparative phylogeographic study of herbaceous-shrubby, Australian endemic plants with short generation times.

Comparative phylogeography can be particularly powerful since congruent patterns found among different lineages may indicate the presence of vicariance. In this way it is possible to distinguish between patterns arising from a common environmental history (irrespective of taxonomy) and those resulting from stochastic change. In 1998, Taberlet *et al*. presented one of the earliest comparative phylogeographic studies of a range of plants and animals in Europe in order to better understand postglacial colonisation routes in Europe. Since then the field has expanded rapidly and many phylogeographic studies now use a comparative approach.

Recent studies have used chloroplast microsatellites (sometimes referred to as simple sequence repeats and abbreviated here as cpSSRs) to infer phylogenetic patterns at the species and population levels (e.g. Cavers *et al.* 2003, Heuertz *et al.* 2004, Kang *et al.* 2007, Fady *et al.* 2008). Multiple loci can be considered as single haplotypes since the loci are linked in the non-recombining chloroplast genome. Data can then be analysed cladistically to retrieve networks of multiple trees, and related haplotypes can be visualised according to geographic locations. In addition, cpDNA can be used to identify past introgression or hybridisation, as demonstrated in phylogeographic studies of the Tasmanian eucalypts (McKinnon *et al.* 2001, McKinnon *et al.* 2004b, Rathbone *et al.* 2007). In these studies extensive chloroplast sharing was more strongly correlated with geographic distribution than morphologically defined species. Such patterns occur through continued backcrossing of hybrid offspring with the parent species and can lead to individuals having an identical chloroplast genome to the maternal parent, and a nuclear genotype of the pollen parent (Avise 1994).

### 1.6.3 Population genetics

Population genetics studies focus on genetic variation within and among populations as well as the geographical structure of genetic variation at different spatial scales. Levels of genetic diversity and patterns of geographic structure of genetic variation are determined by both historical and current processes. An understanding of population genetic structure can elucidate paths of contemporary gene flow within and among populations and thus provide insight into evolutionary changes in species over time. Potential for evolutionary change (including speciation) is based on genetic variation and gene flow between subpopulations: if gene flow becomes inhibited, either by geographical or ecological barriers, populations will be divided into smaller units, which evolve independently. Consideration of genetic variation is therefore imperative for successful management and conservation of rare species, which are often narrowly distributed and at risk of extinction.

Nuclear microsatellite markers (nrSSRs) are popular for studies at the population level. They undergo simple Mendelian inheritance, are co-dominant and have a high mutation rate, thus enabling identification of homozygosity or heterozygosity. Nuclear SSRs are regions of the genome comprising short tandem nucleotide repeat sequences (1-6 bases). The high mutation rate is thought to be due to unequal crossing over or 'slippage' during DNA recombination leading to a variable number of repeat units (Frankham 1995, Page and Holmes 1998, Gaggiotti *et al.* 1999). Nuclear SSRs are abundantly dispersed throughout the genome and their high mutation rate and resultant high degree of polymorphism make them useful molecular markers for estimating genetic diversity, gene flow and spatial structure, particularly at the population level (Avise 1994, Zhang and Hewitt 2003, Gaudeul and Till-Bottraud 2008).

Use of the Polymerase Chain Reaction (PCR) for amplification of nrSSRs means they are relatively quick, cheap, repeatable and easy to apply to large sample numbers. Initial characterisation of the loci, however, can be time consuming and costly. Microsatellite libraries that are transferable across a range of related species are therefore particularly useful (Rossetto 2001). Compared with many other techniques used for population studies, e.g. restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD), SSRs detect higher rates of polymorphism (Frankham *et al.* 2002).

Nuclear microsatellites have been widely applied to studies of Australian plants, particularly for investigating rainforest refugia and population expansion and contractions as a result of glacial cycles (Rossetto *et al.* 2004, Taylor *et al.* 2005, Rossetto *et al.* 2007, Rossetto *et al.* 2008b). Phylogenetic relationships at the population level are not easily determined using nrSSRs due to the high level of homoplasy. Nevertheless with careful interpretation nrSSRs can provide useful evidence for delimitation of species through comparisons of levels of differentiation (Drummond and Hamilton 2007).

### 1.6.4 A hierarchical approach for exploring genetic diversity and distribution patterns

Population level studies are critical to understanding speciation and the distribution of organisms in the landscape because they operate at the junction of macro-evolutionary (genus level) and micro-evolutionary (population level) processes. Diniz-Filho *et al.* (2008) evaluated the interaction of phylogeography, molecular ecology, ecological genetics, geographical genetics, landscape genetics and conservation genetics with biogeography and concluded that a cross-disciplinary approach is likely to be the best way to understand biological distribution patterns at the population level. They argue that since evolutionary processes affect genetic variation at various hierarchical levels and also in a geographic context a single approach is likely to be inadequate. Many recent studies have used a combination of nuclear, mitochondrial and chloroplast markers for studies of population structure and phylogeography in plants and animals (Pope *et al.* 2000, Lexer *et al.* 2005, Jakob and Blattner 2006, Bottin *et al.* 2007e.g. , Edh *et al.* 2007, Kang *et al.* 2007). This cross-disciplinary approach has as yet only been applied to a small number of population level studies of Australian native plants (e.g. Jones *et al.* 2006, Rathbone *et al.* 2007).

## 1.7 Project aims

The primary purpose of this project is to investigate speciation processes in the Australian endemic genus *Tetratheca.* The specific aims toward reaching a broader understanding of the evolutionary history and current processes shaping *Tetratheca* are to:

1. construct the first detailed molecular phylogeny of the genus using plastid and nuclear data to determine relationships between species and investigate (cladistically) biogeographic patterns and possible origins of radiations of lineages

2. investigate phylogeographic patterns within two *Tetratheca* species complexes with uncertain taxonomy, a range of widespread and narrowly distributed taxa, and a wide environmental gradient to look for geographical structure of genetic diversity and identify possible hybridisation events in the evolutionary history of *Tetratheca*

3. examine the population genetics of two closely related species of *Tetratheca* to determine whether current distributions reflect phylogeographic patterns, and to explore possible hybridisation between a widespread, common species and a rare endemic species

4. explore the benefits of a hierarchical approach to investigating genetic diversity

# *Chapter 2* Molecular phylogenetics of *Tetratheca* (Elaeocarpaceae)

## 2.1 Introduction

The relationship between the rainforest elaeocarps and *Tremandra*, *Platytheca* and *Tetratheca* provides an ideal opportunity for investigating the effects of historical processes influencing the evolution of plants characteristic of a broad range of Australian vegetation types. Comparisons of lineages with different life history traits and different ages of diversification gives a broader understanding of the evolutionary history of the Australian flora. This study aims to elucidate evolutionary processes within this dry-adapted lineage, and to provide a basis for comparative studies with rainforest lineages in the Elaeocarpaceae in the future.

There are currently 48 recognised species of *Tetratheca* and two species each of *Platytheca* and *Tremandra* (Thompson 1976, Alford 1995, Jeanes 1996, Butcher and Sage 2005, Bull 2007, Butcher 2007a, Butcher 2007b, Butcher 2007c). *Platytheca* and *Tremandra* are restricted to south-western Western Australia whereas *Tetratheca* is distributed widely throughout south-western Australia and, in the east, from southern Queensland to Tasmania and into South Australia, including Kangaroo Island (Thompson 1976). The known range of the Western Australian species has been extended to the north with the discoveries of *Tetratheca chapmanii* from the Carnarvon Range, Little Sandy Desert (Alford 1995) and *Tetratheca fordiana* from the Pilbara region in Western Australia (Butcher and Sage 2005). Recently, nine new species from south-west Western Australia have been described (Bull 2007, Butcher 2007b, Butcher 2007c, a). There are also several putative new species from Western Australia that remain to be described, including two new species of *Platytheca* (R. Butcher, pers. comm. 2008).

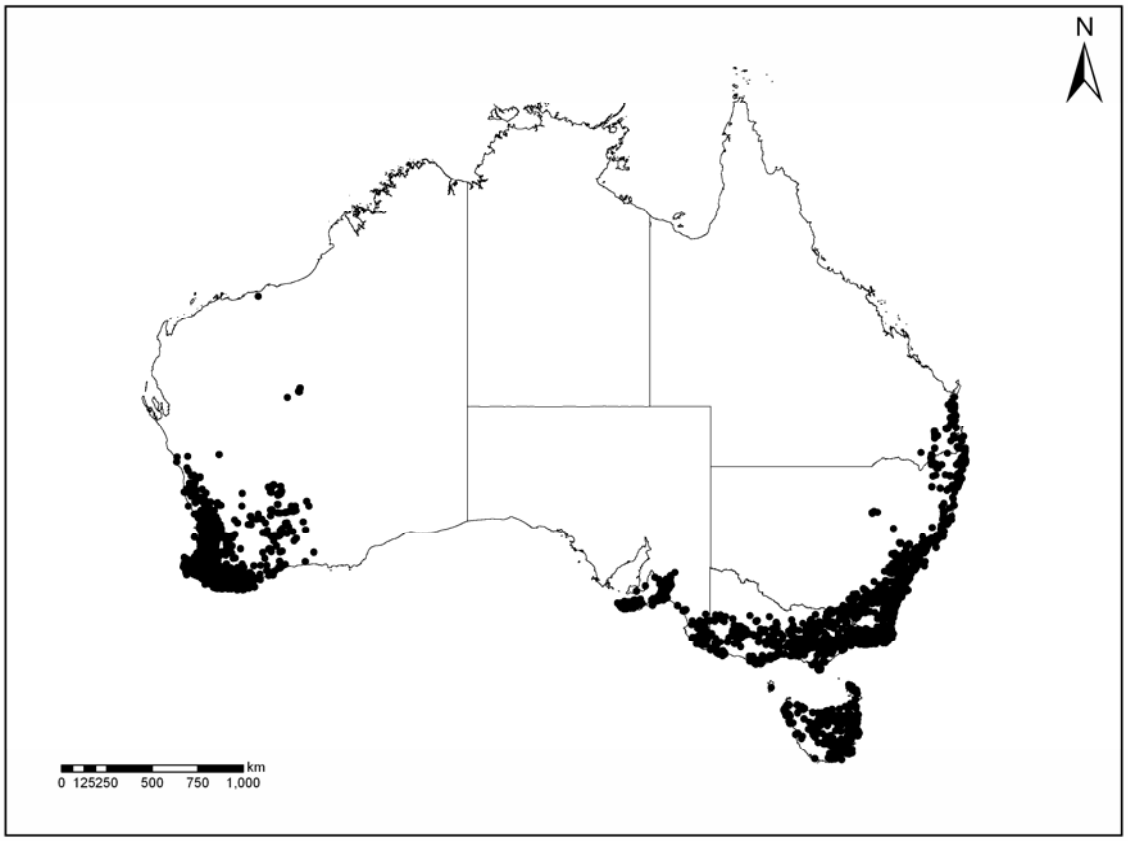



**Figure 2.1 Approximate distribution of *Tetratheca*, *Platytheca* and *Tremandra* generated using collection data from the following herbaria: AD, BRI, CANB, HO, MEL, NE, NSW, PERTH. All maps in the present study were compiled in ArcMap (Version 9.1)**

There is a distinct absence of taxa around the Great Australian Bight and no species occurs in both eastern and western states (Thompson 1976, Alford 1995, Jeanes 1996). Two species, *Tetratheca halmaturina* and *T. insularis*, are endemic to Kangaroo Island. Thompson (1976) suggested that the closest relative to *T. halmaturina* is probably *T. harperi*, a leafless species from outback Western Australia. *Tetratheca insularis* appears to be more closely related to the eastern species *T. pilosa*. Thompson's (1976) treatment provides a solid morphological basis for understanding the taxonomy of this group but she has pointed out that there are problems delimiting taxa in several groups. On the basis of morphological similarity she highlighted the following small groups of taxa whose relationships to one another and other species of *Tetratheca* require further investigation:

1. *T. affinis*, *T. efoliata* and *T. retrorsa*
2. *T. aphylla* and *T. paucifolia*
3. *T. halmaturina* and *T. harperi*
4. *T. hispidissima* and *T. hirsuta*

5. *T. confertifolia* and *T. filiformis*
6. *T. glandulosa* and *T. labillardierei*
7. *T. stenocarpa* and *T. ciliata*
8. *T. rupicola* and *T. thymifolia*
9. *T. pilosa*, *T. gunnii*, *T. neglecta*, *T. rubioides* and *T. insularis*

Affinities of taxa that have been described since Thompson's (1976) treatment also require investigation. The first phenetic and cladistic analyses of *Tremandra*, *Platytheca* and *Tetratheca* based on morphological data was that of Downing (2005). Her study provided a set of hypotheses of relationships within and between these genera that will be tested against a molecular dataset in the present study. Downing's (2005) study supported most of the species boundaries described by Thompson in 1976. Two exceptions are *Tetratheca ciliata* and *T. labillardierei,* each consisting of several distinct clusters of morphological data. Downing (2005) also found considerable overlap between subspecies of *Tetratheca pilosa* and *T. procumbens.* She concluded that the subspecies are probably artificial and that the status of *T. procumbens* requires further investigation.

In Thompson's (1976) revision of *Tetratheca*, she argued that the "greater number of characters in Western Australia, as well as the presence of two other genera sharing some of these characters points to a western origin [for *Tremandra*, *Platytheca* and *Tetratheca*]". This was the first explicit hypothesis for the biogeography of the tremand lineage based on the numbers of different character states in the west versus east of Australia. Thompson's (1976) hypothesis will be tested cladistically by the present molecular study. Utilising a cladistic framework polarises characters, indicating evolutionary directionality, and therefore a more reliable interpretation of biogeographic scenarios.

Previous molecular work on the group (Crayn *et al.* 2006, Butcher 2007a) has suggested relationships such as monophyly of the three genera, and the existence of a clade comprising all the sampled eastern species of *Tetratheca* nested within a grade of western species. These conclusions were based on limited taxon sampling and therefore require testing against a more extensive dataset. This chapter provides the most detailed molecular phylogeny yet for tremand lineage of Elaeocarpaceae.

The specific questions this chapter addresses are:

1. Is *Tetratheca* monophyletic?
2. Do the eastern Australian species form a clade?
3. Is Thompson's (1976) suggestion of a western Australian origin for the group supported by cladistic analyses?
4. Do the Kangaroo Island species represent the overlap of taxa with eastern and western affinities?
5. Are the morphological groupings suggested by previous research supported by molecular data?

## 2.2 Materials and Methods

### 2.2.1 Taxa and plant material

Sampling aimed to represent as much of the known diversity of the genera *Tremandra*, *Platytheca* and *Tetratheca* as possible. Plant material for the present study was fresh or preserved in silica gel for all individuals with the exception of two (*Platytheca juniperina* and *Tetratheca affinis*) that were preserved in CTAB (Thomson 2002).

A total of 72 individuals representing 41 formally described *Tetratheca* species (including three with two subspecies each), four putative new species, both species of *Platytheca* and both species of *Tremandra* were obtained from wild populations (Table 2.1). Two individuals whose species identities could not be verified because voucher specimens could not be located are included in the analysis. One is *Tetratheca* ?*hirsuta* which had been identified as *T. hirsuta* and *T. pubescens* in the past and the other is *T.* ?*setigera* which had been databased as "*T. setigera* or *T. hirsuta".* There are also several individuals with informal names included in the present study as shown in Table 2.1. These are from newly discovered collecting localities in Western Australia and have not yet been assigned to existing or new species. Among them is *T.* ?*spartea* which had previously been recognised as *Tetratheca nuda* var. *spartea* by Bentham (1863), a concept formally discarded by Thompson in 1976. She commented that it may represent a new species

closely related to *T. nuda* or a hybrid between *T. nuda* and possibly *T. hirsuta.* Recently rediscovered in Western Australia it is now considered to be a separate species but has not yet been recircumscribed (R. Butcher, pers. comm. 2008).

Species of *Platytheca* and *Tremandra* were selected as outgroups on the basis of previous studies (Downing 2005, Crayn *et al.* 2006) that had demonstrated that *Tremandra*, *Platytheca* and *Tetratheca* form a monophyletic group within Elaeocarpaceae. *Tetratheca* was also resolved as monophyletic (although sampling was limited) and sister to *Platytheca* by Crayn *et al.* (2006). These two genera form a clade sister to *Tremandra*. Downing's (2005) morphological analysis also resolved *Tremandra* as sister to a clade of *Tetratheca* and *Platytheca,* however *Platytheca* was embedded within *Tetratheca.*

### 2.2.2 Nuclear and chloroplast data

The internal transcribed spacer (ITS) region (comprising ITS1, 5.8S and ITS2) of the nuclear ribosomal DNA and the *trn*L-*trn*F region (including the *trn*L intron and the *trn*L-*trn*F spacer) of plastid DNA were chosen to provide independent molecular estimates of the phylogeny. Inheritance of the chloroplast genome is maternal in the great majority of non-coniferous plants (Dowling *et al.* 1996), while nuclear DNA is biparentally inherited. A comparison of phylogenies based on nuclear and chloroplast data helps to identify possible evolutionary reticulation events, and assists in resolving relationships where data from single regions are insufficient (Comes and Abbott 2001, Linder and Rieseberg 2004). Selection of these molecular regions for sequencing was based on previous studies on Elaeocarpaceae and other comparable groups. These regions proved to be informative on the relationships between species (e.g. Murphy *et al.* 2000, Bradford and Barnes 2001, Berry *et al.* 2005, Yulita *et al.* 2005, Crayn *et al.* 2006).

**Table 2.1 Voucher information**

State/area abbreviations: Flinders Island (FI), New South Wales (NSW), South Australia (SA), Tasmania (TAS), Victoria (VIC), Western Australia (WA); Herbarium accession abbreviations: National Herbarium of New South Wales (NSW), Western Australian Herbarium (PERTH), National Herbarium of Victoria (MEL); sequenced (+); not sequenced (-); sequence obtained from R. Butcher (PERTH) (#); sequenced by D. M. Crayn (NSW) (*); sequenced by P. D. Rymer (NSW) (**); Two sequences of *Tetratheca labillardierei* (T1, T2); sequences downloaded from Genbank are indicated by Genbank accession numbers.

| Species | Locality | State/area | Collector and number | Herbarium accession | ITS | *trn*L-trnF |
|---|---|---|---|---|---|---|
| *Tetratheca affinis* Endl. | Unicup Road, 100 m north of intersection with Wingebellup Road, Kulunilup Nature Reserve | WA | Andrew J. Perkins WA 30 | NSW 709180 | + | + |
| *Tetratheca angulata* R.Butcher | Brand Highway at junction of McNamara Rd, south of Badgingarra | WA | D. M. Crayn 648 | NSW 613140 | + | + |
| *Tetratheca aphylla* subsp. *megacarpa* R.Butcher | SE of Newdegate | WA | R. Butcher TAN_12 | To be lodged at PERTH | # | - |
| *Tetratheca aphylla* F.Muell. subsp. *aphylla* | Bungalbin Hill | WA | R. Butcher TAB_2.6 | To be lodged at PERTH | # | AY237267 |
| *Tetratheca applanata* R.Butcher | From Floater Road, turn left along the track 0.6 km along Archer Drive, past transmission tower to next hill crest | WA | R. Butcher, J.A. Wege, N. Gibson 1206 | To be lodged at PERTH | + | + |
| *Tetratheca bauerifolia* F.Muell. Ex Schuch | Kinglake National Park; on Mt Slide Rd; 1.65 km from the junction of Mt Slide Rd with Steele's Creek Rd and Greenwood Lane | VIC | T Downing, G Downing, V Downing TD38 | To be lodged at MEL | + | + ** |
| *Tetratheca ciliata* Lindl. | Kinglake - Healesville Road, 1.5 km S of Kinglake, edge of Kinglake National Park | VIC | H. McPherson, Anton J. Perkins KH 4 | NSW 807732 | + | - |
| *Tetratheca ciliata* Lindl. | Kinglake National Park; on Mt Slide Rd; 400 m from the intersection of the Mt Slide and Kinglake - Healesville roads | VIC | T Downing, G Downing, V Downing TD33 | To be lodged at MEL | + | + ** |
| *Tetratheca confertifolia* Steetz | Great Northern Highway, 22.8 km north of Bindoon | WA | D. M. Crayn 722 | NSW 700730 | + | + ** |

| Species | Locality | State/area | Collector and number | Herbarium accession | ITS | *trn*L-trnF |
|---|---|---|---|---|---|---|
| *Tetratheca decora* Joy Thomps. | Fans Horizon, Warrumbungles | NSW | H. McPherson, G.M. Towler, S. Skinner W1 | NSW 715144 | + | + |
| *Tetratheca efoliata* F.Muell. | Precise locality unknown | WA | R. Davis s.n. 10/4/96 | PERTH 06208231 | + | + |
| *Tetratheca ericifolia* Sm. | Bay Road, Berrilee (residence of Jocelyn Howell) | NSW | J. Howell s.n. | NSW 619997 | + | + ** |
| *Tetratheca erubescens* J.P.Bull | Koolyanobbing Forest deposit | WA | P. Butcher Te12 | To be lodged at PERTH | + | + |
| *Tetratheca exasperata* R.Butcher | 3 km along Hughes Mill road from Boyup Brook-Arthur Road | WA | R. Butcher, J. A. Wege 1233 | PERTH 07719906 | + | + |
| *Tetratheca filiformis* Benth. | c. 2-2.5 km E of Chesapeake Road on Pingerup Road, SE Northcliffe | WA | R. Butcher 966 | NSW 650574 | + | + |
| *Tetratheca glandulosa* Sm. | Booralie Namba Trail, Duffys Forest | NSW | H. McPherson, L. J. Murray, L. L. Lee DFg 1 | NSW 728290 | + | + |
| *Tetratheca gunnii* Hook.f. | Cultivated: Royal Tasmanian Botanic Gardens, Hobart. Wild source: Tattersalls Road, Harford | TAS | N. Papworth s.n. | NSW 725119 | + | + |
| *Tetratheca halmaturina* J.M.Black | Mays Road, Kangaroo Island | SA | H. McPherson, D. M. Crayn, P. Pisanu MR 16 | NSW 725115 | + | + |
| *Tetratheca harperi* F.Muell. | Mount Jackson | WA | R. Butcher TH_2.5 | To be lodged at PERTH | # | AY237278 |
| *Tetratheca aff. hirsuta* | Mount Cooke, 2.5 km N of Albany Highway along track below powerlines | WA | R. Butcher 1237 | PERTH 07719884 | + | + |
| *Tetratheca hirsuta* Lindl. (pink) | 3.6 km along Teetree Road from Mooliabeene Road, Chittering Shire | WA | R. Butcher, F. Hort, J. Hort 1175 | PERTH 07719973 | + | + |

| Species | Locality | State/area | Collector and number | Herbarium accession | ITS | *trn*L-trnF |
|---|---|---|---|---|---|---|
| *Tetratheca hirsuta* Lindl. (white) | 3.6 km along Teetree Road from Mooliabeene Road, Chittering Shire | WA | R. Butcher, F. Hort, J. Hort 1174 | PERTH 07719981 | + | + |
| *Tetratheca ?hirsuta* | On the Albany Hwy, 28 km north of North Bannister | WA | T. Downing TD39 (P.Ladd) | To be lodged at MEL | + * | + ** |
| *Tetratheca hispidissima* Steetz | 1.1 km W of Tinglewood Road on Shedley Driver, SW Walpole | WA | R. Butcher 964 | NSW 650563 | + | + |
| *Tetratheca insularis* Joy Thomps. | Bark Hut Road 3 Kangaroo Island | SA | H. McPherson, D. M. Crayn, P. Pisanu BHR 3 | NSW 723913 | + | + |
| *Tetratheca juncea* Sm. | Cultivated. Wild source: Vales Point Power Station | NSW | M. Rossetto s.n. | NSW 762959 | + * | + ** |
| *Tetratheca labillardierei* Joy Thomps. | East Bagdad Road, 8.5 km from highway | TAS | H. McPherson, Anton J. Perkins BD 13 | NSW 807733 | + | + T1 |
| *Tetratheca labillardierei* Joy Thomps. | Cultivated: Royal Tasmanian Botanic Gardens, Hobart. Wild source: Waterworks Reserve | TAS | N. Papworth s.n. | NSW 725118 | - | + T2 |
| *Tetratheca labillardierei* Joy Thomps. | 2.3 - 2.6 km N of Clover Flats on Khancoban-Cabramurra road, Snowy Mountains | NSW | H. McPherson, Anton J. Perkins KCFl 16 | NSW 769156 | + | + |
| *Tetratheca labillardierei* Joy Thomps. | Mount Samaria State Park, Bushland near junction of Mount Samaria Road and Williams Road | VIC | H. McPherson, Kathi Downs SSP 4 | NSW 612219 | + | - |
| *Tetratheca neglecta* Joy Thomps. | Royal National Park | NSW | H. McPherson R 7 | NSW 712857 | + | + |
| *Tetratheca nepheliodes* R.Butcher | Eneabba area | WA | R. Butcher TAE_2.10 | To be lodged at PERTH | # | AY237271 |

| Species | Locality | State/area | Collector and number | Herbarium accession | ITS | *trn*L-trnF |
|---|---|---|---|---|---|---|
| *Tetratheca nuda* Lindl. | Darling, Mundaring Weir area, South Ledge, 100 m along track from car park, exposed area | WA | D. M. Crayn, A. Spooner, K. Lemson 731 | NSW 700771 | + | + |
| *Tetratheca parvifolia* Joy Thomps. | 800 m NE along Wade Road from Brookhampton Road (Brookhampton Locality) | WA | R. Butcher 916 | NSW 650561 | + * | + |
| *Tetratheca paynterae* Alford subsp. paynterae | Windarling Range | WA | R. Butcher TW_1.5 | To be lodged at PERTH | # | AY237276 |
| *Tetratheca paynterae* subsp. cremnobata R.Butcher | Windarling Range | WA | R. Butcher TDH_5 | To be lodged at PERTH | # | AY237273 |
| *Tetratheca phoenix* R.Butcher | Mount Cooke, edge of Bibbulmun Track | WA | R. Butcher 1236 | PERTH 07719892 | + | + |
| *Tetratheca pilifera Lindl.* | Darling, Walyunga National Park, Hillslope behind Ranger's residence, Walyunga National Park | WA | R. Butcher 922 | NSW 650569 | + | + |
| *Tetratheca pilosa* subsp. *Latifolia* Joy Thomps. | Junction of Mottle Range Road and Tara Range Road S of Buchan | VIC | H. McPherson, Anton J. Perkins TR 1 | NSW 612731 | + | + |
| *Tetratheca pilosa* subsp. *latifolia* Joy Thomps. | Cultivated: Royal Tasmanian Botanic Gardens, Hobart. Wild source: Five Mile Road | FI | N. Papworth, s.n. Flinders Island | NSW 725120 | + | + |
| *Tetratheca pilosa* subsp. *latifolia* Joy Thomps. | Junction of Mottle Range Road and Tara Range Road S of Buchan | VIC | H. McPherson, Anton J. Perkins TR 4 | NSW612749 | + | - |
| *Tetratheca pilosa* subsp. *pilosa Labill.* | 5 km along Mosquito Hill Road from Willunga-Goolwa turn off | SA | H. McPherson, D. M. Crayn MH 1 | NSW 723878 | + | + |
| *Tetratheca pilosa* subsp. *pilosa Labill.* | Cultivated: Royal Tasmanian Botanic Gardens, Hobart. Wild source: Chimney Pot Hill, near top | TAS | N. Papworth s.n. | NSW 725121 | + | + |

| Species | Locality | State/area | Collector and number | Herbarium accession | ITS | *trn*L-trnF |
|---|---|---|---|---|---|---|
| *Tetratheca pilosa* subsp. *pilosa Labill.* | Nunamara to Lilydale road (C824), between Tasman highway and B81 from Launceston to Lilydale | TAS | H. McPherson, Anton J. Perkins NL 7 | NSW 807734 | + | + |
| *Tetratheca pilosa* subsp. *pilosa Labill.* | Nunamara to Lilydale road (C824), between Tasman highway and B81 from Launceston to Lilydale | TAS | H. McPherson, Anton J. Perkins NL 12 | NSW 807735 | + | - |
| *Tetratheca pilosa* subsp. *pilosa Labill.* | Rubicon Bridge, 20 m from bridge on road verge | TAS | H. McPherson, Anton J. Perkins RB 2 | NSW 807736 | + | - |
| *Tetratheca procumbens* Hook.f. | Road to Butlers Gorge, 5 km from highway turnoff 3 km South of Tarraleah | TAS | H. McPherson, Anton J. Perkins BG 3 | NSW 807737 | + | - |
| *Tetratheca procumbens* Hook.f. | Ida Bay State Reserve | TAS | H. McPherson, Anton J. Perkins, R. Greenhill IB 15 | NSW 807738 | + | - |
| *Tetratheca retrorsa* Joy Thomps. | Tutanning Nature Reserve, c. 50 m in from boundary track on SE side of N part of Tutanning Nature Reserve | WA | R. Butcher 929 | NSW 650573 | + * | + |
| *Tetratheca rubioides* A.Cunn. | Mount Boyce Lookout, Blue Mountains | NSW | H. McPherson, Anton J. Perkins MtB10 | NSW 613095 | + | + |
| *Tetratheca rupicola* Joy Thomps. | Murphy's Glen Blue Mountains | NSW | H. McPherson, Anton J. Perkins MG 8 | NSW 613532 | + | + |
| *Tetratheca ?setigera* | Stirling Range National Park, Stirling Range Drive | WA | D. M. Crayn, K. A. Kron, Andrew J. Perkins 702d | NSW 708005 | + * | + |
| *Tetratheca shiressii* Blakely | Royal National Park, At entrance to Curra Moors management trail on Sir Bertram Stevens Drive | NSW | D. M. Crayn, M. Rossetto 604 | NSW 613011 | + * | + ** |

| Species | Locality | State/area | Collector and number | Herbarium accession | ITS | *trn*L-trnF |
|---|---|---|---|---|---|---|
| *Tetratheca similis* Joy Thomps. | Take Metro Road for 6.1 km S of Brookton Highway, SW track | WA | D. M. Crayn, A. Spooner, K. Lemson 732 | NSW 700773 | + | + |
| *Tetratheca ?spartea.* | Anvil Block, Julimar, Shire of Toodyay. 2.1 km SSE-WSW along the southern boundary track from the western end of Beard Road | WA | R. Butcher, F. Hort, J. Hort 1179 | PERTH 07719590 | + | + |
| *Tetratheca* sp. Boonanarring (F. Hort 1509) | Boonanarring Nature Reserve | WA | R. Butcher, F. Hort, J. Hort 1165A | To be lodged at PERTH | + | + |
| *Tetratheca* sp. Granite (S. Patrick SP1224) | Berry Nature Reserve, Reen Road, Gidgegannup West, c. 300 m W of toilet block | WA | R. Butcher, F. Hort, J. Hort 1187 | To be lodged at PERTH | + | + |
| *Tetratheca aff. virgata/retrorsa* (Mundaring) | Reservoir road, Beraking, 13.8 km SE from Mundaring Weir-Kalamunda Road, west side of road | WA | R. Butcher, F. Hort, J. Hort 1182 | To be lodged at PERTH | - | + |
| *Tetratheca stenocarpa* J.H.Willis | Kurth Kiln Park; on Gembrook - Launching Place Rd; on either side of the clearing made for the Transmission Lines [electricity] | VIC | T Downing, G Downing, V Downing TD53 | To be lodged at MEL | + * | + ** |
| *Tetratheca stenocarpa* J.H.Willis | Old Chum Creek Road, off C72 highway from Kinglake to Healesville, approximately 2.5 km S of Toolangi | VIC | H. McPherson, Anton J. Perkins OCC 2 | NSW 807739 | + | - |
| *Tetratheca subaphylla* Benth. | Walking track to Mount Imlay (approximately 20 km SW of Eden) | NSW | H. McPherson, Anton J. Perkins MI 1 | NSW 614222 | + | + |
| *Tetratheca thymifolia* Sm. | 9.7 km NE of Mongarlowe (near Braidwood) | NSW | H. McPherson, D. McPherson M 2 | NSW 614487 | + | + |
| *Tetratheca virgata* Steetz | c. 800 m along boundary track of unnamed National Park (Wandoo Conservation Park) | WA | R. Butcher 928 | NSW 650572 | - | + |

| Species | Locality | State/area | Collector and number | Herbarium accession | ITS | *trn*L-trnF |
|---|---|---|---|---|---|---|
| *Platytheca galioides* Steetz | Stirling Range National Park, Stirling Range Drive | WA | D.M. Crayn, K. A. Kron, Andrew J. Perkins 701c | NSW 700478 | + | + |
| *Platytheca juniperina* | Scree slope on track up to Mount Toolbrunup peak, Stirling Ranges National Park | WA | Andrew J. Perkins s.n. | To be lodged at NSW | + | + |
| *Tremandra diffusa* R.Br. | 5 km W of Denmark on Walpole Road, c. 100 m E of Lapko Road | WA | R. Butcher 961 | NSW 650550 | + * | + |
| *Tremandra stelligera* R.Br. ex DC. | Walpole-Nornalup National Park, Car park at the entrance to the Treetop Walk, Valley of the Giants | WA | D. M. Crayn, K. A. Kron, Andrew J. Perkins 706 | NSW 700698 | - | +** |

### 2.2.3 DNA isolation

Total genomic DNA was extracted from silica-dried, CTAB-preserved or fresh plant material using DNeasy Plant Mini kits (Qiagen, Hilden, Germany) following the manufacturer's protocol. Tissue was disrupted in liquid nitrogen using a mortar and pestle or in AP1 lysis buffer (Qiagen) using the Qiagen Tissue Lyser. Attempts were also made to extract DNA from herbarium specimens for some of the remaining taxa but amplification of these was not successful. The causes of failure were not investigated experimentally, but DNA degradation of the specimens is likely to be a factor. Attempts by other researchers to isolate DNA from herbarium specimens of other Elaeocarpaceae have only rarely succeeded (D.M. Crayn pers. comm. 2005).

### 2.2.4 Amplification using Polymerase Chain Reaction (PCR)

PCR amplifications were performed in a CP2-03 Thermal Cycler (Corbett Research, Mortlake, Australia). Each 25 μL reaction contained 200 μM of each primer, 200 μM of each dNTP, 2.5 μL 10x NH4 buffer (Bioline, Luckenwalde, Germany), 1.5–3.0 mmol MgCl2, and 0.5 units BIOTAQ™ DNA polymerase (Bioline, Luckenwalde, Germany). Primers GN1 (Scott and Playford 1996) and ITS4 (White *et al.* 1990) were used to amplify the ITS region (Table 2.2). Reaction mixtures were incubated at 94ºC for 3 min then subjected to 30 cycles as follows: denaturation for 30 s at 94ºC; annealing for 30 s at 53, 54 or 55ºC; and extension for 1 min at 72ºC. After cycling, a 4 min incubation was performed at 72ºC.

Primers 'c' and 'f' from Taberlet *et al.* (1991) were used to amplify the *trn*L-*trn*F region (Table 2.2). The cycling profile above was also used for the *trn*L-*trn*F amplifications at an annealing temperature of 55ºC. All PCR products were electrophoresed on 1.5% agarose gels at 130V for 45 min, stained in an ethidium bromide bath and then visualised on an ultra-violet transilluminator. Purification of the PCR products was carried out using Jetquick columns (Genomed, Bad Oeynhausen, Germany) or ExoSAP-IT (Clevelend, Ohio, USA) according to the manufacturers' protocols.

Two distinct ITS products were obtained for *Tetratheca efoliata*. PCR products from one individual were cloned using the pGEM – T Easy Vector System (Promega, Madison, USA) following the manufacturer's instructions. An initial screen PCR was performed to test the success of the cloning procedures. Four colonies for each species were then selected and the DNA insert was amplified via PCR using M13 forward (5'-CGCCAGGGTTTTCCCAGTCAC-3') and reverse (5'-AGGAAACAGCTATGAC-3') primers manufactured by Sigma-Proligo.

**Table 2.2 Details of primers used for amplification of ITS and *trn*L-*trn*F regions in *Tremandra*, *Platytheca* and *Tetratheca***

| Region | Primer | Primer sequence 5' – 3' | Annealing temp. °C | Approx. size (bp) | Reference |
|---|---|---|---|---|---|
| ITS | GN1 | CGCGAGAAGTTCATTGAACC | 53, 54, 55 | 800 | Scott and Playford (1996) |
| | ITS4 | TCCTCCGCTTATTGATATGC | | | White *et al.* (1990) |
| *trn*L-*trn*F | c | CGAAAATCGGTAGACGCTACG | 55 | 1000 | Taberlet *et al.* (1991) |
| | f | ATTTGAACTGGTGACACGAG | | | Taberlet *et al.* (1991) |

## 2.2.5 Sequencing and alignment

Sequences of the ITS region were generated for 57 *Tetratheca* individuals (from approximately 40 species), both species of *Platytheca* and one of *Tremandra*. Sequences for an additional six individuals of *Tetratheca* from Western Australia were obtained from Ryonen Butcher (PERTH). For the *trn*L intron and the *trn*L-*trn*F spacer region, sequences were generated for 53 individuals of *Tetratheca* as well as both species of *Platytheca* and *Tremandra*. The remaining five *trn*L-*trn*F sequences were obtained from GenBank as indicated in Table 2.1.

For most individuals, forward and reverse sequences were obtained for both the ITS and *trn*L-*trn*F regions. Only forward ITS sequences were obtained for *Tetratheca pilosa* subsp. *pilosa* (from Tasmania) and *T. retrorsa*, and clean sequences could not be obtained for *T. labillardierei* (T2, from Tasmania), *T. virgata*, *T.* aff. *virgata*/*retrorsa* or *Tremandra stelligera*. Forward and reverse sequences for *T. rupicola* and *T. thymifolia* were alignable,

but contained a large number of ambiguities. Four cloned PCR products of *T. efoliata* were also sequenced and aligned. Sequence divergence among clones ranged from 2-16 base pair changes. Most occurred as single base changes, however one clone contained a five base insertion. Despite this divergence, preliminary analyses (not presented here) confirmed that the four clones formed a clade with 100% bootstrap support. As a result only one clone was included in the final alignment.

For *trn*L-*trn*F, clean sequence was only obtained in one direction (reverse) for *Tetratheca exasperata* and *T.* aff. *virgata*/*retrorsa*. Clean sequences could not be obtained for the following taxa: *T. aphylla* subsp. *megacarpa*; *T. ciliata* from east of Melbourne, Victoria; *T. labillardierei* (Victoria); *T. pilosa* subsp. *latifolia* from Tara Range, Victoria; *T. pilosa* subsp. *pilosa* from two localities in northern Tasmania; both individuals of *T. procumbens*; and *T. stenocarpa* from Old Chum Creek in Victoria.

Each 20 µL sequencing reaction mixture contained approximately 60 ng of purified template DNA, 1 µL of BigDye version 3.1 (Applied Biosystems, Foster City, California, USA), 1.5 µL of CSA buffer, 0.32 µL of 10 µM primer and purified water (to 20 µL). The reaction mixture was subjected to 25 cycles of: 10 s at 96ºC; 5 s at 50ºC; and 4 min at 60ºC, with a final incubation for 1 min at 60ºC. Sequencing products were purified by ethanol precipitation and dried in a vacuum centrifuge for 5-10 min. Sequences were then visualised using model 3730 capillary sequencing machines (Applied Biosystems, Foster City, California, USA) at the Automated DNA Sequencing Facility of the University of New South Wales (UNSW).

Electropherograms were viewed and edited by eye and consensus sequences for each sample assembled and aligned manually using BioEdit 7.0.1 (Hall 1999). Data were exported as NEXUS files and inferred parsimony informative indels were scored as two-state presence/absence characters with MacClade 4 (Maddison and Maddison 2000) or PAUP*4.0b10 (Swofford 2002) following the "simple" indel coding method described by Simmons and Ochoterena (2000). Bases 119-151 (33 nucleotide positions of the total of 990) of the ITS alignment were excluded from the phylogenetic analysis because the alignment showed considerable ambiguity in this section.

### 2.2.6 Phylogenetic analyses

There are three main approaches for inferring molecular phylogenies: distance methods, character-based and model-based methods. Distance methods calculate pair-wise distances between sequences and in doing so simplify the data by removing information about evolutionary changes at individual sites. An example of a distance method is Neighbour Joining, which is fast, simple and results in a single tree (Page and Holmes 1998). Maximum Parsimony (MP) analysis is a character-based method which searches for the tree or trees with the minimum number of character state changes (Felsenstein 2004). The most parsimonious (or simplest) explanation is that which requires the fewest mutations to explain relationships among taxa. A benefit of MP analysis is that it makes few assumptions about the data, however it will often produce a large number of slightly different, but equally parsimonious trees, particularly when the phylogenetic signal is low. Support for phylogenetic inferences using MP analyses can be assessed by resampling approaches such as bootstrap (Felsenstein 2004) and jackknife (Farris *et al.* 1996).

Model-based methods such as Maximum Likelihood (ML) and Bayesian analysis allow the user to specify an evolutionary model and test the data against it – with the assumption that the model applied appropriately describes the data. Modeltest (Posada and Crandall 1998) is a tool designed to test datasets against a set of sequence evolution models in order to select the most adequate. Even with the appropriate model a disadvantage of ML methods that produce a single tree is that they require extensive computer memory and time. This renders them unfeasible for studies with large numbers of sequences. Bayesian analysis is a modified ML method based on a prior probability of tree topologies. It produces a set of trees with approximately equal likelihoods rather than the single most likely tree (Page and Holmes 1998). Frequencies of clades retrieved by a Bayesian analysis are akin to probabilities and so interpretation is straight forward (Alfaro *et al.* 2003), and computational effort required is much less than standard maximum likelihood methods. Alfaro *et al.* (2003) tested bootstrapping and Bayesian Markov Chain Monte Carlo sampling with a simulation study to determine which was more reliable for assessing phylogenetic confidence. They found that Bayesian analyses retrieved correct relationships more often (and using fewer characters) than bootstrap and as a result posterior probability

values were higher. Provided the model chosen appropriately describes the data, Bayesian analyses are robust (Alfaro *et al.* 2003). Maximum parsimony, on the other hand is convenient because it does not depend on a user-defined model.

Selecting a method for constructing phylogenies is often a compromise between efficiency, computational requirements (speed and available memory), consistency and robustness (Page and Holmes 1998, Hall 2001). Many current studies of molecular data use maximum parsimony and Bayesian approaches and these methods have been selected for the present study. Choosing two or more methods, and two or more independent sources of data (e.g. ITS and *trn*L-*trn*F DNA sequence data in the present study) has advantages over selection of a single method. If outcomes are congruent we can have more confidence that the result more strongly reflects the underlying phylogeny than the inherent biases of any one particular method.

A potential problem in the interpretation of molecular data is base composition bias. If particular bases occur more frequently than others then some substitutions may also be more common than others (Page and Holmes 1998). Some phylogenetic analysis methods assume the frequencies of the four nucleotides to be approximately the same for all sequences. Therefore, a profound imbalance in base frequencies, if uncorrected, either across the dataset as a whole or in pair-wise comparisons can mislead phylogenetic analyses (Pettigrew 1994). Base compositional homogeneity was investigated using PAUP*4.0b10 (Swofford 2002) in order that the appropriate analysis methods could be applied. Mean base frequencies were found for each dataset and a chi-squared test for compositional homogeneity was performed across all taxa.

Maximum parsimony analyses were performed on each dataset using PAUP*4.0b10 (Swofford 2002). Following Fitch (1971) all nucleotide positions were treated as independent, unordered characters and were given equal weight. Heuristic searches were conducted with both indels included and excluded. Simple taxon addition sequence and tree-bisection-reconnection (TBR) branch swapping were applied. Branch lengths were determined under ACCTRAN optimisation. Gaps were treated as missing data and positions at which the base was ambiguous were treated as uncertain rather than polymorphic.

Analyses of each dataset retrieved a large number of optimal trees that exceeded the available computer memory, preventing the analyses from running to completion. A restricted heuristic search of 1000 iterations saving 100 trees per iteration was therefore applied to each dataset. All other criteria were kept the same. The CONDENSE option was chosen to remove identical trees and a strict consensus of all equally parsimonious trees found by the search was produced for each dataset. On the basis of the strict consensus trees produced, the following analyses were applied only to datasets that included indels.

Reverse constraint analyses were performed using PAUP*4.0b10 (Swofford 2002) to test the result achieved by the restricted parsimony analyses. The strict consensus tree for each dataset was loaded as a constraint tree and the analyses were set to find the shortest trees that were not compatible with it. Three thousand iterations of random taxon addition searching were performed and only 100 trees were saved per iteration to avoid memory overrun. All other search criteria were the same as previous heuristic searches. A further 3000 iterations were performed to search for trees either compatible with the strict consensus of the same length, or shorter than the shortest tree obtained by maximum parsimony analysis.

Clade support was assessed by bootstrap analysis (Felsenstein 1985). Heuristic searches incorporated 1000 pseudoreplicates using the same search criteria as for the parsimony analysis saving 100 trees for each pseudoreplicate. Bootstrap support of 100% was assessed as robust; 95-99% strong; 80-94% moderate and 50-79% weak. Branches with bootstrap values of 50% or less were considered unsupported.

Constraint trees were constructed in MacClade 4 (Maddison and Maddison 2000) to test the morphological groups previously identified by Thompson (1976) and to test various species concepts. Constraint trees were loaded into PAUP*4.0b10 (Swofford 2002) and heuristic searches with 100 random taxon addition iterations were performed using each of the constraints, saving a maximum of 100 trees per iteration. Comparisons were made between the lengths of the constraint trees in which those groups were present and the shortest trees from the unconstrained analyses.

Standard maximum likelihood analysis was not feasible for a dataset of this size due to time required to adequately search tree space under a suitably complex evolutionary model. Therefore Bayesian estimates of the phylogeny were obtained for *trn*L-*trn*F and ITS using MrBayes 3.0b4 (Huelsenbeck and Ronquist 2001). The general time-reversible (GTR) model, the most general likelihood model (chosen using Modeltest; Posada and Crandall 1998), was employed. The number of substitution types was set to six and among-site rate variation was modelled as a gamma distribution with four rate categories. All other priors, such as the individual base frequencies, were set flat (Dirichlet priors). Tree space was searched using Markov Chain Monte Carlo analysis with four Markov chains beginning from randomly chosen trees. The chains were run for 200,000 generations with one tree sampled every 100 generations.

Trees found prior to the chains converging on a narrow range of likelihood values were discarded. These trees comprise the "burn-in" (or the number of generations required for the Markov chains to reach a steady value). Convergence was determined by plotting the generation number against the likelihood value of the sampled tree in Microsoft Excel (2002). Trees found prior to the likelihood values reaching a steady state were excluded and the remaining trees used to construct a 50% majority rule consensus tree in PAUP*4.0b10 (Swofford 2002). The proportion of saved trees (post burn-in) whereby a given node is recovered represents the Bayesian posterior probability. Bayesian support is considered significant at posterior probability values of 0.95 or higher (Larget and Simon 1999).

A combined analysis of the datasets was performed using the restricted parsimony method as for the individual datasets. Only the 54 individuals for which both ITS and *trn*L-*trn*F data were available were included in the combined analyses. This dataset was tested with reverse constraint analyses using the strict consensus and various morphological groupings as previously described. Bootstrap assessment and a Bayesian analysis were also applied to the combined data using the same criteria as for the individual datasets.

## 2.3 Results

### 2.3.1 Nuclear ITS data

#### Compositional homogeneity

The mean base frequencies across the ITS dataset were A = 0.22383 (range 0.20651 – 0.26462), C = 0.28931 (0.25692 – 0.30899), G = 0.30193 (0.26395 – 0.32305) and T = 0.18493 (0.16446 – 0.21394). A chi-squared test across all taxa indicated that there was no significant departure from compositional homogeneity ($\chi 2 = 59.390921$, df = 186, p = 1.00), therefore compositional bias is not a concern for these data. This test ignores correlation due to phylogenetic structure so caution must be taken in interpretation.

#### Maximum parsimony and bootstrap support

The complete ITS dataset comprised 63 individuals (Table 2.1) and 912 characters (after 33 positions of ambiguous alignment were excluded): 183 were parsimony informative and 200 variable but parsimony uninformative. Forty-five indels, 38 of which were informative in the final ITS dataset, were scored and added to the alignment bringing the total number of characters to 957. Of these 218 were parsimony informative and 207 variable but parsimony uninformative. More than half (21) of the indels were insertions ranging from 1-8 base pairs (bp). Deletions ranged from 1-18 base pairs. One unique indel supports the position of the outgroups and the monophyly of *Tetratheca* and 14 unique indels support the two *Platytheca* species. Details of indel types, the number of bases, and the groups of taxa that the indels occur in can be found in Appendix 1. Problems obtaining clean sequence for *Tetratheca pilosa* subsp. *pilosa*, *T. retrorsa*, *T. rupicola* and *T. thymifolia* may have been due to paralogous copies of this region so caution must be used in interpreting the positions of these species in the strict consensus for ITS.

Sequence divergence between outgroups and ingroups ranged from 80-135 substitutions, compared with a range of 1-69 within *Tetratheca.* Substitutions among eastern Australian taxa ranged from 0-26, on average much lower than among western Australian taxa which ranged from 1-69. Fewer than ten substitutions occurred between individuals of the same species or subspecies within the western taxa, whereas there were zero substitutions between several eastern taxa.

The restricted analyses ran to completion retrieving 96597 trees (after the CONDENSE option was applied). The shortest trees had a length of 756 steps with a consistency index (excluding uninformative characters) of 0.5867 and retention index of 0.7497. This result indicates high levels of homoplasy, or characters that have arisen by convergent evolution rather than shared ancestry, and may account for some of the lack of resolution retrieved. The strict consensus of these trees is presented in Figure 2.2. The structure of the strict consensus tree generated with indels was identical to that without indels but bootstrap support values were higher when indels were included so this dataset was used for the remaining analyses.

The reverse constraint analyses did not find any shorter trees than the initial parsimony analysis. Neither was any tree found showing different relationships from the strict consensus at the same length. Therefore, the strict consensus of trees found by the restricted parsimony analysis appears to be a reliable summary of relationships based on these data.

The strict consensus tree is for the most part poorly resolved, however a few clades are supported. Taxa from eastern Australia form a weakly supported clade (labelled E) arising from the grade of western taxa. The bootstrap value (bs) for the eastern Australian clade is 78% however a large subclade comprising most of the eastern taxa in a polytomy has high support (91% bs). The remaining eastern Australian taxa *Tetratheca ericifolia* with *T. juncea* (100% bs and four indels) and two individuals of *T. ciliata* from Victoria (100% bs) are sister clades with weak bootstrap support (69%).

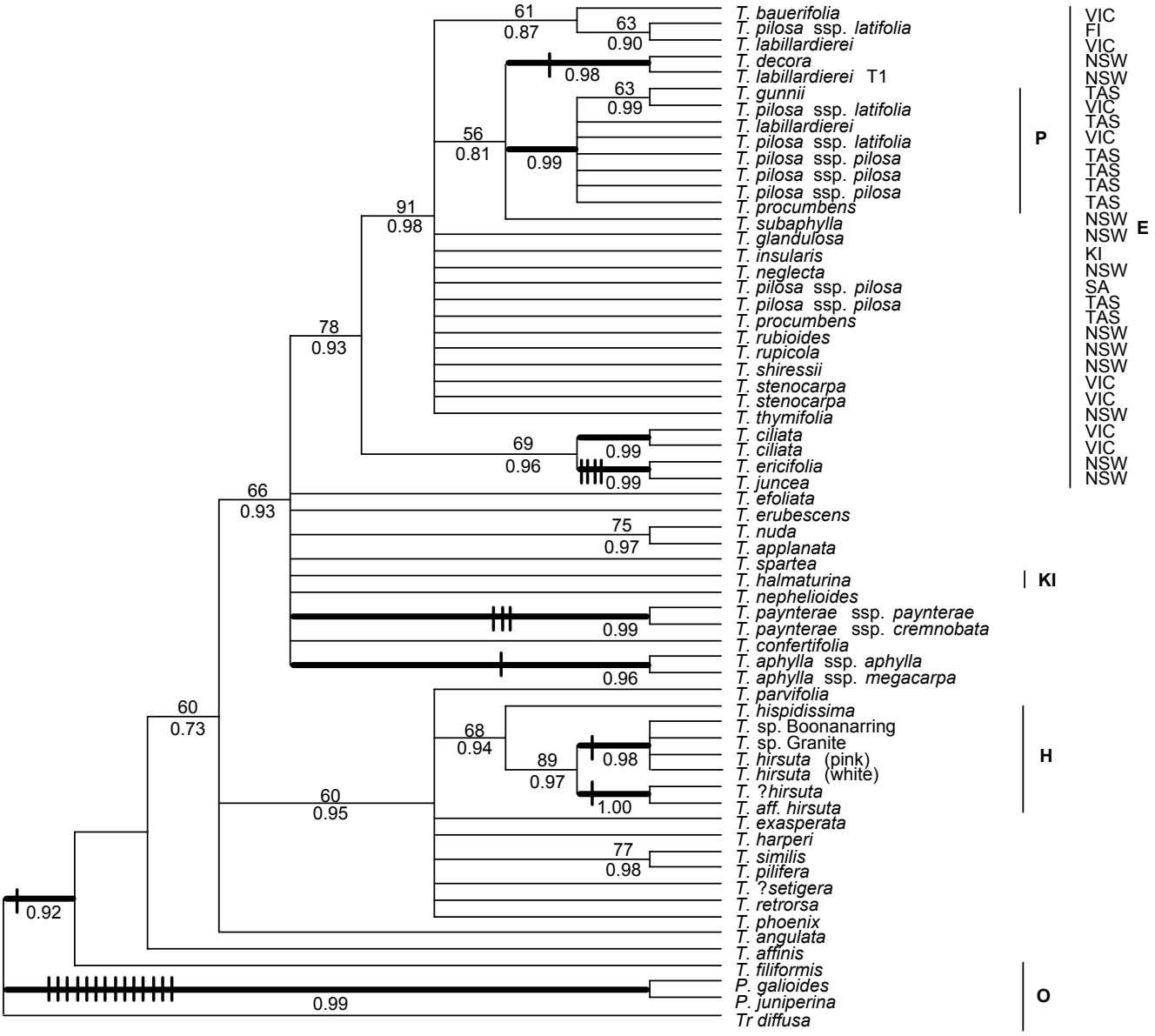



**Figure 2.2 Phylogenetic relationships based on parsimony analysis of the ITS data.**

Strict consensus of 96597 trees (L=756, CI=0.5867, RI=0.7497, RC=0.5345). Bootstrap values from 50-94% are shown above the branches, bootstrap values 95% and over indicated by bold lines, and Bayesian posterior probabilities below the branches. Bars represent unique indels. Abbreviations: *Tetratheca* (*T.*), *Tremandra* (Tr.), *Platytheca* (P.), Flinders Island (FI), Kangaroo Island (KI), New South Wales (NSW), Tasmania (TAS), Victoria (VIC). Clades that will be discussed in some detail are: Outgroups (O), *Tetratheca hirsuta* clade (H), *Tetratheca pilosa* clade (P), Eastern Australian clade (E), *Tetratheca labillardierei* from Tasmania (T1)

The clade containing *Tetratheca pilosa* subsp. *latifolia* from Victoria with *T. gunnii* from Tasmania (63% bs) in a polytomy with *T. pilosa* subsp. *latifolia* from eastern Victoria, three individuals of *T. pilosa* subsp. *pilosa* and one *T. procumbens* and one *T. labillardierei* (T1) from northern Tasmania (labelled P) has strong bootstrap support (95%). A second individual of *T. labillardierei* from the Snowy Mountains in southern NSW is strongly supported (99% bs) as sister to *T. decora* from north-western NSW. This relationship is also supported by a unique indel. An individual of *T. pilosa* subsp. *latifolia* (from Flinders Island) is weakly supported as sister to *T. labillardierei* from Victoria (63% bs). These individuals are in turn sister to *T. bauerifolia* with poor support (61% bs). The placement of this clade within the eastern subclade is uncertain, as are the relationships among the remaining eastern taxa. Although there are several individuals each of *T. pilosa* (including two subspecies), *T. labillardierei, T. ciliata* and *T. stenocarpa* included in the analysis no individuals within a species group together.

The western Australian taxa display greater phylogenetic structure than the eastern taxa, however only a few clades have robust (100%) bootstrap support: *Tetratheca aphylla* subsp. *aphylla* and *T. aphylla* subsp. *megacarpa* (also supported by one indel); the two subspecies of *T. paynterae* (supported by three indels); *T.* ?*hirsuta* with *T.* aff. *hirsuta* (one indel); and two individuals of *T. hirsuta* (one with pink and the other with white flowers) with *T.* sp. Boonanarring and *T.* sp. Granite (one indel). The last two clades form a monophyletic group (labelled H) with moderate bootstrap support (89%). Weakly supported relationships are indicated between *T. similis* and *T. pilifera* (77%), and *T. nuda* and *T. applanata* (75%).

*Tetratheca insularis* from Kangaroo Island is within the well supported eastern sub-clade while the placement of the other Kangaroo island species, *T. halmaturina*, is uncertain among the grade of western Australian taxa. The two individuals are labelled KI in the strict consensus tree. The monophyly of *Tetratheca* (and the position of the outgroups) is supported by a bootstrap value of 100% and one unique indel. The two *Platytheca* species are also robustly supported by 14 indels and a bootstrap value of 100%. *Tetratheca filiformis* then *T. affinis* are basal to the rest of the genus however the positions are not supported, and poorly supported (60% bs) respectively.

**Bayesian Analysis**

The four Markov chains reached a steady value after 6000 generations. The posterior probabilities (pp) of nodes based on trees sampled after the burn-in was discarded are presented below the branches on the strict consensus tree (Figure 2.2). Patterns of support are similar to those obtained by bootstrap. However some branches with only weak to moderate bootstrap support have significant posterior probability values. For example the eastern Australian subclade (excluding *Tetratheca ericifolia* and *T. ciliata*) has a posterior probability of 0.98. The sister relationship between *T. gunnii* and *T. pilosa* subsp. *latifolia* is supported by 0.99 pp. The two *T. ciliata* individuals (0.99 pp) and *T. ericifolia* with *T. juncea* (0.99 pp) are supported in a sister relationship (0.96 pp) where they had been only weakly supported by bootstrap. Clade H is supported by a posterior probability of 0.94 and a subclade of western taxa (including clade H) is supported by 0.95 pp. The weakly supported relationship between *T. similis* and *T. pilifera* is supported by a posterior probability value of 0.98, as is the relationship between *T. nuda* and *T. applanata.* Again the placements of *T. filiformis* followed by *T. affinis* at the base of *Tetratheca* are not supported.

A number of relationships that were not present in the strict consensus tree are retrieved by the Bayesian analysis, but only one is supported. The two individuals of *Tetratheca stenocarpa* are sister taxa; a clade is formed comprising *T. nuda* with *T. applanata* and the two subspecies of *T. aphylla* as well as a clade including these taxa along with *T.* ?*spartea*. Another subset of the western taxa including *T* ?*setigera*, *T. parvifolia*, *T. similis* with *T. pilifera*, *T. harperi*, *T. phoenix* and *T. exasperata* form a supported clade (0.96 pp).

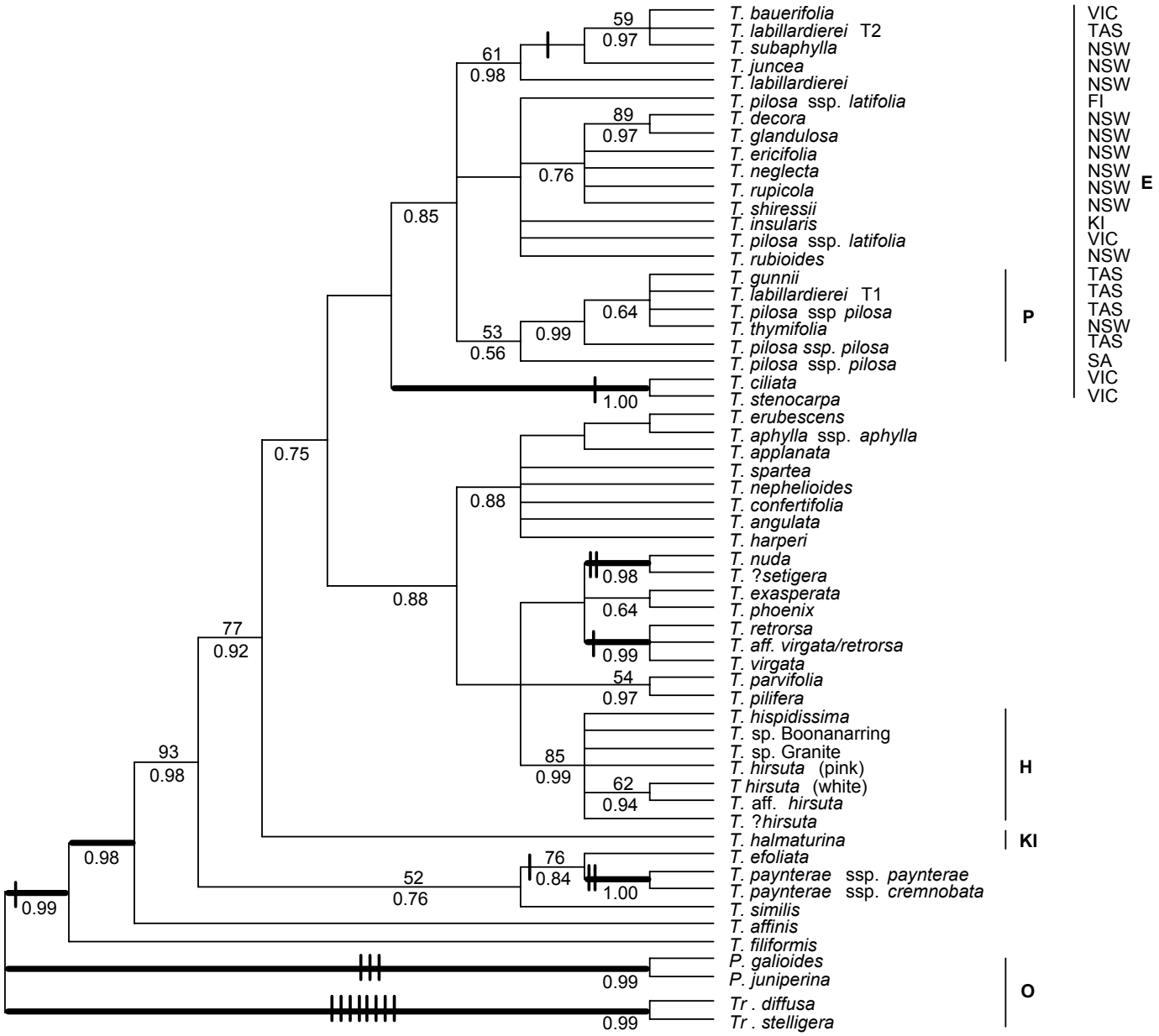



**Figure 2.3 Phylogenetic relationships based on parsimony analysis of the *trn*L-*trn*F data.**

Strict consensus of 97890 trees (L=216, CI=0.7632, RI=0.8879, RC=0.5345). Bootstrap values from 50-94% are shown above the branches, bootstrap values 95% and over indicated by bold lines, and Bayesian posterior probabilities below the branches. Bars represent unique indels. Abbreviations: *Tetratheca* (T.), *Tremandra* (Tr.), *Platytheca* (P.), Flinders Island (FI), Kangaroo Island (KI), New South Wales (NSW), Tasmania (TAS), Victoria (VIC). Clades that will be discussed in some detail are: Outgroups (O), *Tetratheca hirsuta* clade (H), *Tetratheca pilosa* clade (P), Eastern Australian clade (E), two individuals of *Tetratheca labillardierei* from Tasmania (T1 and T2)

### 2.3.2 Chloroplast *trn*L-*trn*F data

**Compositional homogeneity**

Mean base frequencies across the dataset were A = 0.37805 (range 0.36431 – 0.40475), C = 0.16995 (0.15942 – 0.18142), G = 0.15493 (0.14407 – 0.17273) and T = 0.29708 (0.25889 – 0.31105). A chi-squared test across all the taxa indicated that there was no significant difference from compositional homogeneity ($\chi 2 = 22.503624$, df = 171, p = 1.00).

**Maximum parsimony and bootstrap support**

The complete *trn*L-*trn*F dataset included 58 individuals as shown in Figure 2.3. The total number of characters was 999, 78 of which were parsimony informative and 61 variable but parsimony uninformative. Twenty-six indels, 23 of which were included in the final *trn*L-*trn*F dataset, were scored and added to the alignment bringing the total number of characters to 1025, 103 parsimony informative and 62 variable but parsimony uninformative. Approximately half (11) of the indels were insertions ranging from 2-28 base pairs long and most were repeats of adjacent sequence. Deletions ranged from 1-10 base pairs. One unique indel supports the position of the outgroups and the monophyly of *Tetratheca,* eight unite the two species of *Tremandra* and three support the two species of *Platytheca.* Details of indel types, number of bases and groups of taxa they occur in can be found in Appendix 2.

Sequence divergence among outgroups and ingroups ranged from 20-47 substitutions compared with a range of 0-31 within *Tetratheca.* Substitutions among eastern Australian taxa ranged from 0-10, lower on average than among western Australian taxa which ranged from 0-31 (with most values between 10 and 20). Some pair-wise comparisons of distance in both the eastern and western taxa show little or no sequence divergence. *Tetratheca* ?*hirsuta*, *T.* sp. Boonanarring, *T.* sp. Granite and *T. hispidissima* are identical to one

another, as is *T. virgata* to *T. retrorsa* and *T.* aff. *virgata*/*retrorsa*; and *T. stenocarpa* to *T. ciliata*.

The structure of the strict consensus tree generated with indels was identical to that without indels. Bootstrap support values were higher for the analysis with indels included therefore this dataset was used for the remaining analyses. The restricted analysis ran to completion saving 97890 trees after the CONDENSE option was applied. The shortest trees found had a length of 216 steps with a consistency index (excluding uninformative characters) of 0.7632 and retention index of 0.8879. The strict consensus of these trees is presented in Figure 2.3. The reverse constraint analyses did not alter the tree length and no trees were found that showed different relationships from the strict consensus at the same length. The shortest tree length found by the initial search seems a representitive estimate based on these data.

The strict consensus tree from analysis of *trn*L-*trn*F data is generally poorly resolved and although a clade of all eastern Australian taxa is retrieved it is not supported. All eastern taxa (excluding *Tetratheca ciliata* and *T. stenocarpa*) form an unsupported clade sister to a strongly supported sister relationship between *T. ciliata* and *T. stenocarpa* (96% bs). A poorly supported clade, comprising *T. bauerifolia* with an individual of *T. labillardierei* and *T. subaphylla* (59 % bs), is sister to *T. juncea* with no bootstrap support but united by one unique indel. A second individual of *T. labillardierei* is poorly supported as sister to this clade (61% bs). *Tetratheca decora* and *T. glandulosa* are united by a bootstrap value of 89%. A weakly supported (53% bs) clade comprises *T. gunnii, T. labillardierei,* three individuals of *T. pilosa* ssp. *pilosa* and *T. thymifolia. T ciliata* and *T. stenocarpa* are resolved as sister taxa with high support (96% bs). This last finding conflicts with the ITS results which resolved *T. ciliata* as sister to a clade of *T. ericifolia* with *T. juncea* supported by a bootstrap value of 69% and a posterior probability value of 0.96.

The western Australian taxa display some similar relationships to those found in the ITS analysis, for example, the two subspecies of *Tetratheca paynterae* form a strongly supported clade (96%) which is also supported by two unique indels. In this analysis, however, they are further resolved as sister to *T. efoliata* with weak support (76% bs). The species in the *T. hirsuta* complex again form a clade (H) with moderate support (85% bs),

however an internal relationship between *T.* aff. *hirsuta* and *T. hirsuta* (white) is resolved, albeit with poor support (62% bs). *Tetratheca* aff. *hirsuta* was robustly supported as sister to *T.* ?*hirsuta* in the ITS analysis. Also in contrast to the ITS results *T. nuda* is robustly supported as sister to *T.* ?*setigera* (100% bs). A highly supported (98% bs) polytomy of *T. retrorsa*, *T.* aff. *virgata*/*retrorsa* and *T. virgata* is retrieved, however two of these taxa were not included in the ITS dataset therefore no comparison can be made. *Tetratheca parvifolia* is united with *T. pilifera* with poor bootstrap support (54%). The Kangaroo Island taxa, *Tetratheca insularis* and *T. halmaturina*, do not form a clade. *Tetratheca insularis* falls within an unsupported polytomy of the eastern Australian taxa and the position of *T. halmaturina* is also unresolved within an unsupported clade of western taxa.

The positions of *Tetratheca filiformis* basal to the rest of *Tetratheca* and *T. affinis* at the base of the genus excluding *T. filiformis* have high and moderate bootstrap supports of 97% and 93% respectively. This pattern concurs with the structure of the ITS strict consensus tree, although support for ITS was weak at best. Again *Tetratheca* is resolved as monophyletic with the position of the outgroups strongly supported by a bootstrap value of 95%. Relationships of the two species of *Platytheca* together and both *Tremandra* together are each robustly supported (100% bs).

**Bayesian Analysis**

The four Markov chains reached a steady state after 6000 generations. The posterior probabilities of nodes are presented below the branches on the strict consensus tree (Figure 2.3). For the most part, relationships retrieved by the Bayesian analysis reflect those of the *trn*L-*trn*F strict consensus parsimony analysis. Clades strongly supported by bootstrap generally have high posterior probabilities. Several relationships that had weak or moderate bootstrap support are supported by posterior probability values. One example is a clade comprising *Tetratheca bauerifolia*, *T. labillardierei* (T2) and *T. subaphylla* (59% bs; 0.97 pp) and this clade with *T. juncea* and *T. labillardierei* from NSW (61% bs; 0.98 pp). *Tetratheca decora* is supported as sister to *T. glandulosa* by a posterior probability of 0.97 (89% bs) and the relationship between *T. parvifolia* with *T. pilifera* is also supported (54% bs 0.97 pp). Clade H is strongly supported by a posterior probability of 0.99 (85% bs).

There are also a number of clades supported by posterior probability values that had no bootstrap support: *Tetratheca gunnii, T. labillardierei* (T1) from Tasmania and two individuals of *T. pilosa* subsp. *pilosa* from Tasmania and *T. thymifolia* (0.99 pp). There are several clades that were retrieved by both analyses but unsupported including a clade of eastern Australian taxa and a large subclade of western taxa.

### 2.3.3 Combined analysis of ITS and *trn*L-*trn*F

**Maximum parsimony and bootstrap support**

The complete dataset comprised all individuals that were common to both datasets (54 in total) and corresponding indels. The restricted parsimony analysis ran to completion, retrieving 95476 trees after the CONDENSE option was applied. The shortest trees had a length of 1004 steps with a consistency index (excluding uninformative characters) of 0.5790 and retention index of 0.7193. The strict consensus of these trees is presented in Figure 2.4.

All sampled eastern Australian taxa form a moderately supported clade (94% bs) containing two strongly supported subclades. The first comprises three taxa from Tasmania *Tetratheca gunnii*, *T. labillardierei* (T1) and *T. pilosa* subsp. *pilosa* plus *T. pilosa* subsp. *latifolia* from Victoria (95% bs). The second is a sister relationship between *T. ericifolia* and *T. juncea* (100% bs). There is weak support for a subclade of eastern taxa (69% bs) that excludes a weakly supported clade comprising *T. ciliata* and *T. ericifolia* with *T. juncea* (56% bs).

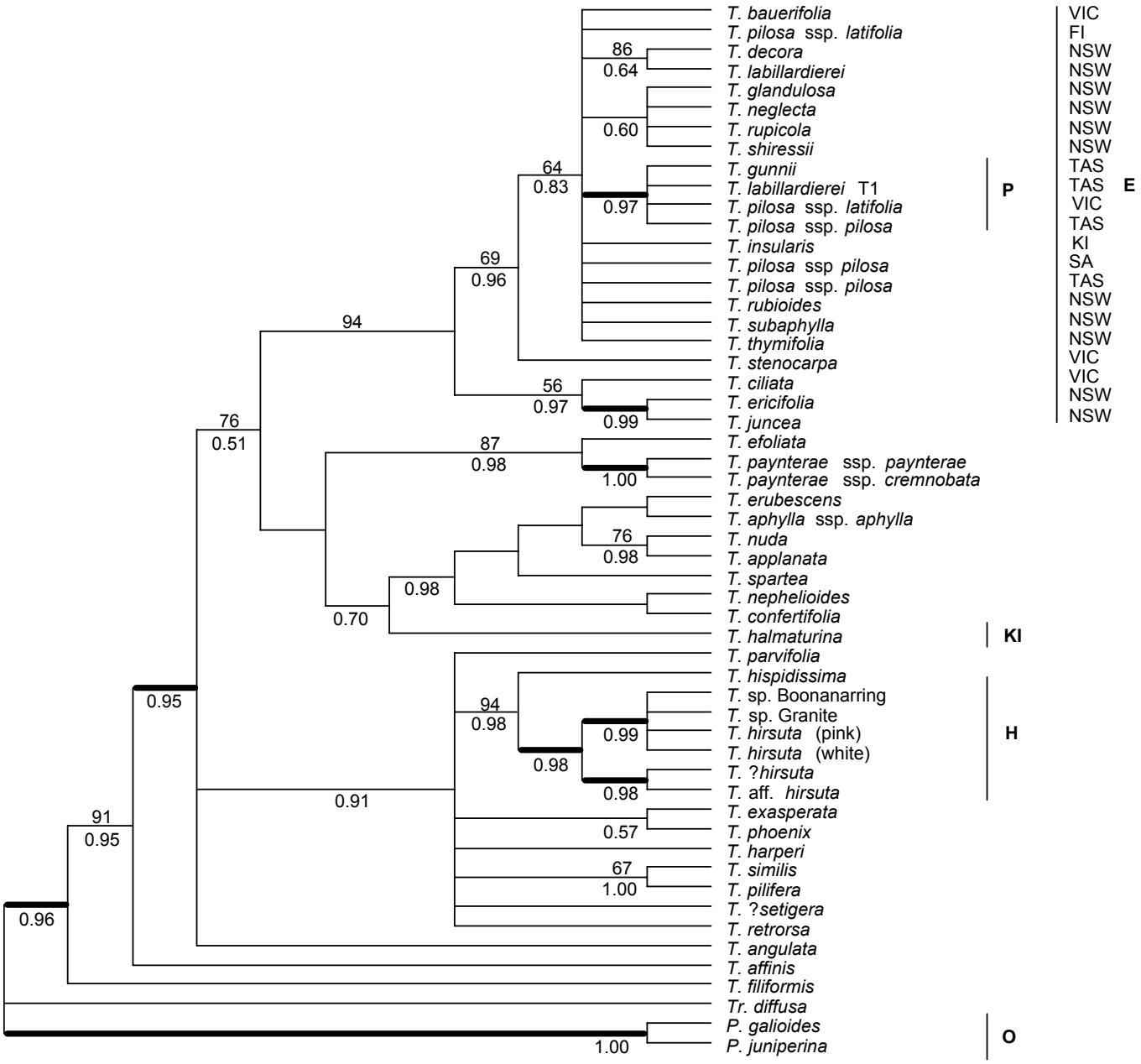



**Figure 2.4 Phylogenetic relationships based on parsimony analysis of combined data.**

Strict consensus of 95476 trees (L=1004, CI=0.5790, RI=0.7193, RC=0.5094). Bootstrap values from 50-94% are shown above the branches, bootstrap values 95% and over indicated by bold lines, and Bayesian posterior probabilities below the branches. Bars represent unique indels. Abbreviations: *Tetratheca* (T.), *Tremandra* (Tr.), *Platytheca* (P.), Flinders Island (FI), Kangaroo Island (KI), New South Wales (NSW), Tasmania (TAS), Victoria (VIC). Clades that will be discussed in some detail are: Outgroups (O), *Tetratheca hirsuta* clade (H), *Tetratheca pilosa* clade (P), Eastern Australian clade (E), *Tetratheca labillardierei* from Tasmania (T1)

There are several clades with robust (100% bs) support among western taxa: two clades within clade H, and the *Tetratheca paynterae* subspecies are sister to one another. There is strong support (95% bs) to unite the two clades within clade H, and clade H as a whole is also moderately supported (94% bs). *Tetratheca efoliata* has moderate bootstrap support as sister to the *T. paynterae* subspecies (87%) and there is weak support uniting *T. similis* and *T. pilifera* (67%). The Kangaroo Island taxa do not form a clade: *T. insularis* is placed with the eastern Australian clade, whereas *T. halmaturina* is placed within an unsupported clade of Western Australian taxa. Monophyly of *Tetratheca* is robustly supported and the positions of *T. filiformis* and *T. affinis* basal to the rest of *Tetratheca* are moderately (91% bs) and strongly (99% bs) supported respectively.

There are some minor points of conflict between the ITS and *trn*L-*trn*F trees. For example, *Tetratheca decora* with *T. labillardierei* was present in the ITS analysis with strong support (99% bs) but is not retrieved by the *trn*L-*trn*F analysis. Instead *T. decora* is united with *T. glandulosa* with moderate bootstrap support (89%). *Tetratheca ciliata* with *T. stenocarpa* occurred in the *trn*L-*trn*F analysis with strong support (96% bs) and is not resolved by ITS. None of the datasets resolves the individuals of *T. pilosa* together nor individuals of *T. labillardierei* as a clade, however each analysis has high support for different individuals of *T. pilosa* with *T. gunnii* and the northern Tasmanian individual of *T. labillardierei* (T1).

**Constraint analyses**

The results of the constraint analyses are presented in Table 2.3. Two of Thompson's (1976) morphological groups were present in the strict consensus tree for *trn*L-*trn*F, one of which was also present in the ITS and combined analyses. The remaining constraint analyses produced trees longer than the original parsimony analysis. Group two (Thompson 1976, p. 141) could not be tested as none of the analyses contained *Tetratheca paucifolia*.

**Table 2.3 Results of constraint analyses**

| Group | Number of steps longer than original tree | | |
|---|---|---|---|
| | **ITS** | ***trn*L-*trn*F** | **combined** |
| 1. *T. affinis*, *T. efoliata* and *T. retrorsa* | 14 | 16 | 25 |
| 2. *T. aphylla* and *T. paucifolia* | N/A | N/A | N/A |
| 3. *T. halmaturina* and *T. harperi* | 8 | 1 | 10 |
| 4. *T. hispidissima* and *T. hirsuta* | 0 | 0 | 0 |
| 5. *T. confertifolia* and *T. filiformis* | 8 | 12 | 27 |
| 6. *T. glandulosa* and *T. labillardierei* | 15 | 8 | 15 |
| 7. *T. stenocarpa* and *T. ciliata* | 7 | + | 2 |
| 8. *T. rupicola* and *T. thymifolia* | 11 | 4 | 4 |
| 9a. *T. pilosa*, *T. gunnii*, *T. neglecta*, *T. rubioides, T. procumbens,* and *T. insularis* | 9 | 5 | 12 |
| 9b. *T. pilosa* and *T. procumbens* | 11 | 5 | 12 |
| 9c. *T. pilosa* subspecies | 11 | 4 | 12 |
| 9d. *T. pilosa* subsp. *pilosa* and *T. insularis* | 8 | 4 | 11 |
| 10. *T. labillardierei* clade | 13 | 4 | 11 |

## Bayesian Analysis

The Bayesian tree reflects the relationships of the combined parsimony tree, however the posterior probability values are generally higher than the bootstrap values. For example, a subclade of eastern taxa including all species except *Tetratheca ciliata*, *T. ericifolia* and *T. juncea* has a posterior probability of 0.96. A clade comprising *T. erubescens*, *T. aphylla* subsp. *aphylla*, *T. applanata*, *T. nephelioides*, *T. confertifolia*, and the two subspecies of *T. nuda*, is supported (0.98 pp). A relationship between *T. nuda* and *T. applanata* is supported (0.98 pp). The positions of *T. filiformis* and *T. affinis* at the base of *Tetratheca* are both supported by posterior probability values of 0.95. There are no moderately or strongly supported relationships in the Bayesian tree that differ from the strict consensus.

## 2.4 Discussion

Thompson's (1976) revision provides a solid morphological basis for understanding the taxonomy of this group. She pointed out that there are problems in the delimitation of some species and relationships are "…puzzling and the evolutionary trends are obscure" (Thompson 1976 p.141). Downing (2005) tested species limits with a phenetic analysis. For the most part she found good separation between taxa however there was considerable overlap between *Tetratheca pilosa* subsp. *pilosa*, *T. pilosa* subsp. *latifolia* and *T. procumbens*. She also found some overlap between *T. insularis*, *T. neglecta* and *T. rubioides*. The only member of the *T. pilosa* group identified by Thompson (1976) that was clearly separated from all others was *T. gunnii*. *Tetratheca ciliata* and *T. labillardierei* each comprised several morphological clusters in Downing's (2005) analysis and she suggested that these taxa also require further investigation.

Downing's (2005) morphometric analysis also tested relationships within *Tetratheca* cladistically. The cladograms were generally poorly resolved, and the relationships that are indicated in the strict consensus are without support. *Tetratheca ciliata* is sister to *T. stenocarpa* and a clade comprising all eastern Australian taxa plus *T. insularis*, *T. pilifera* and *T. similis* is shown in her majority rule tree. Her strict consensus of 169985 equally most parsimonious trees however does not resolve this clade. The only relationships present in the strict consensus in the eastern taxa are *T. stenocarpa* with *T. ciliata*, and a clade comprising *T. neglecta*, *T. rubioides*, *T. rupicola*, *T. juncea* and *T. decora*. The following sister relationships are present among the western Australian taxa: *T. pubescens* with *T. setigera*, *T. pilifera* with *T. similis* and *T. hirsuta* with *T. hispidissima*. This last pair is sister to *Platytheca*, thus rendering *Tetratheca* paraphyletic. None of these relationships, however, includes any support values. Downing's (2005) results will be discussed further in the context of morphological and taxonomic groupings tested by the present molecular analysis.

### 2.4.1 Monophyly of *Tetratheca*

The monophyly of the tremand lineage, sister to a clade comprising *Aceratium*, *Elaeocarpus* and *Sericolea* within Elaeocarpaceae, has been established by several recent studies (e.g. Savolainen *et al.* 2000, Crayn *et al.* 2006). In the analysis of *trn*L-*trn*F data by Crayn *et al.* (2006) *Tremandra* is basal to the group and *Platytheca* is resolved as sister to the rest of *Tetratheca*, rendering *Tetratheca* monophyletic. Downing (2005) used *Tremandra* only as the outgroup in her analysis as the addition of *Elaeocarpus* had no effect on tree topology. In contrast to work by Crayn *et al.* (2006), Downing's study found *Platytheca* nested within *Tetratheca*.

For the most part the tree topologies retrieved by the two phylogenetic inference methods were congruent, though the results of the Bayesian analyses produced trees with more supported branches on the strict consensus trees than bootstrap. The maximum parsimony results presented here support the monophyly of *Tetratheca* with nucleotide sequence divergence from *Platytheca* and *Tremandra* in the order of 2-5% for *trn*L-*trn*F and 9-15% for ITS. *Tremandra* is supported by eight unique indels in *trn*L-*trn*F but only one individual was sequenced for ITS so indels could not be scored. Three and 14 unique indels support *Platytheca* in *trn*L-*trn*F and ITS respectively. The position of all the outgroups with respect to *Tetratheca* is supported in each case by one unique indel. High bootstrap values and posterior probabilities support the position of the outgroups in each analysis as previously discussed. The ranges of substitutions in the *trn*L-*trn*F from *Tetratheca* to *Platytheca* compared with *Tetratheca* to *Tremandra* are similar: approximately 20-47 in each case. For the ITS dataset, however, the difference between the two ranges is large: 80-103 compared with 106-135 respectively. In both datasets the sequence divergence from *Tetratheca* to the outgroups is much higher than sequence divergence within *Tetratheca.*

### 2.4.2 Western origin for *Tetratheca* and differentiation of an eastern clade

Thompson (1976) suggested a western Australian origin for *Tetratheca* with subsequent dispersal to the east, each group then radiating independently over a long period of isolation. The strong to moderate support for the placement of the western taxa *Tetratheca filiformis* and *T. affinis* at the base of the *trn*L-*trn*F tree is consistent with this notion of a western origin for the group. The eastern clade arising from a grade of western taxa proposed by Crayn *et al.* (2006) is supported by the present study which uses a much larger dataset. Although the eastern group is not monophyletic in the *trn*L-*trn*F strict consensus tree, the data do not conflict with the concept of an eastern clade since *T. stenocarpa* and *T. ciliata* arise from a polytomy with a clade comprising the rest of the eastern taxa, *T. halmaturina*, and a western clade.

For *trn*L-*trn*F the number of substitutions across the eastern clade is low (average=5) compared with the sequence divergence across western taxa (average=13). There is an average of eight substitutions among eastern taxa for ITS. Compared with divergence among the western species (37 substitutions) this value is low. These results suggest that the eastern species of *Tetratheca* are of relatively recent origin. An alternative explanation is that the evolutionary rate has slowed in the eastern taxa but there is no evidence to support this.

Both ITS and *trn*L-*trn*F data robustly support the sister relationship of the two subspecies of *Tetratheca paynterae*. The two *T. aphylla* subspecies are similarly supported in the ITS tree, however *trn*L-*trn*F sequence data were only available for one of these individuals. There are several individuals of each of *T. pilosa* subsp. *pilosa* and *T. pilosa* subsp. *latifolia* included in both datasets, and two individuals of *T. procumbens* in the ITS dataset. In neither of the trees do these taxa form clades representing specific or subspecific boundaries. These results support Downing's (2005) conclusion that the subspecies of *T. pilosa* seem to be artificial, and their relationship with *T. procumbens* is unclear. A clade comprising *T. hispidissima* and the six individuals in the *T. hirsuta* complex is recognised

by all datasets (clade H). Internal structure within this clade differs between datasets with poor support in the *trn*L-*trn*F tree.

Ladiges (2006) issued a cautionary reminder that to explain the present day distributions of single lineages by dispersal events may not be appropriate. She suggests that vicariance should be considered when reconstructing biogeographical scenarios for parts of the Australian flora. Congruent patterns across multiple plant lineages as found by Crisp and Cook (2007), indicate that the disjunctions between many groups within the south-western and south-eastern floras correlate with an ancient vicariant event: the establishment of the Nullarbor Plain.

The Nullarbor Plain remains a barrier to migration between south-western and south-eastern floras as indicated by the high instance of endemism at the species level. Molecular data presented here support the conclusions of Downing *et al*. (2008) that several lineages (including *Tremandra, Platytheca* and *Tetratheca*) diverged first in Western Australia. *Tetratheca halmaturina* falls among one of these Western Australian lineages and may have been isolated on Kangaroo Island by the barrier of the Nullarbor Plain. Crisp and Cook (2007) concluded that vicariance has had a strong impact on the evolution of the southern Australian flora. The timing of diversification of *Tetratheca* during the Miocene was determined by Crayn *et al.* (2006), and it corresponds with rapid diversification of other sclerophyllous groups such as *Banksia*, *Eucalyptus*, *Allocasuarina* and some lineages of Fabaceae (Crisp *et al.* 2004) indicating that the Nullarbor Plain may have had an effect on the evolution of *Tetratheca.* This is also supported by the absence of any shared species between eastern and western Australia. Nevertheless, to explain the current distribution only by vicariance would require a large number of lineage extinctions in the east. This is a less parsimonious hypothesis than dispersal of at least two lineages to the east followed by isolation due to the formation of the Nullarbor Plain.

### 2.4.3 Affinities of the Kangaroo Island taxa

Thompson (1976) suggested that the tremand lineage has colonised Kangaroo Island from both east and west of the Australian mainland. Indeed, on the basis of both ITS and *trn*L-

*trn*F data, *Tetratheca insularis* has a greater number of nucleotide base changes from *T. halmaturina* than either *T. ciliata* or *T. ericifolia* (the most basal of the eastern species), and less divergence (1-16 bases less) than *T. halmaturina* from either of these taxa. In all analyses performed, *T. insularis* falls within a well supported subclade of eastern taxa that does not include *T. halmaturina*. Thompson's (1976) suggestion that *T. insularis* shows closer affinities with eastern than western taxa, and that *T. halmaturina* is more closely allied with western species, is supported by the present analyses.

### 2.4.4 Testing morphological groups with molecular data

The species groups presented by Thompson (1976) and tested here represent only a subset of the genus. Since Thompson's treatment, several new species have been described which may necessitate revision of some of the originally proposed relationships. Nevertheless, her nine morphological groups provide a good basis for discussion of relationships within *Tetratheca*. An additional species concept (*Tetratheca labillardierei*) was tested by constraint analyses and will be discussed below.

1. ***Tetratheca affinis*, *T. efoliata* and *T. retrorsa***

Group one comprises *Tetratheca affinis*, *T. efoliata* and *T. retrorsa* but is not supported by any of the analyses conducted in the present study. *Tetratheca filiformis* and *T. affinis* are basal to the rest of *Tetratheca* in all analyses. The position of *T. efoliata* is not resolved by ITS data, but it is resolved as sister to the two *T. paynterae* subspecies by t*rn*L-*trn*F data. In both datasets sequence divergence is higher between *T. efoliata* and the other two taxa than among most of the western taxa. Sequence divergence is similarly high between *T. filiformis* and *T. affinis.* The constraint analysis testing the monophyly of these three taxa produced trees substantially longer than the strict consensus trees: 14, 16 and 25 steps for t*rn*L-*trn*F, ITS and combined analyses respectively. There is no evidence from these data that these three taxa are closely related.

### 2. *T. aphylla* and *T. paucifolia*

Morphological group two was not tested here as sequence data could not be obtained for *Tetratheca paucifolia*.

### 3. *T. halmaturina* and *T. harperi*

Group three pairs two leafless Western Australian species. Recent morphological and molecular studies of leafless species from Western Australia (Butcher 2007a, Butcher *et al.* 2007) describe a number of new species that alter original ideas about this relationship. In a morphological and molecular study of relationships among rare Western Australian *Tetratheca* species, Butcher *et al.* (2007) concluded that *Tetratheca harperi, T. aphylla* and *T. paynterae* belong to three separate lineages and that 'leaflessness' is due to adaptive convergence in response to environmental conditions. The molecular analyses presented here support the convergent evolution of 'leaflessness' with leafless species arising in at least five different clades with varying degrees of support.

The positions of *Tetratheca halmaturina* and *T. harperi* in the ITS and *trn*L-*trn*F analyses presented are poorly resolved. For *trn*L-*trn*F there are 12 substitutions between them and 30 for ITS. Constraint analysis for ITS produces a tree eight steps longer than the strict consensus, although for *trn*L-*trn*F only one extra step is required to unite the taxa. Evidence to unite these taxa is not strong, however, and more resolution for the western groups is needed to fully investigate the placement of these species.

### 4. *T. hispidissima* and *T. hirsuta*

*Tetratheca hispidissima* and *T. hirsuta* appear to be closely related according to the molecular data presented here with all analyses uniting these taxa. In each case they occur in the clade denoted H which includes a number of newly discovered taxa showing morphological affinities with *T. hirsuta* (R. Butcher, pers. comm. 2007). The ITS strict consensus places *T. hispidissima* sister to the *T. hirsuta* complex with weak support (68% bs, 0.94 pp). These taxa are supported in a polytomy with moderate (85%) bootstrap support and 0.99 posterior probability in the *trn*L-*trn*F dataset. Work is currently underway

by Ryonen Butcher (PERTH) and colleagues to determine the relationships of a number of the newly discovered taxa (*T.* sp. Boonanarring, *T.* sp. Granite, *T.* aff. *hirsuta*, *T.* ?*hirsuta* (pink) and *T.* ?*hirsuta* (white)). The present study indicates that these taxa form a monophyletic group sister to *T. hispidissima* (clade H) using ITS data and a polytomy including *T. hispidissima* for *trn*L-*trn*F data. In both cases, *T.* aff. *hirsuta* differs by a greater number of substitutions and is resolved in different sister relationships in the analyses: with *T.* ?*hirsuta* in the ITS tree and *T. hirsuta* (white) in the *trn*L-*trn*F tree.

### *5. T. confertifolia* and *T. filiformis*

Group five pairs *Tetratheca confertifolia* with *T. filiformis* on the basis of morphology, however molecular data do not support this relationship. The placement of *Tetratheca filiformis* as sister to the rest of the genus is supported by high bootstrap and posterior probability values (97% and 0.98 respectively) for *trn*L-*trn*F. Although this position is resolved but unsupported for ITS, both datasets show high sequence divergence from the rest of *Tetratheca* and the constraint analysis grouping the two species produces trees much longer than the original strict consensus trees (8, 12 and 27 steps more for ITS, *trn*L-*trn*F and combined analyses respectively). The congruence of molecular phylogenies presented here is evidence that this relationship is artificial; however, the precise position of *T. confertifolia* remains unclear.

In Downing's (2005) morphometric analysis a clade comprising *Tetratheca filiformis,* the two species of *Platytheca, T. hirsuta and T. hispidissima* is resolved as sister to *T. confertifolia* but there is very little resolution among the remainder of the genus and the homoplasy index is high (0.61). In a recent cladistic study of trichome and floral characters however, Downing *et al.* (2008) resolve *T. filiformis* as basal to a clade including *Platytheca* and *Tetratheca*, and hypothesise that it could represent a fourth genus of the tremand lineage. Their analysis supports the conclusion of the present study that the relationship between *T. filiformis* and *T. confertifolia* is unlikely.

### 6. *T. glandulosa* and *T. labillardierei*

*Tetratheca labillardierei* and *T. glandulosa* comprise Thompson's (1976) sixth group. They have been traditionally paired on the basis of their glandular hairs. None of the analyses in the present study resolve this sister relationship. ITS data do not resolve the position of *T. glandulosa* within the eastern subclade and *trn*L-*trn*F data indicate a sister relationship with *T. decora* with moderate support (89% bs, 0.97 pp). The constraint analyses require trees 15 (ITS), 8 (*trn*L-*trn*F) and 15 (combined) steps longer than the MP tree to retrieve *T. glandulosa* and *T. labillardierei* as sister taxa, strong evidence against the relationship.

Base differences between *Tetratheca glandulosa* and individuals of *T. labillardierei* are in the range of 9-11 and 3-5 for ITS and *trn*L-*trn*F respectively. These values are similar to divergence among the rest of the eastern taxa. Base divergence is slightly higher between individuals of *T. labillardierei*: 6-12 (ITS) and 1-4 (*trn*L-*trn*F), and both ITS and *trn*L-*trn*F data resolve one individual only of *T. labillardierei* (T1) with *T. gunnii* and individuals of both subspecies of *T. pilosa*. Downing's (2005) phenetic analysis found two morphological clusters within *T. labillardierei* suggesting that the current circumscription of the species may be inadequate. That they do not form a clade in any of the present molecular analyses would also suggest that *T. labillardierei* requires further investigation. Three of the four individuals sequenced for the present study fall into one of Downing's clusters, and the individual from NSW into the other. The latter individual forms a strongly supported clade with *T. decora* (also from NSW) in the combined and ITS analyses, conflicting with the *trn*L-*trn*F result which resolved *T. decora* and *T. glandulosa* as sisters.

Given that other species (e.g. *Tetratheca affinis*, *T. pilosa*, *T. rupicola* and *T. stenocarpa*) have glandular hairs, albeit with lower density, it could be that traditional notions concerning this relationship have relied too heavily on this character. Thompson (1976) suggested that hair-type and combination of hair types are important characters for identification of species within *Tetratheca*. On the basis of this hypothesis, Downing (2005) performed a trichome study of *Tetratheca* to include in her morphometric analysis. She recognised five of six hair types defined by (Thompson 1976) as independent character states. The sixth character "glandular-tipped setae" was combined with "non-

glandular-tipped setae" for Downing's cladistic analysis because there was considerable homoplasy displayed by setae leading to concern that separating "glandular-tipped setae" and "non-glandular-tipped setae" would result in two homoplasious, uninformative character states. The recent trichome study by Downing *et al.* (2008) places the two species in the same morphological group (one of six among the tremand clade) along with eleven other eastern taxa but to the exclusion of *T. decora.*

### *7. T. stenocarpa* and *T. ciliata*

These two species are strongly supported as sister taxa in the *trn*L-*trn*F analysis but this relationship conflicts with the ITS results. Two individuals of each of *Tetratheca ciliata* and *T. stenocarpa* were included in the ITS dataset but only one of each for *trn*L-*trn*F. This may have influenced the results somewhat. An analysis of the ITS dataset without the extra individuals was performed but not presented here since the same relationship between *T. ciliata* and *T. ericifolia* with *T. juncea* to the exclusion of *T. stenocarpa* was retrieved. Constraint analysis of ITS data produced a tree seven steps longer than the strict consensus.

The chloroplast sequences for these taxa are identical while sequences diverge by 15-16 bases for ITS. This is higher than divergence between *Tetratheca stenocarpa* and all other eastern taxa apart from *T. juncea* and *T. glandulosa.* Hybridisation has been suggested as a possibility within this group on the basis of morphology (J. Thompson, pers. comm. 2005), an hypothesis that will be tested with a population analysis in the study using these species. Downing (2005) also resolved a sister relationship between *T. ciliata* and *T. stenocarpa* on the basis of morphology and Downing *et al.* (2008) place them together along with eleven other taxa on the basis of trichome and floral characters.

A phylogeographic study of these two species (Chapter 3), as well as a study of population structure, and presence and direction of inter-population gene flow within and among them (Chapter 4), will clarify the taxonomic boundaries of these species. The likelihood of hybridisation will be investigated in both studies, and each will provide insights into the evolutionary processes shaping current distribution pattern of *T. ciliata* and *T. stenocarpa.*

### *8. T. rupicola* and *T. thymifolia*

The positions of *Tetratheca rupicola* and *T. thymifolia* are incongruent across the two datasets, although there is little resolution in either analysis. For ITS both species reside within a polytomy of eastern taxa. For *trn*L-*trn*F, *T. thymifolia* is supported (0% bs, 0.99 pp) in a polytomy with *T. gunnii* and two individuals of *T. pilosa* subsp. *pilosa* and one individual of *T. labillardierei*. In the combined analysis *T. rupicola* is part of a small eastern clade with no support and *T. thymifolia* is among the large eastern polytomy. Eleven extra steps would be required to resolve these taxa as monophyletic according to the ITS constraint analysis. This renders the association unlikely, however, only four extra steps would be necessary in the *trn*L-*trn*F and combined analyses. *Tetratheca thymifolia* is the most widely distributed species of the genus and displays significant morphological variation across its range (Thompson, 1976, Downing 2005), therefore specific limits may warrant further investigation. In Downing's (2005) cladistic analysis *T. rupicola* is resolved sister to *T. juncea* within a small eastern clade also including *T. neglecta*, *T. rubioides* and *T. decora*. These results also conflict with Thompson's (1976) proposal that *Tetratheca rupicola* is most closely related to *T. thymifolia*. More resolution is required to investigate this relationship further.

### *9. T. pilosa, T. gunnii, T. neglecta, T. rubioides* and *T. insularis*

Thompson's final grouping is the *Tetratheca pilosa* group comprising *Tetratheca pilosa*, *T. gunnii*, *T. neglecta*, *T. rubioides* and *T. insularis*. *Tetratheca procumbens*, a species not recognised by Thompson (1976) but reinstated by Jeanes in 1995, has been included in the present study since both authors agree it is closely related to *T. pilosa*. Thompson suggested it is a procumbent, mountainous form of *T. pilosa.* While these taxa do not form a clade in any of the strict consensus trees, subsets of the group are supported in each (see clade P in each of the phylogenetic trees). Due to the complexity of this group a number of constraint analyses were run with subgroups of the *T. pilosa* group as follows: *T. pilosa* with *T. procumbens;* the two species of *T. pilosa* together; *T. pilosa* subsp. *pilosa* with *T. procumbens* and *T. pilosa* subsp. *latifolia* with *T. procumbens.* The extra steps required to resolve these groups were 8-11 for ITS, 4-5 for *trn*L-*trn*F and 11-12 for the combined analysis: quite strong evidence against the groupings.

On the basis of trichome and floral morphology (Downing *et al.* 2008) the *Tetratheca pilosa* group is divided into two of six morphological groups: *T. pilosa, T. gunnii* and *T. insularis* fall in a group with eight other eastern taxa; *T. rubioides* and *T. neglecta* group with four other eastern and four western species. The phenetic analysis performed by Downing in 2005 found that *T. pilosa* subsp. *pilosa, T. pilosa* subsp. *latifolia* and *T. procumbens* formed overlapping morphological clusters. She suggested that the subspecific division could be artificial and the taxonomic status of *T. procumbens* required more work. In Chapter 3 of the present study, chloroplast microsatellites are used to investigate phylogeography and population structure of the *T. pilosa* group in order to clarify taxonomic boundaries and investigate the possible role of hybridisation in the evolution of this group.

#### *10. T. labillardierei*

The final constraint analyses were undertaken to investigate support for relationships among individuals of *Tetratheca labillardierei.* Trees were 13, 4 and 11 steps longer than the ITS, *trn*L-*trn*F and combined strict consensus trees respectively. As outlined above, this species clustered in two separate groups in Downing's (2005) morphometric analysis and individuals T1 and T2 from Tasmania did not form a clade in any of the molecular analyses presented here. *T. labillardierei* is a widely distributed species, occurring in southern NSW and throughout Victoria and Tasmania (Gardner and Murray 1992, Jeanes 1999, Australia's Virtual Herbarium), and morphology varies greatly (pers. obs. 2004, 2005, 2006). Further investigation is needed to determine whether the *T. labillardierei,* as it is currently circumscribed, is a meaningful species concept.

### 2.4.5 Evidence of incomplete lineage sorting or hybridisation in the evolutionary history of *Tetratheca*

There are several points of conflict between the ITS and *trn*L-*trn*F datasets. Of particular note is the conflict between placements of *Tetratheca ciliata* discussed previously. This conflict occurs between taxa with identical chloroplast sequences. Thompson (1976) stated that hybridisation in *Tetratheca* is extremely rare: she found only three or four specimens

in approximately 3000 examined that displayed morphological characters suggesting hybridisation. More recently, after examining new collections of *T. ciliata* and *T. stenocarpa*, she has proposed that hybridisation may occur more often in *Tetratheca* than previously thought. She has also suggested possible hybrids among several Western Australian taxa. (J. Thompson, pers. comm. 2005).

Further investigation is needed to determine whether the conflicting results between ITS and *trn*L-*trn*F constitute problems with current taxonomic species concepts, or are the result of incomplete lineage sorting, hybridisation or other reticulation events. Given the relatively young age and rapid diversification of the tremand lineage (compared with the rest of Elaeocarpaceae), incomplete lineage sorting could be influencing the molecular results, however, this hypothesis remains to be tested. Chloroplast and nuclear microsatellites are employed in Chapters 3 and 4 to examine phylogeography and population genetic structure within and among populations of the *T. pilosa* group and *T. ciliata*, *T. stenocarpa* in order to investigate processes that may be causing conflict between independent phylogenetic inferences.

## 2.5 Conclusions

Molecular phylogenetic studies have revealed some important relationships within *Tetratheca.* The genus is monophyletic, and the eastern Australian taxa form a clade arising from a grade of western Australian taxa. A Western Australian origin for the lineage is hypothesised and vicariant events that have influenced the southern Australian flora have probably influenced the present distribution of *Tetratheca.* The endemic species on Kangaroo Island do not form a clade, supporting Thompson's (1976) suggestion that *Tetratheca halmaturina* is more closely related to Western Australian species and *T. insularis* to species in the east.

Only two of the eight tested morphological groups proposed by Thompson (1976) are retrieved by the molecular analyses. The remainder of the species groups are either unlikely (i.e. requiring many more steps than the strict consensus trees) or there is insufficient resolution to determine relationships at the species level. Population studies of

two groups will be carried out in Chapters 3 and 4 to determine whether alternative tools can elucidate relationships within *Tetratheca.*

Hybridisation is often put forward as an explanation for conflict between plastid and nuclear analyses. Given the age and relatively rapid diversification of *Tetratheca* incomplete lineage sorting is another possible hypothesis to explain the lack of resolution of the phylogenies as well as points of conflict. The following chapters will investigate phylogeography, population diversity and differentiation in two species complexes in order to explore the influences of these processes on speciation within *Tetratheca.*

# *Chapter 3* Comparative phylogeography of two species complexes within *Tetratheca*

## 3.1 Introduction

Evidence from this study and others (Downing 2005, Crayn et al. 2006) indicates that species of *Tetratheca* in eastern Australia are closely related, and current distribution patterns are the result of complex evolutionary histories. The eastern Australian species of *Tetratheca* are mostly localised endemics, and many are rare or endangered. Only a few species are widespread, some with disjunctions between populations. In the past 220 years, human activities have probably played a role in shaping current distribution patterns of *Tetratheca*. The main centres of diversity are in areas where there has been extensive land clearing and exclusion of fire: around the Sydney Basin and throughout rural Victoria and South Australia.

Conflict between the ITS and *trn*L-*trn*F datasets (presented in Chapter 2) indicates that hybridisation may play a role in the evolution of the genus. The ploidy of *Tetratheca* is unknown, however J. Thompson (pers. comm. 2005) suggested of the eastern Australian taxa that hybridisation may be more common in *Tetratheca* than previously thought. The study of species complexes (groups of morphologically poorly differentiated taxa) provides an ideal opportunity to investigate the utility of novel molecular tools to delineate taxa. The levels of gene flow and strength and distribution of genetic differentiation within and among members of *Tetratheca* have not previously been investigated and may help to resolve taxonomic boundaries in species complexes where previous morphological and molecular study has yielded little resolution. Two complexes from south-eastern Australia, the *Tetratheca pilosa* group and *T. ciliata* plus *T. stenocarpa*, provide a range of distribution patterns, environmental and morphological gradients for investigating the evolutionary history of *Tetratheca*.

An understanding of the distribution and structure of genetic variation of rare or threatened species is particularly important for implementation of conservation strategies since different approaches will be required for genetically heterogeneous and relatively uniform species (Pons and Petit 1995). Both species complexes investigated here are represented by both common and rare species. Comparisons of genetic variation among them will therefore provide insights into the genetic complexities of the genus as a whole.

### 3.1.1 Chloroplast microsatellites

There are many molecular tools available for studies of genetic diversity. Each varies in potential resolution, type of data generated, cost and time effectiveness, and also the taxonomic level at which they are most useful (Avise 1994). Ideally for population studies, a quick, simple and cost effective method that will be variable at the population level is needed, due in particular to the large sample numbers usually required.

Chloroplast markers have been increasingly applied to phylogeographic studies of plants (Taberlet *et al.* 1998, Cavers *et al.* 2003, Aoki *et al.* 2004, Heuertz *et al.* 2006, Pardo *et al.* 2008) and chloroplast microsatellites (cpSSRs) in particular are becoming more widely applied in a large number of genetic and phylogeographic studies. Like the mitochondrial markers widely applied in animal studies, cpSSRs are usually uniparentally inherited, highly polymorphic and abundant. A limitation of chloroplast markers is that the level of variation can often be low due to the slow rate of evolution of the chloroplast genome (Schaal *et al.* 1998, Byrne *et al.* 2002).

In angiosperms, chloroplast DNA is generally maternally inherited (exceptions include *Passiflora* (Hansen *et al.* 2007) and many conifers (Dowling et al. 1996)). Since the chloroplast genome does not recombine it can provide clearer patterns of migration than biparentally inherited, nuclear DNA. Genetic variation detected using maternally inherited chloroplast markers is often more strongly geographically structured than that found with nuclear markers. This is due to chloroplast DNA inheritance occurring via seeds, the movement of which are limited by dispersal mechanisms. In contrast nuclear gene flow results also from pollen dispersal, so the potential for admixture is much higher. Geographic structure is likely also to be more pronounced in chloroplast data because the

effective population size of the chloroplast genome is approximately one quarter that of the nuclear genome (Schaal *et al.* 1998). This means that there is higher potential for sorting of haplotype lineages due to bottlenecks.

The poricidal anthers of *Tetratheca* are indicative of the buzz pollination syndrome (Matthews and Endress 2002), and studies of floral structure and breeding systems suggest that autogamy is rare (Bartier et al. 2001, Gross et al. 2003). Plants are low to the ground (from 5-150 cm), seeds are released as the fruit dessicates and dehisces, and the primary dispersers are ants (Boesewinkel 1999, Bartier et al. 2001). Given the reproductive biology of *Tetratheca* one would expect to find more geographical structure with chloroplast markers than nuclear markers since their seeds are unlikely to be dispersed far.

Since individual chloroplast microsatellite length variants are linked, they can be combined into single haplotypes. Haplotypes can then be analysed cladistically and networks constructed to determine intraspecific phylogenetic relationships (Posada and Crandall 2001). CpSSRs are also useful for detecting hybridisation since haplotypes will be shared among the maternal parent and hybrid offspring (McKinnon *et al.* 2001, McKinnon *et al.* 2004a, McKinnon *et al.* 2004b, Heuertz *et al.* 2006).

Development of universal chloroplast primer sets (e.g. Taberlet *et al.* 1991, Weising and Gardner 1999) has facilitated many studies of intraspecific variation in plants using chloroplast markers. Weising and Gardner's (1999) set of conserved cpSSR PCR primers have been successfully applied to studies in a variety of angiosperm genera including: *Nicotiana* L., *Brassica* L., *Cordyline* Comm. ex R.Br. (Weising and Gardner 1999); *Cytisus* L. (Kang *et al.* 2007) and *Betula* L. (Maliouchenko *et al.* 2007) to name a few. The present study is the first to apply these markers to investigations of phylogeographic patterns in endemic Australian species.

The ten conserved primer pairs, ccmp1-10 (Weising and Gardner 1999), and one pair specifically designed from *trn*L-*trn*F sequence data (Tecp01) are here tested for amplification success and variability. Five loci are used for phylogeographic studies in two species complexes within *Tetratheca*. The first is a study of members of the *Tetratheca pilosa* group (defined in the previous chapter): *T. pilosa* subsp. *pilosa*, *T. pilosa* subsp. *latifolia*, *T. procumbens* and *T. insularis*. The second focuses on *T. ciliata* and *T.*

*stenocarpa*. The results and discussions for each species complex will be presented separately, and then discussed together at the end of the chapter. The specific aim of this chapter is to investigate the utility of chloroplast markers for studies of genetic variability within and among populations of *Tetratheca* in order to:

1. determine whether further taxonomic clarification is possible in closely related groups of species of *Tetratheca*
2. detect phylogeographic patterns in *Tetratheca* to better understand the evolutionary history of the genus
3. determine whether hybridisation is a factor influencing speciation in *Tetratheca*.

## 3.2 Materials and Methods

### 3.2.1 Sampling

There is always a compromise between cost, time and outcome for studies based on a large number of individuals. Muirhead *et al.*(2008) highlight the importance of sampling strategies in chloroplast DNA studies, stating that results and interpretations can be influenced by insufficient sampling. In Pons and Petit's (1995) analysis of chloroplast DNA in oak species, optimal sampling regimes for detecting genetic variation using a single locus are those aiming to capture higher numbers of populations rather than more individuals per population. Since this study seeks to detect genetic structure at a landscape scale, Pons and Petit's (1995) recommendation was followed.

Fifty-nine populations were sampled across the two species complexes. Herbarium data (accessed via Australia's Virtual Herbarium) were used to determine distributions of the target groups so that sampling could aim to capture the geographic ranges of each species as well as their environmental and morphological gradients. Three individuals were sampled at approximately equal intervals along transects through each population. Transects varied in length according to apparent boundaries of each population. Since this study aims to investigate large scale patterns within and among populations of closely

related taxa the sampling strategy was devised to maximise the number of populations across the ranges of each species and each complex.

### 3.2.2 Plant material and DNA isolation

Vouchers for each population were collected and lodged at the National Herbarium of New South Wales. Population information used in this chapter is presented in Table 3.1. Leaf tissue (silica-dried or fresh) was disrupted in the AP1 lysis buffer (Qiagen) using the Qiagen Tissue Lyser and total genomic DNA was extracted using DNeasy plant mini kit or 96DNeasy Plant kit (Qiagen) following the manufacturer's instructions.

### 3.2.3 Chloroplast microsatellites

Universal chloroplast microsatellite primers, ccmp1-10 (Weising and Gardner 1999), were selected for testing (see table 3.2 for details). The *trn*L-*trn*F sequence alignment used for the molecular phylogenetic analyses in Chapter 2 contained a microsatellite region that showed considerable variation among and within species of *Tetratheca* and its allies. An additional primer pair (Tecp01 – see Table 3.2) was designed from the *trn*L-*trn*F region using PRIMER 3 (Rozen and Skaletsky 2000) and analysed for compatibility using NETPRIMER (PREMIER Biosoft International). An M13(-21) universal sequence (5'-TGTAAAACGACGGCCAGT-3') was appended to each of the eleven forward primers to facilitate fluorescent labelling following the method of Schuelke (2000). To avoid expensive custom-made fluorescently labelled primers Schuelke (2000) uses the forward primer with the universal M13(-21) sequence to attach an independent fluorescent label. A benefit of Schuelke's (2000) approach is that much of the optimisation can be done without the fluorescent dyes. Once optimised, the ability to choose any fluorescent label for any forward primer provides much more flexibility for multiplexing and greatly reduces the costs in large scale studies.

**Table 3.1 Population information for the chloroplast microsatellite analysis of the *Tetratheca pilosa* group, *T. ciliata* and *T. stenocarpa***

| Taxon | Pop. code | Locality | State | Latitude | Longitude |
|---|---|---|---|---|---|
| *T. ciliata* Lindl. | BL | Batlow | NSW | -35.56527778 | 148.09083333 |
| *T. ciliata* Lindl. | PR | Paddy River Falls | NSW | -35.86583333 | 148.11361111 |
| *T. ciliata* Lindl. | SW | Saltwater Creek Road | NSW | -37.20194444 | 149.95694444 |
| *T. ciliata* Lindl. | TT | Tumbarumba-Tumut Road | NSW | -35.71194444 | 148.04250000 |
| *T. ciliata* Lindl. | DH | Dukes Highway | SA | -36.58555556 | 140.72333333 |
| *T. ciliata* Lindl. | CB | Canadian Ballarat | VIC | -37.58083333 | 143.89472222 |
| *T. ciliata* Lindl. | CR | Cann River | VIC | -37.59305556 | 148.96694444 |
| *T. ciliata* Lindl. | DR | Daylesford Road | VIC | -37.36027778 | 144.06611111 |
| *T. ciliata* Lindl. | FH | Frog Hollow | VIC | -37.30722222 | 144.16305556 |
| *T. ciliata* Lindl. | KH | Kinglake to Hurstville Road | VIC | -37.54691667 | 145.33613889 |
| *T. ciliata* Lindl. | LS | Lima South | VIC | -36.83000000 | 145.94888889 |
| *T. ciliata* Lindl. | MF | Mackenzies Falls | VIC | -37.11166667 | 142.39916667 |
| *T. ciliata* Lindl. | MtD | Mount Difficult 2 | VIC | -37.12638889 | 142.49944444 |
| *T. ciliata* Lindl. | MV | Murray Valley Highway | VIC | -36.15083333 | 147.46194444 |
| *T. ciliata* Lindl. | PG | Poverty Gully | VIC | -37.08277778 | 144.22388889 |
| *T. ciliata* Lindl. | RH | Reef Hills State Park | VIC | -36.60444444 | 145.93777778 |
| *T. ciliata* Lindl. | SCR | Sardine Creek Road | VIC | -37.39083333 | 148.67861111 |
| *T. ciliata* Lindl. | VRR | Victoria Range Road | VIC | -37.23333333 | 142.30000000 |
| *T. ciliata* Lindl. | WR | Wallaby Rocks | VIC | -37.12472222 | 142.30138889 |
| *T. insularis* Joy Thomps. | BHR | Bark Hut Road 1 | KI | -35.71277778 | 137.30277778 |
| *T. insularis* Joy Thomps. | K2 | Playford to South Coast Road | KI | -35.81638889 | 136.97750000 |
| *T. insularis* Joy Thomps. | SR1 | Shackle Road 1 | KI | -35.91611111 | 136.72194444 |
| *T. insularis* Joy Thomps. | SR3 | Shackle Road 3 | KI | -35.79777778 | 136.76583333 |
| *T. insularis* Joy Thomps. | SB | Stokes Bay Road | KI | -35.90944444 | 136.99055556 |
| *T. pilosa* subsp. *latifolia* Joy Thomps. | GR1l | Gray | TAS | -41.80980556 | 148.11275000 |
| *T. pilosa* subsp. *latifolia* Joy Thomps. | KR | Kerrisons Road | TAS | -41.23172222 | 146.71161111 |
| *T. pilosa* subsp. *latifolia* Joy Thomps. | NR | Nugent Road | TAS | -42.75116667 | 147.79077778 |
| *T. pilosa* subsp. *latifolia* Joy Thomps. | NL | Nunamara to Lilydale Road | TAS | -41.36725000 | 147.25480556 |
| *T. pilosa* subsp. *latifolia* Joy Thomps. | BT | Bobs Track | VIC | -37.54769444 | 149.70086111 |

| Taxon | Pop. code | Locality | State | Latitude | Longitude |
|---|---|---|---|---|---|
| *T. pilosa* subsp. *latifolia* Joy Thomps. | CCJ | Captain Creek Jetty Road | VIC | -37.54486111 | 149.71005556 |
| *T. pilosa* subsp. *latifolia* Joy Thomps. | CT | Collins Track | VIC | -37.52305556 | 147.88472222 |
| *T. pilosa* subsp. *latifolia* Joy Thomps. | GCT | Genoa Creek Track | VIC | -37.48377778 | 149.54875000 |
| *T. pilosa* subsp. *latifolia* Joy Thomps. | PL | Painted Line Track | VIC | -37.65166667 | 148.22444444 |
| *T. pilosa* subsp. *latifolia* Joy Thomps. | TR | Tara Range | VIC | -37.58833333 | 148.18833333 |
| *T. pilosa* subsp. *pilosa* Labill. | VH | 23.5 km from Victor Harbour | SA | -35.55055556 | 138.40472222 |
| *T. pilosa* subsp. *pilosa* Labill. | MH | Mosquito Hill | SA | -35.40555556 | 138.67166667 |
| *T. pilosa* subsp. *pilosa* Labill. | ML | Mount Lofty | SA | -34.98777778 | 138.70444444 |
| *T. pilosa* subsp. *pilosa* Labill. | RR | Range Road | SA | -35.57361111 | 138.27722222 |
| *T. pilosa* subsp. *pilosa* Labill. | WP | Waitpinga turnoff | SA | -35.57916667 | 138.56333333 |
| *T. pilosa* subsp. *pilosa* Labill. | SS | 3 km W of Swansea | TAS | -42.01308333 | 147.90147222 |
| *T. pilosa* subsp. *pilosa* Labill. | BR | Bensemans Road | TAS | -41.41097222 | 146.79161111 |
| *T. pilosa* subsp. *pilosa* Labill. | BBR | Black Bobs Rivulet | TAS | -42.38858333 | 146.58877778 |
| *T. pilosa* subsp. *pilosa* Labill. | CV | Collinsvale | TAS | -42.84452778 | 147.17386111 |
| *T. pilosa* subsp. *pilosa* Labill. | CRT | Coronation Road | TAS | -43.08569444 | 147.87419444 |
| *T. pilosa* subsp. *pilosa* Labill. | GR1p | Gray | TAS | -41.80980556 | 148.11275000 |
| *T. pilosa* subsp. *pilosa* Labill. | KD | Kallista Drive | TAS | -43.03144444 | 147.07525000 |
| *T. pilosa* subsp. *pilosa* Labill. | OH | Longley | TAS | -42.96800000 | 147.20175000 |
| *T. pilosa* subsp. *pilosa* Labill. | PT | Pipeline Track | TAS | -42.91994444 | 147.26925000 |
| *T. pilosa* subsp. *pilosa* Labill. | RB | Rubicon Bridge | TAS | -41.23941667 | 146.55927778 |
| *T. pilosa* subsp. *pilosa* Labill. | WRT | Waterworks Road | TAS | -42.91708333 | 147.29211111 |
| *T. procumbens* Hook.f. | BLT | Bronte Lagoon | TAS | -42.15969444 | 146.38330556 |
| *T. procumbens* Hook.f. | BG | Butlers Gorge | TAS | -42.29188889 | 146.36163889 |
| *T. procumbens* Hook.f. | FG | Franklin-Gordon Rivers NP | TAS | -42.17219444 | 146.18147222 |
| *T. procumbens* Hook.f. | IB | Ida Bay State Reserve | TAS | -43.44411111 | 146.90275000 |
| *T. procumbens* Hook.f. | LR | Lune River | TAS | -43.43891667 | 146.91519444 |
| *T. stenocarpa* J.H. Willis | EB | East Beenak Road | VIC | -37.91936111 | 145.62980556 |
| *T. stenocarpa* J.H. Willis | FRV | Forest Road | VIC | -37.99863889 | 145.81169444 |
| *T. stenocarpa* J.H. Willis | OCC | Old Chum Creek Road | VIC | -37.57061111 | 145.47622222 |
| *T. stenocarpa* J.H. Willis | TP | Tentpole Road | VIC | -37.91094444 | 145.59791667 |

**Table 3.2 Details of eleven chloroplast microsatellite loci used in the present study**

| Primer name | Primer sequence 5' – 3' | Estimated size (bp) |
|---|---|---|
| ccmp1 | Fwd: CAGGTAAACTTCTCAACGGA<br>Rev: CCGAAGTCAAAAGAGCGATT | 139* |
| ccmp2 | Fwd: GATCCCGGACGTAATCCTG<br>Rev: ATCGTACCGAGGGTTCGAAT | 189* |
| ccmp3 | Fwd: CAGACCAAAAGCTGACATAG<br>Rev: GTTTCATTCGGCTCCTTTAT | 112* |
| ccmp4 | Fwd: AATGCTGAATCGAYGACCTA<br>Rev: CCAAAATATTBGGAGGACTCT | 126* |
| ccmp5 | Fwd: TGTTCCAATATCTTCTTGTCATTT<br>Rev: AGGTTCCATCGGAACAATTAT | 121* |
| ccmp6 | Fwd: CGATGCATATGTAGAAAGCC<br>Rev: CATTACGTGCGACTATCTCC | 103* |
| ccmp7 | Fwd: CAACATATACCACTGTCAAG<br>Rev: ACATCATTATTGTATACTCTTTC | 133* |
| ccmp8 | Fwd: TTGGCTACTCTAACCTTCCC<br>Rev: TTCTTTCTTATTTCGCAGDGAA | 77* |
| ccmp9 | Fwd: GGATTTGTACATATAGGACA<br>Rev: CTCAACTCTAAGAAATACTTG | 98* |
| ccmp10 | Fwd: TTTTTTTTTAGTGAACGTGTCA<br>Rev: TTCGTCGDCGTAGTAAATAG | 103* |
| Tecp01 | Fwd: CGGGACTCTATCTTTATTCTTG<br>Rev: CGTACTGAAATACTATCTCAAATG | 221** |

*based on *Nicotiana* **based on *Tetratheca*

### 3.2.4 Locus screening I: amplification using PCR

Initial PCR tests were conducted to determine amplification success and product size for each amplifiable locus. PCR amplifications followed a protocol modified from (Schuelke 2000) in which PCR products are fluorescently labelled with a third, dye-labelled M13 primer. DNA from one to three individuals of *Tetratheca ciliata*, *T. stenocarpa*, *T. pilosa* subsp. *latifolia* and *T. procumbens* was tested. A further 24 individuals were amplified for loci ccmp7 and ccmp10 to determine whether polymorphisms could be detected within and among populations and species.

Each 15 μL PCR reaction contained 1.5 μL 10x NH4 buffer (Bioline, Luckenwalde, Germany), 1.5–2.0 mmol MgCl2, 0.2 mM of each dNTP and 0.06 units BIOTAQ™ DNA polymerase (Bioline, Luckenwalde, Germany), 0.05 μM of the M13 forward primer and 0.2uM each of the reverse primer and fluorescent M13 primer (labelled with FAM, NED or VIC). The PCR profile used was: one cycle of 94ºC incubation for 5 min; 30 cycles of

94ºC denaturation for 30 s; annealing at 55ºC for 45 s; and extension at 72ºC for 45 s; eight denaturation cycles of 94ºC for 30 s; annealing at 53ºC for 45 s; and extension at 72ºC for 45 s. A final extension was performed at 72ºC for 5 min.

All PCR amplifications used a CP2-03 Thermal Cycler (Corbett Research, Mortlake, Australia) or Veriti Thermal Cycler (Applied Biosystems, Foster City, California, USA). Two of three amplification products were multiplexed and diluted 1 in 40. They were then detected on an ABI 3730 Sequencer (Applied Biosystems) at the Automated DNA Sequencing Facility at UNSW. Microsatellite profiles were examined using Genemapper (v3.7, Applied Biosystems).

### 3.2.5 Locus screening II: multiplexing PCR reactions

Multiplexing PCR reactions (as well as amplification products) can minimise costs so a test of Qiagen Multiplex mix was conducted. PCR amplification followed a method modified from Schuelke (2000) and the manufacturer's protocol as outlined in the subsequent paragraph. Eight microsatellite loci were selected after initial screening and multiplexed in three combinations as follows: Tecp01, ccmp2, ccmp10; ccmp1, ccmp8, ccmp9; ccmp3, ccmp4. Eight individuals of *Tetratheca ciliata* were tested for multiplexing using a gradient of annealing temperatures (in three degree increments) from 57 - 63ºC.

Each 10µL multiplex reaction contained 5µL Multiplex PCR mix (Qiagen), 0.05µM of each forward primer, 0.2µM of each reverse primer, and fluorescent M13 primer (FAM, NED or VIC dyes) at half the total concentration of the reverse primers. The cycling profile was: 15min incubation at 94ºC; 38 cycles of denaturation (30s at 94ºC), annealing (1min 30s at 57, 60 or 63ºC for 30 cycles and 53ºC for the last eight cycles) and extension (45s at 72ºC); final 30min incubation at 60ºC. Electrophoresis of products was carried out at 150V for 30 min in 2% agarose gels. After staining in ethidium bromide products were visualised on an ultra-violet transilluminator.

### 3.2.6 Chloroplast microsatellite analysis

One hundred and seventy-seven individuals from the two species complexes were used for cpSSR analysis of five loci. Locus ccmp3 was amplified individually, ccmp2 multiplexed with ccmp10 and ccmp4 with Tecp01. A further 12 individuals, including three to six from each of *Tetratheca neglecta*, *T. rubioides* and *T. halmaturina*, were also tested for amplification. The PCR profile used the multiplexing profile outlined in the previous section with annealing temperature set at 57ºC. PCR products were then diluted 1 in 40 and multiplexed (all 5 loci together) for genotyping. Amplification products were detected on an ABI 3730 Sequencer as outlined previously and microsatellite profiles were sized and scored manually using Genemapper (v3.7, Applied Biosystems, Foster City, California, USA).

Pompanon *et al.* (2005) highlighted the importance of considering, testing and reporting errors in genotyping studies. They recommend conducting blind replicates of 5-10% of the samples depending on the nature of the study. In some cases (particularly where DNA quality is low) they suggest that systematic replication of some samples is required to adequately assess error rates. Thirty-two to 54 individuals (16-30 percent of the total sampled) from the present study were repeated for each locus to determine reliability of the genotyping results. The error rate was 1.04%.

### 3.2.7 Data analysis

**Population diversity indices and haplotype sharing**

Alleles for each individual at each locus were visualised by mapping genotyping data onto distribution maps for each of the species complexes. Since the chloroplast genome does not recombine, a unique combination of alleles for the five loci was defined as a haplotype. The following population diversity indices were calculated using GenAlEx 6.1 (Peakall and Smouse 2006): number of haplotypes ($N$), number of private haplotypes ($N_P$), and unbiased diversity (uh – equivalent to (N / (N-1)) * h, or Nei's (1987) $H_E$).

**Geographical structure of genetic variation**

Geographic structure is defined by the following hypothesis: that closely related haplotypes are found in the same geographic areas more often than would be expected by chance. An understanding of the geographic patterns of genetic diversity, particularly within and among closely related taxa, can provide insights into the historical and ecological processes that have shaped their evolution, particularly if concurrent patterns are found across a range of lineages.

The programs PERMUT and CPSSR (Pons and Petit 1996, Burban *et al.* 1999) were used to determine whether phylogeographical structure is present within the target species and species complexes. The tests apply a permutation approach to determine whether $N_{ST}$ or $R_{ST}$ are significantly greater than $G_{ST}$, in which case phylogeographic structure is present in the dataset. Since only five loci are used in the present study both $R_{ST}$ and $N_{ST}$ were calculated; significance was determined over 1000 permutations of $N_{ST}$ and $R_{ST.}$

Weir and Cockerham's (1984) measure, $F_{ST,}$ is commonly used to determine the degree of population structure between demes. When sample sizes are identical for all populations, $G_{ST}$ (Pons and Petit 1995) is equivalent to Weir and Cockerham's $F_{ST.}$ $N_{ST}$ and $R_{ST}$ are alternative estimators for $F_{ST}$ and are used to detect diversity in microsatellite data. Unlike $F_{ST}$ or $G_{ST}$, which are based on frequency of haplotypes, parameters $N_{ST}$ and $R_{ST}$ also take into account the distances between haplotypes. $R_{ST}$ (Slatkin 1995) also accounts for the step-wise mode of evolution of microsatellites, therefore is often applied to studies employing chloroplast microsatellites (Petit *et al.* 2005). When the number of loci analysed is low (<20) Gaggiotti *et al*. (1999) suggest that $R_{ST}$ may be less reliable than $F_{ST.}$

Values for $N_{ST}$ and $R_{ST}$ are generally higher than $G_{ST}$ (Petit et al. 2005) and this comparison can be tested for significance using the permutation approach of Pons and Petit (1996) and Burban *et al.* (1999). If $N_{ST}$ or $R_{ST}$ is larger than $G_{ST}$ and $N_{ST}$ or $R_{ST}$ is significantly greater than the permuted $N_{ST}$ or $R_{ST}$ values, phylogeographical structure is present in the dataset. This approach was used to analyse populations of each species and subspecies individually, apart from *Tetratheca procumbens* since all populations shared a single

haplotype. *Tetratheca pilosa* with *T. procumbens*, and the *T. pilosa* species complex as a whole, were also tested since the taxa appear to be very closely related and the specific limits are unclear. *Tetratheca ciliata* and *T. stenocarpa* were analysed individually and together.

**Analysis of molecular variance (AMOVA)**

Analysis of Molecular Variance (AMOVA) was introduced by (Excoffier *et al.* 1992) to determine partitioning of variance components in haploid allele frequencies. AMOVA tests (based on pair-wise $F_{ST}$ values) were conducted using GenAlEx 6.1 (Peakall and Smouse 2006) to determine the relative contributions of variance components within and between populations, species, species complexes, and geographic regions, to the structure of genetic variation. Graphical outputs were obtained as pie charts from GenAlEx.

**Spatial AMOVA (SAMOVA)**

Spatial Analysis of Molecular Variance (SAMOVA) was developed in 2002 by Dupanloup *et al.* to define population groups (number of groups = K) that are maximally differentiated from one another. That is, groups of populations that maximise the proportion of total genetic variance ($F_{CT}$ index). Groups are defined on the basis of genetic and spatial data with no *a priori* assumptions about group boundaries. Populations that are genetically homogeneous and geographically adjacent are thus assigned to groups. Since this method makes no assumptions about Hardy-Weinberg equilibrium within populations, nor linkage equilibrium between loci, it can be applied to both genotypic and haplotypic data.

SAMOVA was used to define population groups for the two species complexes, and to identify the location of genetically unique groups among the populations tested. Since the species appear to be very closely related and taxonomic boundaries are unclear, species were analysed with species pooled into complexes as well as most species individually. *Tetratheca procumbens* was not analysed individually since only two haplotypes are present. *Tetratheca insularis* and *T. stenocarpa* were not analysed individually since they are only represented by five and four populations respectively. The program SAMOVA 1.0

(Dupanloup *et al.* 2002) was used to perform 10,000 iterations for K=2-8 using 100 initial conditions. SAMOVA requires two input files: one for genetic data of individuals grouped into populations, and the other containing the geographic coordinates of each population. Genetic input files were created using microsatellite allele length data from which SAMOVA computes a distance matrix (sum of squared size differences – $R_{ST}$) for subsequent analyses.

**Median spanning Networks**

To determine phylogenetic relationships between populations and closely related species median spanning networks (Bandelt *et al.* 1999) were constructed using the microsatellite tandem repeat (YSTR) format in Network 4.2. Network 4.2 combines alleles into single haplotypes for the analysis. The parameter epsilon (ε) was set to zero so that each branch constitutes the minimum number of mutational differences between haplotypes under a step-wise mutation model (Bandelt et al. 1999). One haplotype network was coloured according to current specific and subspecific concepts, and a second network for each species complex was coloured according to geography. Branch lengths were not drawn proportional to the number of mutations between haplotypes. All unmarked branches represent single mutations, and for all remaining branches the number of mutations is indicated. Each haplotype is represented in the network by a circle, and circle areas are proportional to the frequency of individuals sharing the haplotype. Intermediate evolutionary steps invoked between haplotypes are indicated as simple connections between lines rather than circles.

**Fu's F test of selective neutrality**

Fu's (1997) F test of selective neutrality calculates the statistic $F_S$ and then tests, using a coalescent simulation algorithm, whether $F_S$ calculated from observed data differs significantly from $F_S$ calculated using random samples under the assumptions of selective neutrality and population equilibrium. P-values are determined by proportion of random $F_S$ less than or equal to the observed statistics and p-values below 0.02 are considered

significant at the 5% level. Large negative $F_S$ values indicate population demographic expansion due to a large number of recent mutations (indicated by excess rare haplotypes).

Fu's $F_S$ was calculated using Arlequin 2.0 (Schneider *et al.* 1999). Microsatellite data were binary coded following Pereira *et al.* (2002) and significance was assessed using 1000 simulated samples.

## 3.3 *Tetratheca pilosa* group

### 3.3.1 Introduction

*Tetratheca pilosa* currently comprises two subspecies: the first, *T. pilosa* subsp. *pilosa* is common on the Fleurieu Peninsula in South Australia and widespread throughout Tasmania. Herbarium data indicates that *T. pilosa* subsp. *pilosa* also occurs across the western half of Victoria, however, despite targeted searches no plants of this species were found in Victoria. The second subspecies, *T. pilosa* subsp. *latifolia*, is distributed throughout eastern Victoria and is less commonly found in northern and eastern Tasmania. The two subspecies are morphologically similar, separated by leaf shape and ovary pubescence (Thompson 1976, Jeanes 1996). Both characters vary widely within each subspecies. For the most part they are morphologically distinct on the mainland of Australia but in Tasmania there is considerable morphological overlap. On the mainland of Australia the two subspecies are geographically separated, however their ranges coincide in Tasmania (Thompson 1976, Jeanes 1996, Australia's Virtual Herbarium). Jeanes (1996) states that intermediates between the two have been observed in Tasmania.

Thompson (1976) suggested that the taxonomy of *Tetratheca pilosa* and a number of closely related taxa require further investigation as the species boundaries are unclear. The remaining taxa in this complex are: *T. rubioides*, a narrow endemic in the Blue Mountains; *T. neglecta*, restricted to the southern part of the Central Coast Botanical Division (*sensu* Harden 1990); *T. insularis*, one of two endemic species from Kangaroo Island; and *T. gunnii*, a critically endangered species (listed under the Environment Protection and Biodiversity Conservation Act 1999) narrowly distributed in northern Tasmania. In 1996,

Jeanes reinstated *T. procumbens*, another closely related species that occurs widely in central and southern Tasmania and is known from only a few collections from alpine areas in Victoria. This is included among the *Tetratheca pilosa* group for the purposes of the present study. Examples of *T. pilosa* and *T. procumbens* are shown in Figure 3.1 and Figure 3.2.

Thompson argued that *Tetratheca procumbens* represents a procumbent form of *T. pilosa*, and she suggested that morphological differences could be a product of environment. Most specimens she sighted for her 1976 treatment of *Tetratheca* appeared closely to resemble *T. pilosa* subsp. *pilosa* but samples from the Moroka Range in the highlands of Victoria were more similar to *T. pilosa* subsp. *latifolia*. Downing's (2005) phenetic analysis of the two *T. pilosa* subspecies and *T. procumbens* found considerable overlap. She suggested that the boundaries, particularly between the two *T. pilosa* subspecies, may be artificial. The phylogenetic analyses in chapter two of the present study did not resolve any of these taxa as monophyletic.

The species in the *Tetratheca pilosa* group represent a group of closely related taxa with a range of distribution patterns from widespread, discontinuous and narrow to isolated populations. An understanding of genetic diversification within and between species in this complex will help to elucidate the evolutionary history that has shaped the current taxonomic and distribution patterns of *Tetratheca*.

**Figure 3.1** ***Tetratheca pilosa*** **habitat (left) and habit (right)**

**Figure 3.2** ***Tetratheca procumbens***

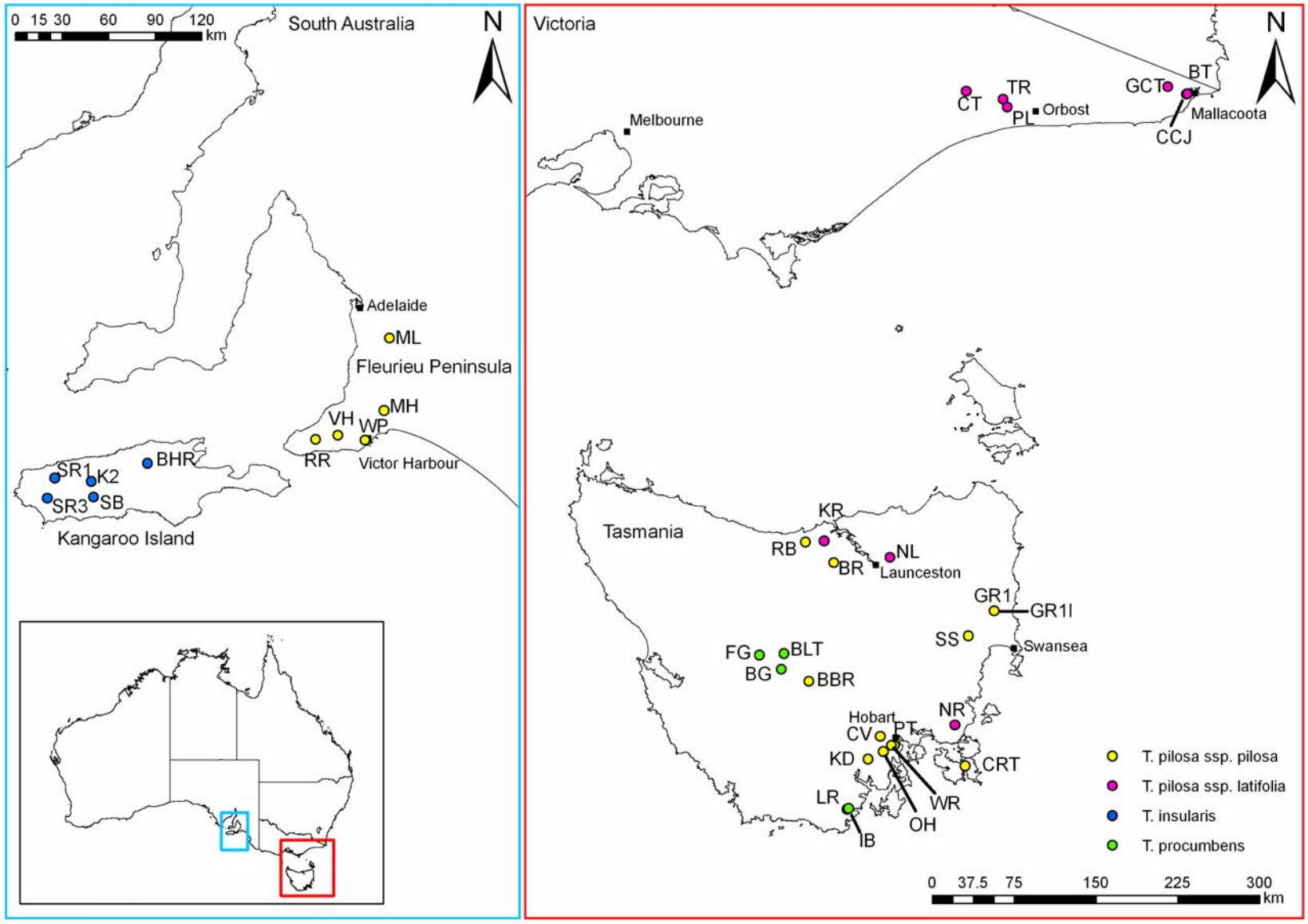



**Figure 3.3 Locations of populations sampled for chloroplast microsatellite study of the *Tetratheca pilosa* group.**

### 3.3.2 Sampling

Thirty-six populations (three individuals from each) were sampled from the *Tetratheca pilosa* group including sixteen populations of *T. pilosa* subsp. *pilosa* and ten of *T. pilosa* subspecies *latifolia*; five of *T. procumbens* and five populations of *T. insularis* (see Figure 3.3). *Tetratheca gunnii* from Northern Tasmania was not sampled due to its national status as critically endangered. Only one population of *T. neglecta* was located and only part of the range of *T. rubioides* was sampled, therefore these taxa were omitted from population-level studies. The four taxa included, however, reflect wide morphological gradients and medium to extreme environmental and geographic gradients, and therefore provide a good overview of patterns within the group as a whole. No samples of *T. procumbens* were located in Victoria despite targeted searches. *Tetratheca* species are often difficult to locate when not in flower and *T. procumbens* is small and rare so it is possible that it was over-looked.

### 3.3.3 Chloroplast microsatellite analysis

A total of one hundred and eight individuals from 36 populations among the four taxa were genotyped. Data analyses were performed as outlined in section 3.2.7 of the current chapter. For alleles that did not amplify, missing data points were estimated as the most common allele in the population. Where this was not clear the most common allele occurring among populations was used. An additional three taxa (*Tetratheca neglecta* and *T. rubioides* from the *Tetratheca pilosa* group and *T. halmaturina* from Kangaroo Island) were genotyped to test the utility of the markers but were not included in any of the data analyses due to insufficient sampling as outlined previously.

### 3.3.4 Results

All five loci were polymorphic across the four taxa, except for ccmp10 which was polymorphic within one population of *Tetratheca insularis* only (see Appendix 3).

Thirty-one alleles were identified across the five loci for the 108 individuals. Alleles occurred as mononucleotide length variants at ccmp3, ccmp4 and Tecp01. Lengths of alleles at ccmp2 and ccmp10 varied from 144-182 and 109-122 base pairs respectively, with large disjunctions between the extremes of the ranges. It may be that length variants at these two loci are not simple step-wise mutations of a repeat unit. Locus ccmp2 was the most variable with 11 alleles. Eight alleles were identified for Tecp01, five for each of ccmp3 and ccmp4 and two for ccmp10.

An average of 6.8% of individuals failed to amplify across the five loci. The highest percentages of missing data were recorded from ccmp10 (11%) and ccmp2 (15%), approximately 4% of individuals failed to amplify at ccmp3 and ccmp4, and all individuals at Tecp01 amplified successfully. Missing data were interpolated conservatively as described in the previous section. It is therefore possible that diversity levels detected by this study represent a slight underestimate of actual diversity levels. The extra taxa tested, *Tetratheca neglecta, T. rubioides* and *T. halmaturina* also amplified successfully with polymorphic fragments detected at one, three and two loci respectively (see Appendix 3). These tools would be appropriate for a future, expanded chloroplast microsatellite study.

The distributions of alleles by locus and population are presented in Figure 3.4, Figure 3.5, Figure 3.6, Figure 3.7 and Figure 3.8. Each circle represents a population and a third of each circle an individual. Shared alleles are indicated by like colours at individual loci. There are 11 private alleles across the five loci: six occur in populations of *T. insularis*, two in each of two populations of *T. pilosa* subsp. *pilosa* and *T. pilosa* subsp. *latifolia*, and one in *T. procumbens*.

There are very few geographic patterns evident in the distribution of alleles. The most common allele at each of four loci occurs in all of the major areas sampled: Kangaroo Island, the Fleurieu Peninsula, eastern Victoria and Tasmania. At ccmp3 the most common allele is present in all populations except on the Fleurieu Peninsula. This is one of several examples where the peninsula is distinct from Kangaroo Island. Despite some allele-sharing between the two regions there are alleles at every locus that occur on Kangaroo Island and not on the Fleurieu Peninsula. At all loci except ccmp4, 1-3 of the differences are due to unique alleles on Kangaroo Island. At all loci except

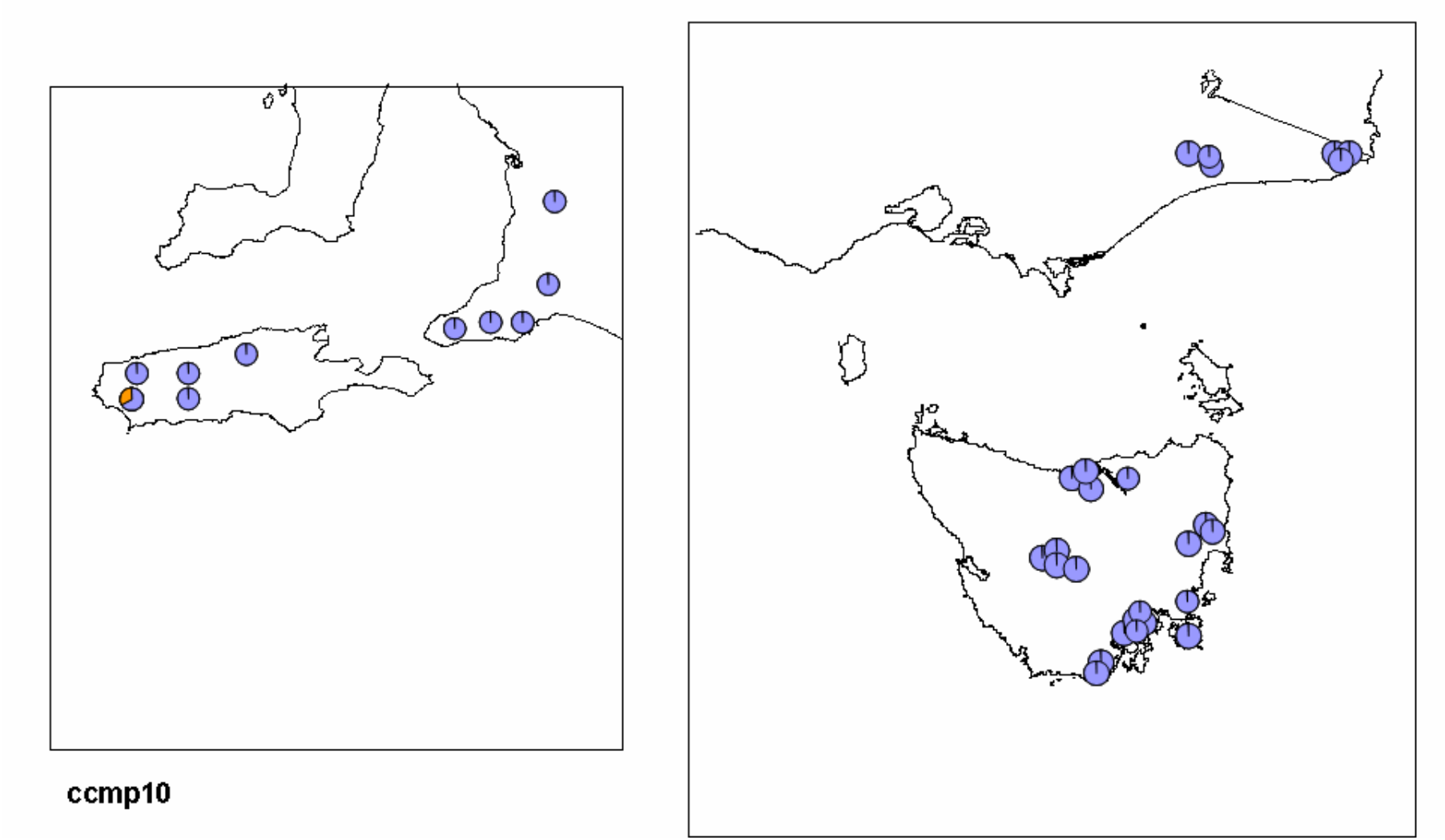



**Figure 3.4 Distribution of alleles at locus ccmp10**

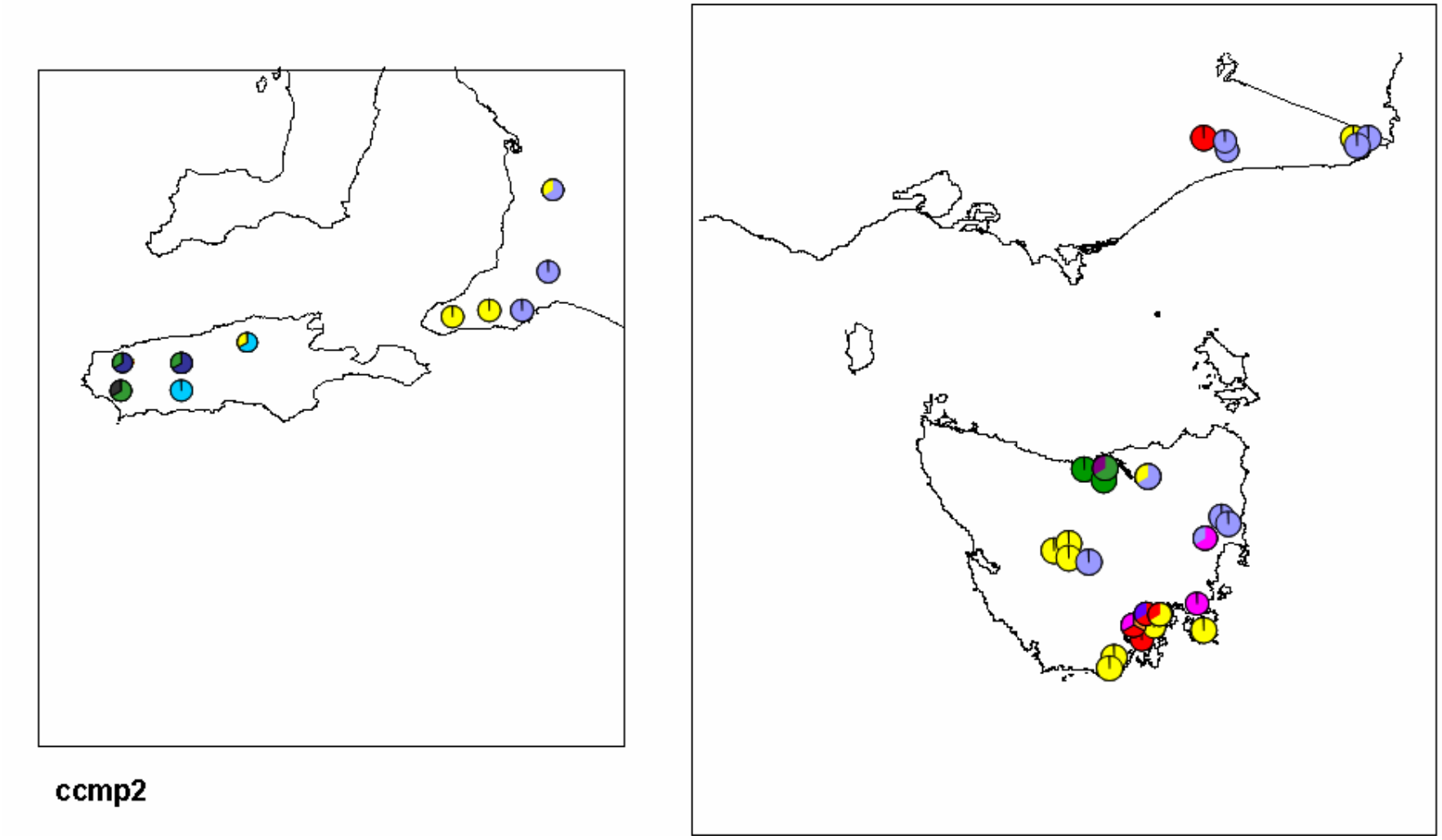



**Figure 3.5 Distribution of alleles at locus ccmp2**

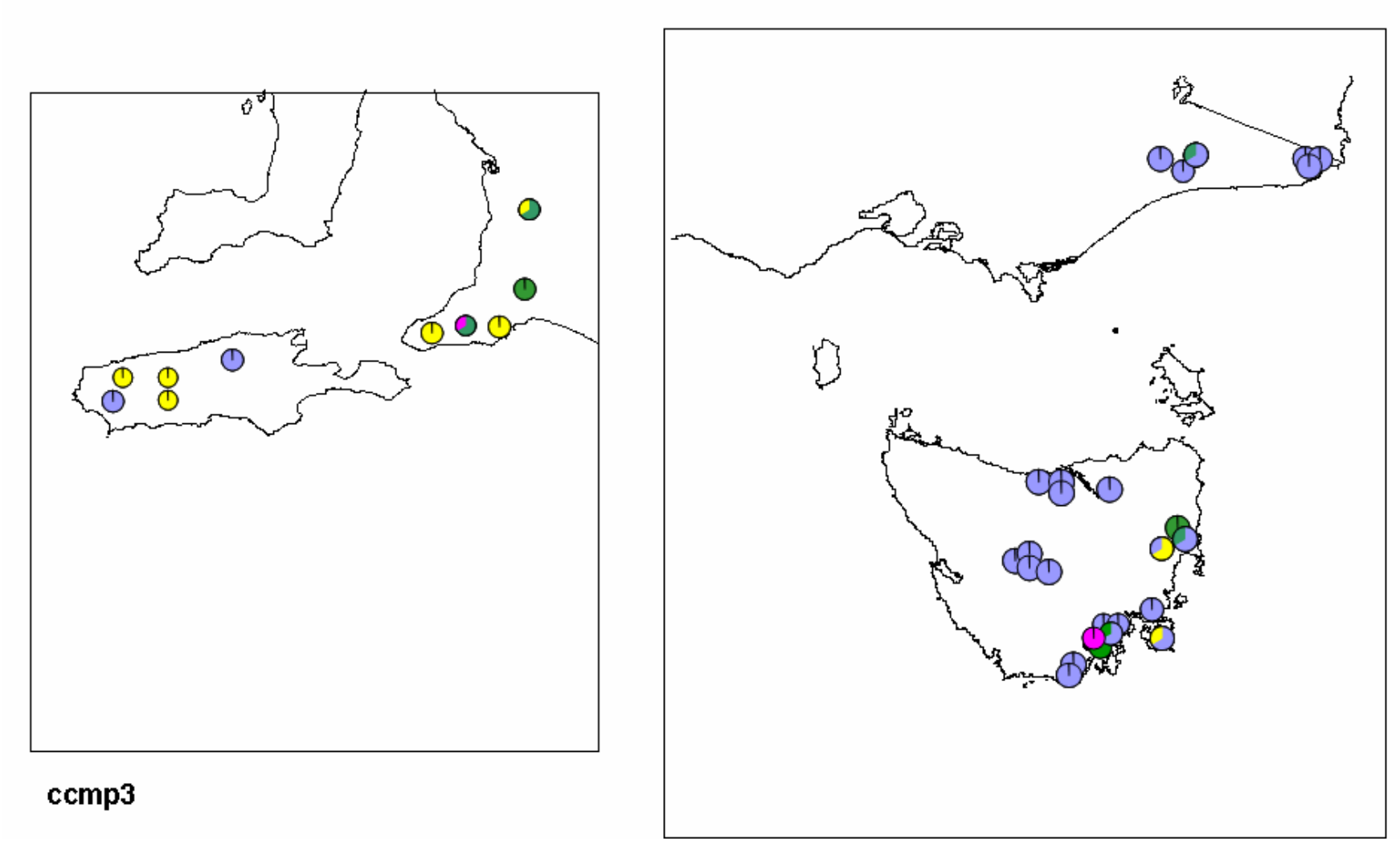



**Figure 3.6 Distribution of alleles at locus ccmp3**

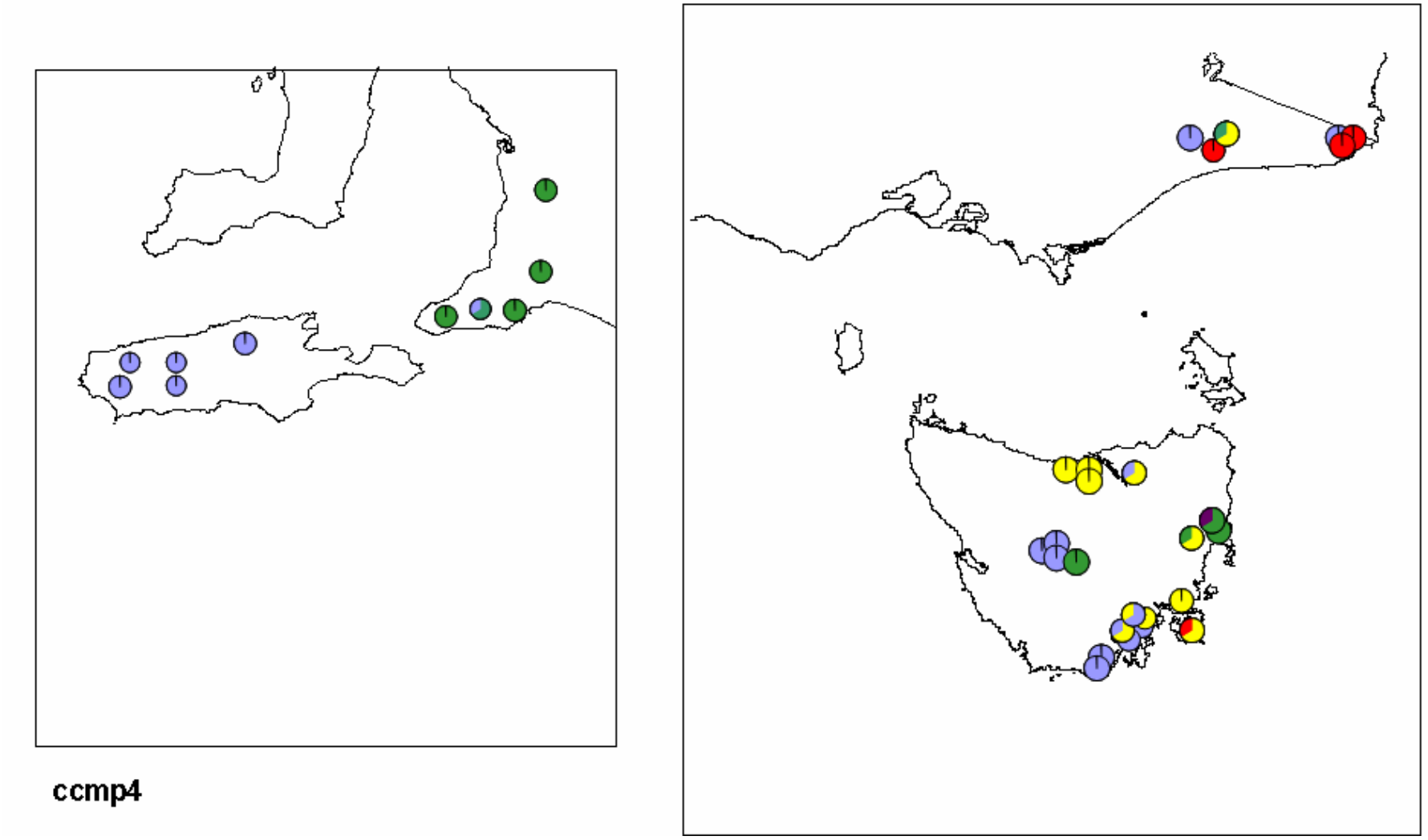



**Figure 3.7 Distribution of alleles at locus ccmp4**

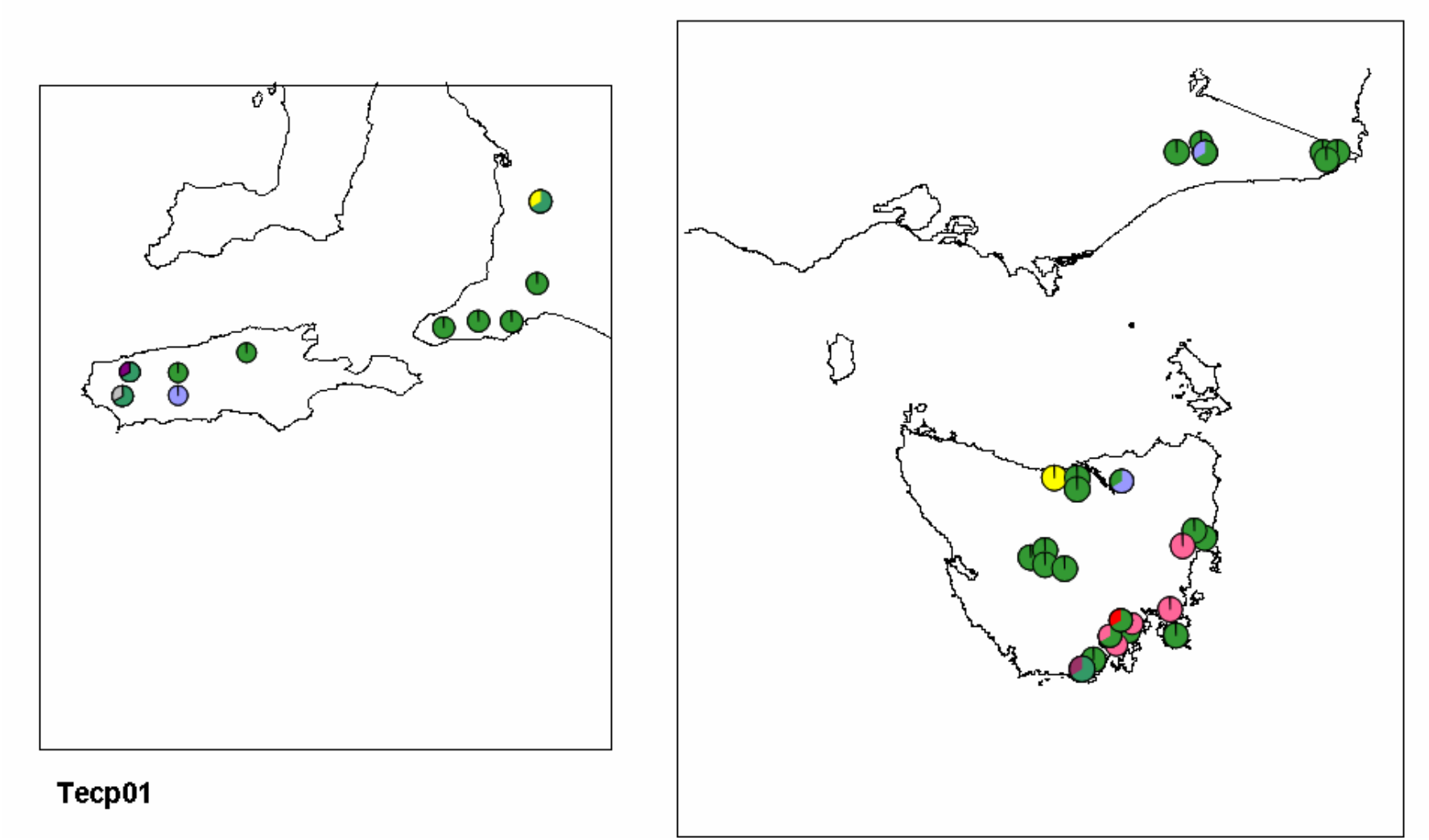



**Figure 3.8 Distribution of alleles at locus Tecp01**

ccmp10 there are alleles present on Kangaroo Island (and not the Fleurieu Peninsula) that are shared with populations of both subspecies of *T. pilosa* in Victoria and/or Tasmania.

Only two alleles were detected at locus ccmp10: both are present on Kangaroo Island but only one among the Fleurieu Peninsula, eastern Victoria and Tasmania. At all remaining loci allelic diversity is highest on Kangaroo Island and Tasmania.

Thirty-six haplotypes were defined by combining the 31 alleles among the five loci (see Appendix 4 for haplotype descriptions and Table 3.3 for distribution and frequency of haplotypes within and among populations and taxa). Haplotype 16 is the most common haplotype and occurs in all populations of *Tetratheca procumbens*, two populations each of *T. pilosa* subsp. *pilosa* and *T. pilosa* subsp. *pilosa* but is not present in any populations of *T. insularis*. Only three other haplotypes (haplotypes 6, 22 and 28) were shared among taxa: each occurring in both *T. pilosa* subsp. *pilosa* and *T. pilosa* subsp. *latifolia*. There are eight haplotypes shared among two or more populations and 18 haplotypes shared among individuals within one or more populations.

Table 3.4 presents diversity statistics by population. The number of haplotypes per population varied from 1-3 with averages of 1.8, 1.6, 1.6 and 1.2 for *Tetratheca insularis*, *T. pilosa* subsp. *latifolia*, *T. pilosa* subsp. *pilosa* and *T. procumbens* respectively. All populations of *T. insularis* contained private haplotypes (average 1.5) and private haplotypes were also detected in most populations of *T. pilosa* subsp. *latifolia* and *T. pilosa* subsp. *pilosa* (each averaging 0.9). The average number of private alleles for *T. procumbens* was much lower (0.2) since only one population contained more than one haplotype.

Unbiased diversity ranged from 0-0.667. The average diversity for *Tetratheca insularis* was highest (0.556) while diversity indices for *T. pilosa* subsp. *latifolia* and *T. pilosa* subsp. *pilosa* were 0.367 and 0.333 respectively. Diversity in *T. procumbens* was much lower than the other taxa with a value of 0.133. This is because four of five populations were fixed for a single haplotype. There was also zero genetic diversity in five populations of *T. pilosa* subsp. *latifolia* and nine populations of *T. pilosa* subsp. *pilosa*.

**Table 3.3 Number and distribution of haplotypes within and among populations and taxa.**

| | *T. insularis* | | | | | *T. pilosa* ssp. *latifolia* | | | | | | | | | *T. pilosa* ssp. *pilosa* | | | | | | | | | | | | | | | *T. procumbens* | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **H** | **BHR** | **K2** | **SB** | **SR1** | **SR3** | **BT** | **CCJ** | **CT** | **GCT** | **GR11** | **KR** | **NL** | **NR** | **PL** | **TR** | **BBR** | **BR** | **CRT** | **CV** | **GR1p** | **KD** | **MH** | **ML** | **OH** | **PT** | **RB** | **RR** | **SS** | **VH** | **WP** | **WR** | **BG** | **BLT** | **FG** | **IB** | **LR** |
| 1 | | | | 1 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 2 | | | | | | | | | | | 1 | | | | | | | | | | | | | | | | | | | | | | | | | |
| 3 | | | | | 1 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 4 | | 1 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 5 | | | | 2 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 6 | | | | | | | | | | | 2 | | | | | | 3 | | | | | | | | | | | | | | | | | | | |
| 7 | | | | | | | | | | | | | | | | | | | | | | | | | 3 | | | | | | | | | | | |
| 8 | | 2 | | | 2 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 9 | 2 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 10 | | | 1 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 11 | 1 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 12 | | | | | | | | | | | | | | | | | | | 1 | | | | | | | | | | | | | | | | | |
| 13 | | | | | | | | | | | | | | | | | | | | | | | | 1 | | | | | | | | | | | | |
| 14 | | | | | | | | | | | | | | | | | | | | | | | 1 | | | | | | | | | | | | | |
| 15 | | | | | | | | | | | | | | | | | | 1 | | | | | | | | | | | | | | | | | | |
| 16 | | | | | | | | | 3 | | | 1 | | | | | | | | | | | | | 2 | | | | | | 3 | 3 | 3 | 3 | 3 | 2 |
| 17 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | 1 |
| 18 | | | | | | | | | | | | | | | | | | 2 | | | | | | | | | | | | | | | | | | |
| 19 | | | | | | | | | | | | | | | | | | | | | | | | | | | 3 | | 2 | | | | | | | |
| 20 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | 1 | | | | | | | |
| 21 | | | | | | | | | | | | | | | | | | | | | | | | | | | | 1 | | | | | | | | |
| 22 | | | | | | | | | | 1 | | | | | | 3 | | | | | | | | | | | | | | | | | | | | |
| 23 | | | | | | | | | | | | 2 | | | | | | | | | | | | | | | | | | | | | | | | |
| 24 | | | | | | | | | | | | | | | 2 | | | | | | | | | | | | | | | | | | | | | |
| 25 | | | | | | | | | | | | | | 1 | | | | | | | | | | | | | | | | | | | | | | |
| 26 | | | | | | 3 | 3 | | | | | | | 2 | | | | | | | | | | | | | | | | | | | | | | |

| | *T. insularis* | | | | | *T. pilosa* ssp. *latifolia* | | | | | | | | | *T. pilosa* ssp. *pilosa* | | | | | | | | | | | | | | | *T. procumbens* | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **H** | **BHR** | **K2** | **SB** | **SR1** | **SR3** | **BT** | **CCJ** | **CT** | **GCT** | **GR1l** | **KR** | **NL** | **NR** | **PL** | **TR** | **BBR** | **BR** | **CRT** | **CV** | **GR1p** | **KD** | **MH** | **ML** | **OH** | **PT** | **RB** | **RR** | **SS** | **VH** | **WP** | **WR** | **BG** | **BLT** | **FG** | **IB** | **LR** |
| 27 | | | | | | | | | | 1 | | | | | | | | | | | | | | | | | | | | | | | | | |
| 28 | | | | | | | | | | 1 | | | | | 1 | | | | | 3 | | 3 | 1 | | | | | | | | 3 | | | | | |
| 29 | | | | | | | | | | | | | | | | | | | | | | | 1 | | | | | | | | | | | | |
| 30 | | | | | | | | | | | | | | | | | | | | | | | | | | | | 2 | | | | | | | |
| 31 | | | | | | | | | | | | | 3 | | | | | | | | | | | | | | | | | | | | | | |
| 32 | | | | | | | | | | | | | | | | | | | | | | | | 1 | | | | | | | | | | | |
| 33 | | | | | | | | | | | | | | | | | | | | | | | | 1 | 1 | | | | | | | | | | |
| 34 | | | | | | | | 3 | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 35 | | | | | | | | | | | | | | | | | | | | | 3 | | | | | | | | | | | | | | |
| 36 | | | | | | | | | | | | | | | | | | | 2 | | | | | | | | | | | | | | | | |

**Table 3.4 Diversity indices by population: sample size (*N*); number of haplotypes ($N_H$); number of private haplotypes ($N_P$); and unbiased diversity (uh)**

| Species and population | *N* | $N_H$ | $N_P$ | uh |
|---|---|---|---|---|
| *T. insularis* | 3 | 1.8 | 1.5 | 0.556 |
| BHR | 3 | 2 | 2 | 0.667 |
| K2 | 3 | 2 | 1 | 0.667 |
| SB | 3 | 1 | 1 | 0.000 |
| SR1 | 3 | 2 | 2 | 0.667 |
| SR3 | 3 | 2 | 1 | 0.667 |
| *T. pilosa* ssp. *latifolia* | 3 | 1.6 | 0.9 | 0.367 |
| BT | 3 | 1 | 0 | 0.000 |
| CCJ | 3 | 1 | 0 | 0.000 |
| CT | 3 | 1 | 1 | 0.000 |
| GCT | 3 | 1 | 0 | 0.000 |
| GR1 | 3 | 3 | 2 | 1.000 |
| KR | 3 | 2 | 2 | 0.667 |
| NL | 3 | 2 | 1 | 0.667 |
| NR | 3 | 1 | 1 | 0.000 |
| PL | 3 | 2 | 1 | 0.667 |
| TR | 3 | 2 | 1 | 0.667 |
| T. *pilosa* ssp. *pilosa* | 3 | 1.6 | 0.9 | 0.333 |
| BR | 3 | 1 | 1 | 0.000 |
| CRT | 3 | 2 | 2 | 0.667 |
| BBR | 3 | 1 | 1 | 0.000 |
| CV | 3 | 2 | 2 | 0.667 |
| GR1p | 3 | 1 | 0 | 0.000 |
| KD | 3 | 1 | 1 | 0.000 |
| MH | 3 | 1 | 0 | 0.000 |
| ML | 3 | 3 | 2 | 1.000 |
| OH | 3 | 3 | 2 | 1.000 |
| PT | 3 | 2 | 0 | 0.667 |
| RB | 3 | 1 | 1 | 0.000 |
| RR | 3 | 1 | 0 | 0.000 |
| SS | 3 | 2 | 2 | 0.667 |
| VH | 3 | 2 | 1 | 0.667 |
| WP | 3 | 1 | 0 | 0.000 |
| WR | 3 | 1 | 0 | 0.000 |
| *T. procumbens* | 3 | 1.2 | 0.2 | 0.133 |
| BG | 3 | 1 | 0 | 0.000 |
| BLT | 3 | 1 | 0 | 0.000 |
| FG | 3 | 1 | 0 | 0.000 |
| IB | 3 | 1 | 0 | 0.000 |
| LR | 3 | 2 | 1 | 0.667 |

## Geographical structure of genetic variation

Genetic differentiation varied between populations for the individual taxa and taxonomic groups tested (see Table 3.5). $G_{ST}$ values ranged from 0.442 for *Tetratheca insularis* to 0.662 for the two *T. pilosa* subspecies together with *T. procumbens*. $G_{ST}$ for *Tetratheca procumbens* was zero because four populations were fixed for a single haplotype. The fifth population also contained two individuals with the same haplotype. $R_{ST}$ and $N_{ST}$ were therefore not tested for *T. procumbens*. In all cases $R_{ST}$ and $N_{ST}$ were higher than $G_{ST}$ and in most cases this result was significant ($P<0.05$), indicating the genetic variation was geographically structured. Only *T. pilosa* subsp. *pilosa* showed no significant geographic structure after permutations of $R_{ST}$ and $N_{ST.}$

**Table 3.5 Comparison of genetic differentiation statistics for various species concepts.**

| Taxa | No. pops | No. indivs | $N_A$ | $G_{ST}$ | $N_{ST}$ | $R_{ST}$ |
|---|---|---|---|---|---|---|
| *T. pilosa* ssp. *pilosa*, *T. pilosa* ssp. *latifolia*, *T. procumbens*, *T. insularis* | 36 | 108 | 36 | 0.636 | 0.758** | 0.821* |
| *T. pilosa* ssp. *pilosa*, *T. pilosa* ssp. *latifolia*, *T. procumbens* | 31 | 93 | 28 | 0.662 | 0.746** | 0.889* |
| *T. pilosa* ssp. *pilosa*, *T. pilosa* ssp. *latifolia* | 26 | 78 | 27 | 0.639 | 0.722** | 0.891* |
| *T. pilosa* ssp. *pilosa* | 16 | 48 | 19 | 0.651 | 0.712 | 0.844 |
| *T. pilosa* ssp. *latifolia* | 10 | 30 | 12 | 0.609 | 0.693 | 0.994* |
| *T. insularis* | 6 | 18 | 10 | 0.442 | 0.556* | 0 |

* $p \geq 0.05$ ** $p \geq 0.01$

## Geographic and taxonomic patterns

Haplotype networks for the *Tetratheca pilosa* group are presented in Figure 3.9 and Figure 3.10. There are two main clusters of haplotypes separated by 30 evolutionary steps. This large divergence is probably overstated due to the large jump in allele lengths at ccmp2 and ccmp10, however, even when the large jump was scored as a single step event and the dataset reanalysed, the same left and right clusters are still resolved. The results of the

analysis with single steps scored at ccmp2 and ccmp10 are not presented here since the structure of networks was unchanged. The cluster to the left of Figure 3.9 contains all individuals of *T. insularis* with several individuals of *T. pilosa* subsp. *pilosa* and *T. pilosa* subsp. *latifolia*. *Tetratheca pilosa* subsp. *pilosa* is the most disparate group within the dataset with individuals and populations scattered throughout the network. None of the currently recognised species or subspecies within the *Tetratheca pilosa* group is separated completely from the others, however all populations of *T. procumbens* share a single haplotype with one another and with a few individuals each of *T. pilosa* subsp. *pilosa* and *T. pilosa* subsp. *latifolia*. One individual of *T. procumbens* is distinct from the rest by five mutations.

There is geographic structure evident in the network, albeit very little. All of the Kangaroo Island individuals are present in the right hand cluster of the network and all Victorian and South Australian individuals are in the left hand cluster. The Tasmanian individuals are scattered throughout. The nine individuals of *Tetratheca pilosa* that cluster with the Kangaroo Island samples are all from northern Tasmania. Although there is no distinct geographical separation in the network, only the two most common haplotypes are shared among regions.

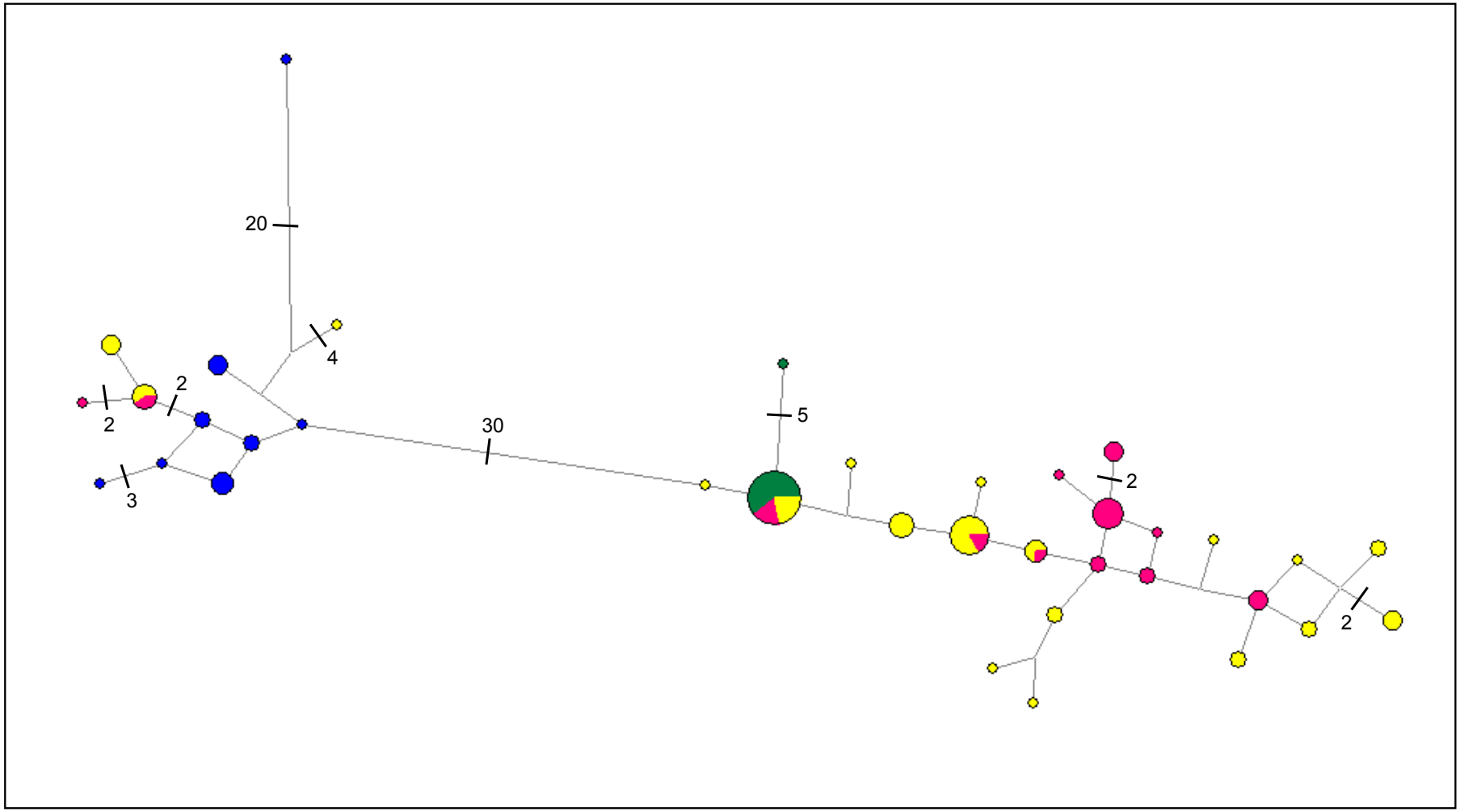



**Figure 3.9 Median-joining network for the *Tetratheca pilosa* group coloured according to current taxonomy. *Tetratheca pilosa* ssp. *pilosa* – yellow; *T. pilosa* ssp. *latifolia* – pink; *T. procumbens* – green; *T. insularis* – blue.**

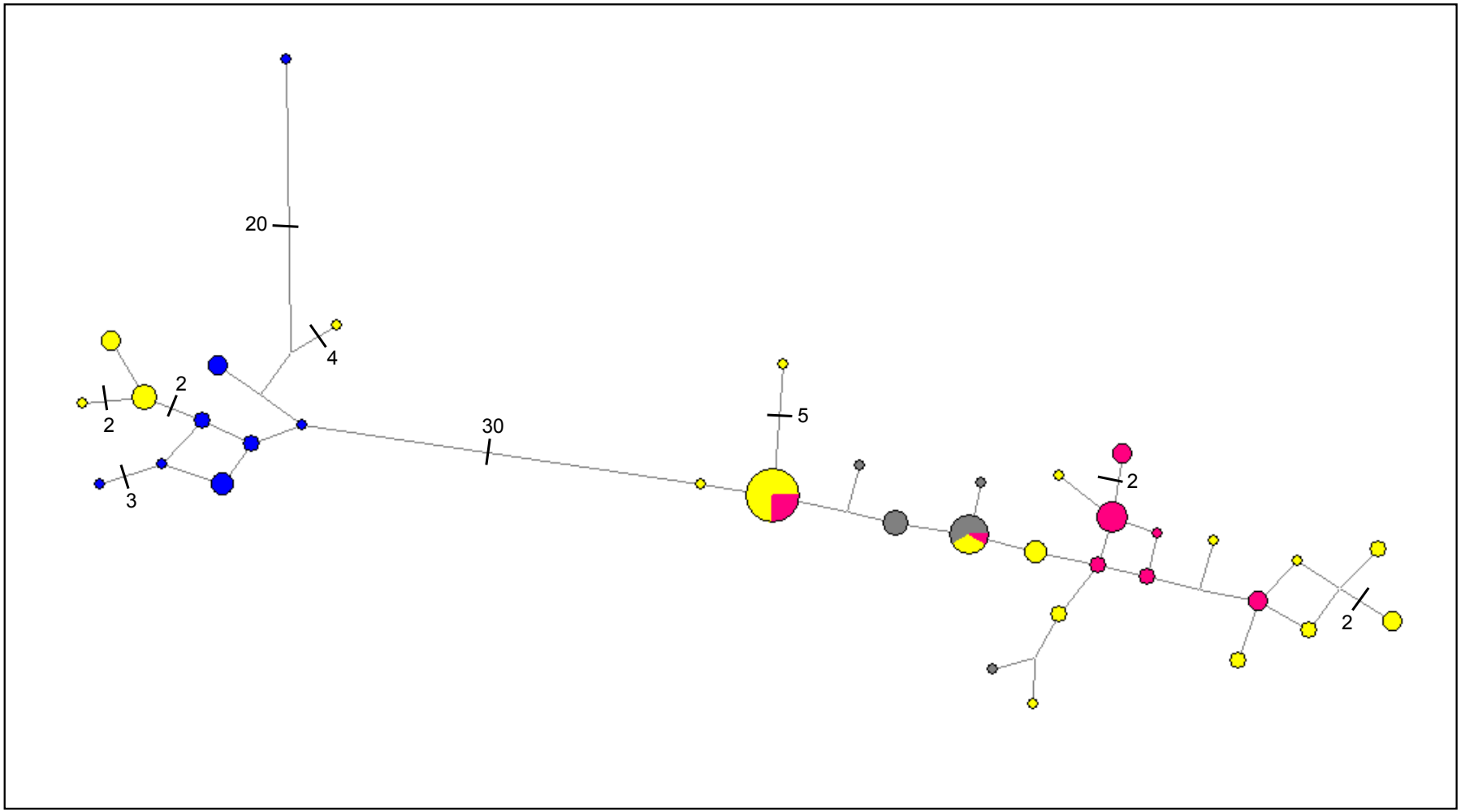


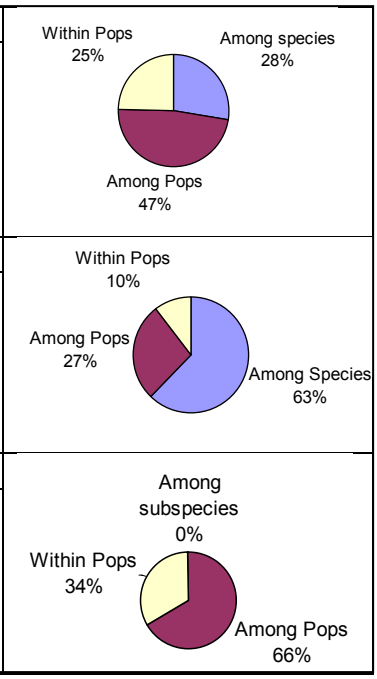

**Figure 3.10 Median-joining network for the *Tetratheca pilosa* group coloured according to geography: Kangaroo Island – blue; South Australia – grey; eastern Victoria – pink; Tasmania – yellow.**

## Analysis of molecular variance

The results of the AMOVA tests are presented in Table **3.6**. Current taxonomic concepts were tested at the specific and subspecific levels. Variance among *Tetratheca pilosa* and *T. procumbens* was fairly high (28%) and $\Phi_{PT}$ was highly significant. From the network results presented in the previous section the specific status of *T. insularis* appears to be appropriate. For comparison an AMOVA test was conducted to examine the partitioning of variance between *T. insularis* and *T. pilosa.* Variance between species was much higher in this case (63%). No variance was detected among the two subspecies of *T. pilosa* supporting Downing's (2005) suggestion that the division is artificial.

**Table 3.6 Partitioning of variance among species and subspecies within the *Tetratheca pilosa* group.**

| **Source of variance** | **$\Phi_{PT}$** | |
|---|---|---|
| *Tetratheca pilosa* and *T. procumbens* | | |
| Among species | 0.752*** | |
| Among populations | | |
| Within populations | | |
| **Source of variance** | **$\Phi_{PT}$** | |
| *Tetratheca insularis* and *T. pilosa* | | |
| Among species | 0.896*** | |
| Among populations | | |
| Within populations | | |
| **Source of variance** | **$\Phi_{PT}$** | |
| *T. pilosa* ssp. *pilosa* and *T. pilosa* ssp. *latifolia* | | |
| Among subspecies | 0.645*** | |
| Among populations | | |
| Within populations | | |




***$p \leq 0.001$

AMOVA tests were also performed to investigate partitioning of variance among different parts of the geographical range of *Tetratheca pilosa* (pooling the two subspecies first and pooling *T. pilosa* with *T. procumbens* second). Populations from Tasmania, Victoria and South Australia were each tested against the rest of the distribution of *T. pilosa* but in all cases the variance among regions was 0%. Similarly, no distinction was found between the

two mainland areas (Fleurieu Peninsula and Eastern Victoria) when Tasmanian populations were removed (results not shown).

**Spatial AMOVA (SAMOVA)**

For the *Tetratheca pilosa* group as a whole, each of the groupings K=2-8 had high $F_{CT}$ values and all were significant. The highest $F_{CT}$ value ($F_{CT}$ = 0.97332) occurred when two groups were resolved. This result separated *Tetratheca insularis* and three Northern Tasmanian populations (RB, BR and KR) from the remainder of the group and this result concurs with the Network results shown in Figures 3.9 and 3.10. At K=8 *Tetratheca procumbens* still clustered together with some populations of *Tetratheca pilosa* subsp. *pilosa* and *T. pilosa* subsp. *latifolia.* Again this result reflects the patterns found by the Network analyses. None of the groupings resolves current taxonomic concepts or distinct geographic areas.

When the two subspecies of *Tetratheca pilosa* were analysed together the highest $F_{CT}$ value ($F_{CT}$ = 0.97343) occurred when the three Northern Tasmanian populations (RB, BR and KR) were separated from the rest of the sample. There was no distinction between subspecies at any value of K tested.

For each of the taxa analysed individually the results were not obviously geographically arranged despite the assumptions of the SAMOVA program that groups are constrained by geography.

**Fu's F test of selective neutrality**

There was no evidence for population demographic expansion in the *Tetratheca pilosa* group. A negative value for Fu's $F_S$ was detected only in the *Tetratheca pilosa* subsp. *latifolia* population GR1 but this result was not significant.

### 3.3.5 Discussion

**Testing current taxonomic concepts**

Results from the cpSSR study presented here do not fully reflect current taxonomic concepts. Of the four recognised taxa in the *Tetratheca pilosa* group, diversity is highest in *Tetratheca insularis,* which is endemic to Kangaroo Island. It is clearly separated from *T. pilosa* on the adjacent mainland, but an affinity with Tasmanian populations of *T. pilosa* is indicated by a shared insertion (approximately 30 base-pairs long). *Tetratheca procumbens* is the only taxonomic unit that shares the same haplotype among all populations. Considering the controversial taxonomic status of this species taxonomically, this result is surprising. As stated earlier, J. Thompson (pers. comm., 2005) does not recognise *T. procumbens* as a species but suggested it is form of *T. pilosa*, procumbent due to altitude and/or climatic conditions. Jeanes (1996) reinstated it as a species on the basis that the Victorian specimens occur in a distinct geographical area and habitat, and they are morphologically distinguishable from the subspecies of *T. pilosa.* He indicated that separation of the Tasmanian samples of *T. procumbens* from *T. pilosa* is more complex since intermediate forms have been observed there.

Unfortunately no samples of *Tetratheca procumbens* from Victoria were collected for the present study. If Victorian individuals were found to share the same haplotype(s) as the Tasmanian samples then Jeanes' (1996) concept of *T. procumbens* as a distinct species would seem appropriate. Otherwise, subspecific or varietal ranking, or Thompson's (1976) suggestion that it is a form of *T. pilosa*, would seem more appropriate. Without a comparison of chloroplast data from both geographically disjunct populations taxonomic conclusions are not clear.

Thompson (1976) recognised two subspecies of *Tetratheca pilosa* but she indicated that further study of the variation within the species is needed since they are very closely related and subspecific boundaries are not clear. She stated that on the mainland the subspecies were distinct and much more uniform than in Tasmania. This distinction between morphological subspecies of *T. pilosa* on the mainland was not detected with

chloroplast microsatellite data, even when Tasmanian populations were removed from the analyses. This result supports Downing's (2005) proposal that the subspecific ranking may be artificial due to overlapping morphology.

**Phylogeographic patterns in the *Tetratheca pilosa* group**

This study of the *Tetratheca pilosa* group has shown that chloroplast diversity is not clearly organised geographically. *Tetratheca pilosa* subsp. *pilosa* was the only taxon tested that showed no significant structuring of genetic variation. It is by far the most widespread so high diversity of haplotypes is not unexpected. Since the boundary between the subspecies would appear to be artificial this result can be disregarded. When the two subspecies of *T. pilosa* are pooled geographic structure is detected. Nevertheless, the patterns are more complex and less obviously structured than expected given that *Tetratheca* species are dispersed by ants and therefore not expected to move far.

Thompson (1976) suggested a Tasmanian origin for the *Tetratheca pilosa* group based on morphology. Presumably this is due to the presence of a greater number of taxa in the group and more shared morphological traits in Tasmania than the mainland. Thompson (1976) hypothesised that the species spread north-eastward and north-westward from Tasmania onto the mainland, followed by the eastern and western clusters diverging into morphologically distinct subspecies each more uniform than in Tasmania. She suggested that *T. insularis* on Kangaroo Island and *T. neglecta* and *T. rubioides* in NSW diverged later from *T. pilosa.*

Coalescent theory states that ancestral alleles retained in populations will be represented in interior positions in haplotype networks with higher numbers of descending lineages than more recently derived ones. The converse is that the more recently derived alleles will be represented by tip positions in the network (Crandall and Templeton 1993). Crandall and Templeton (1993) also suggest that the ancestral alleles will represent a wider distribution. In the networks of the *Tetratheca pilosa* group (Figure 3.9 and Figure 3.10) the most widely distributed haplotypes also occur in interior positions. The two most common haplotypes (Haplotypes 16 and 28) are found in Tasmania and Victoria, and in Tasmania, Victoria and South Australia respectively. Tasmanian haplotypes are most widely

distributed throughout the network, followed by the Victorian ones, whereas South Australian haplotypes are narrowly distributed (see Figure 3.10). These patterns do not necessarily conflict with Thompson's (1976) hypothesis of a Tasmanian origin however the chloroplast patterns are complex. Given that a land bridge has connected Tasmania and mainland Australia at least eight times since the Miocene (McKinnon *et al.* 2004a, Rathbone *et al.* 2007) it is possible that there have been periods of both isolation and exchange. This may account for some of the complexity, particularly in the left-hand cluster of the network. The position of *T. insularis* towards the tips of the network support Thompson's (1976) proposal that *T. insularis* was derived from *T. pilosa* later than the other Tasmanian taxa (in this study represented by only *T. procumbens*).

The close relationships between all the eastern Australian species of *Tetratheca* are indicated by lack of resolution in the phylogenetic analyses (Chapter 2), along with evidence that Thompson's (1976) morphological groupings are for the most part unsupported (Chapter 2). The complexity of patterns detected in the present cpSSR study suggest that focussing on the *Tetratheca pilosa* group may not be sufficient for a complete understanding of migration patterns in *Tetratheca.* Broader sampling within and between species may help to elucidate migration patterns since the *Tetratheca pilosa* group may be based on incorrect assumptions about relationships within the species complex. Sampling higher numbers of individuals within populations may also be more appropriate given the unexpectedly high level of diversity detected in only three individuals.

**Evidence for historic hybridisation events or incomplete lineage sorting**

Haplotype sharing between taxa may occur as a result of hybridisation or incomplete lineage sorting and to distinguish between these two causes can be difficult (Schaal and Olsen 2000, Comes and Abbott 2001, Byrne *et al.* 2002). Incomplete lineage sorting is the retention of ancestral polymorphisms as a result of multiple speciation events (Avise 1994, Avise 2000). Distribution patterns of shared haplotypes can help to identify the most parsimonious explanation of phylogenetic conflict. Hybridisation has been detected in many phylogeographic studies (Steane *et al.* 1998, McKinnon *et al.* 2001, McKinnon *et al.* 2004a, McKinnon *et al.* 2004b, Petit *et al.* 2004, Heuertz *et al.* 2006). In each case shared haplotypes among taxa were based on geographical proximity irrespective of taxonomic

boundaries. When incomplete lineage sorting occurs, the distribution of shared haplotypes is not necessarily restricted to areas that are geographically close. Shared haplotypes are often positioned in the older, interior parts of the haplotype network rather than in more recent, tip positions (Byrne *et al.* 2002).

In the *Tetratheca pilosa* group there are no haplotypes shared between *T. insularis* and any of the other taxa, therefore there is no indication that either hybridisation or incomplete lineage sorting is relevant in this case. There is no strong evidence, based on this cpSSR study, for hybridisation in the evolutionary history of the *Tetratheca pilosa* group. Only two haplotypes are shared between *T. pilosa* and *T. procumbens* (the two most common ones in the network), and they are not geographically close. One is shared among *T. procumbens* and populations of *T. pilosa* from near Hobart, Launceston and Swansea in Tasmania, and another among populations of *T. pilosa* from South Australia and near Swansea in Tasmania.

Jakob and Blattner (2006) state that incomplete lineage sorting is reflected in two ways: a high number of haplotypes shared among species (including shared haplotypes in interior network positions), and high chloroplast diversity. Both of these patterns occur to some extent in the present study. Although in the *Tetratheca pilosa* group there are only two haplotypes shared among species, the distribution of shared haplotypes in the interior of the network, and unique haplotypes throughout and towards the tips, indicates retention of ancestral haplotypes occurring along with descended lineages. This can occur when population sizes remain high over a long period of time. Fu's test of neutrality did not indicate population demographic expansion which concurs with the hypothesis of consistently large population sizes. The uniqueness of populations was also detected with SAMOVA analyses which separated out individual populations or small groups of populations that were not clearly taxonomically or geographically structured. The identical *trn*L-*trn*F sequences found between most *T. pilosa* individuals in clade P (Figure 2.3, Chapter 2) are further support for the hypothesis of incomplete lineage sorting, as is the high diversity of haplotypes detected.

## 3.4 *Tetratheca ciliata* and *T. stenocarpa*

### 3.4.1 Introduction

*Tetratheca ciliata* is widely distributed from the Snowy Mountains and the south coast of New South Wales, throughout Victoria, into south-eastern South Australia and Tasmania, and displays great morphological variation across its range. *T.stenocarpa* is narrowly distributed to the east of Melbourne in Victoria and much more uniform morphologically (J. Thompson pers. comm. 2005; personal observation 2005, 2006). The two species form one of only two resolved clades of eastern Australian taxa in Downing's (2005) cladistic analysis of morphology. Downing's (2005) phenetic study showed the two taxa as distinct species but retrieved three morphological clusters within *T. ciliata*. Her first and second clusters are geographically separated, however the third overlaps with the others at the edges of their ranges. Thompson (1976) lists these taxa as a pair that requires further taxonomic investigation and suggested hybridisation may have had some influence on their evolution (J. Thompson pers. comm. 2005).

The phylogenetic analyses from plastid and nuclear datasets (Chapter 2) conflict with respect to the positions of these taxa. The *trn*L-*trn*F data for *Tetratheca ciliata* and *T. stenocarpa* are identical whereas the ITS data resolve *T. ciliata* as sister to *T. ericifolia*, albeit with only moderate support (69% bs; 0.96 pp). The conflict between the plastid and nuclear analyses suggests that hybridisation or incomplete lineage sorting may be affecting phylogenetic resolution of these species.

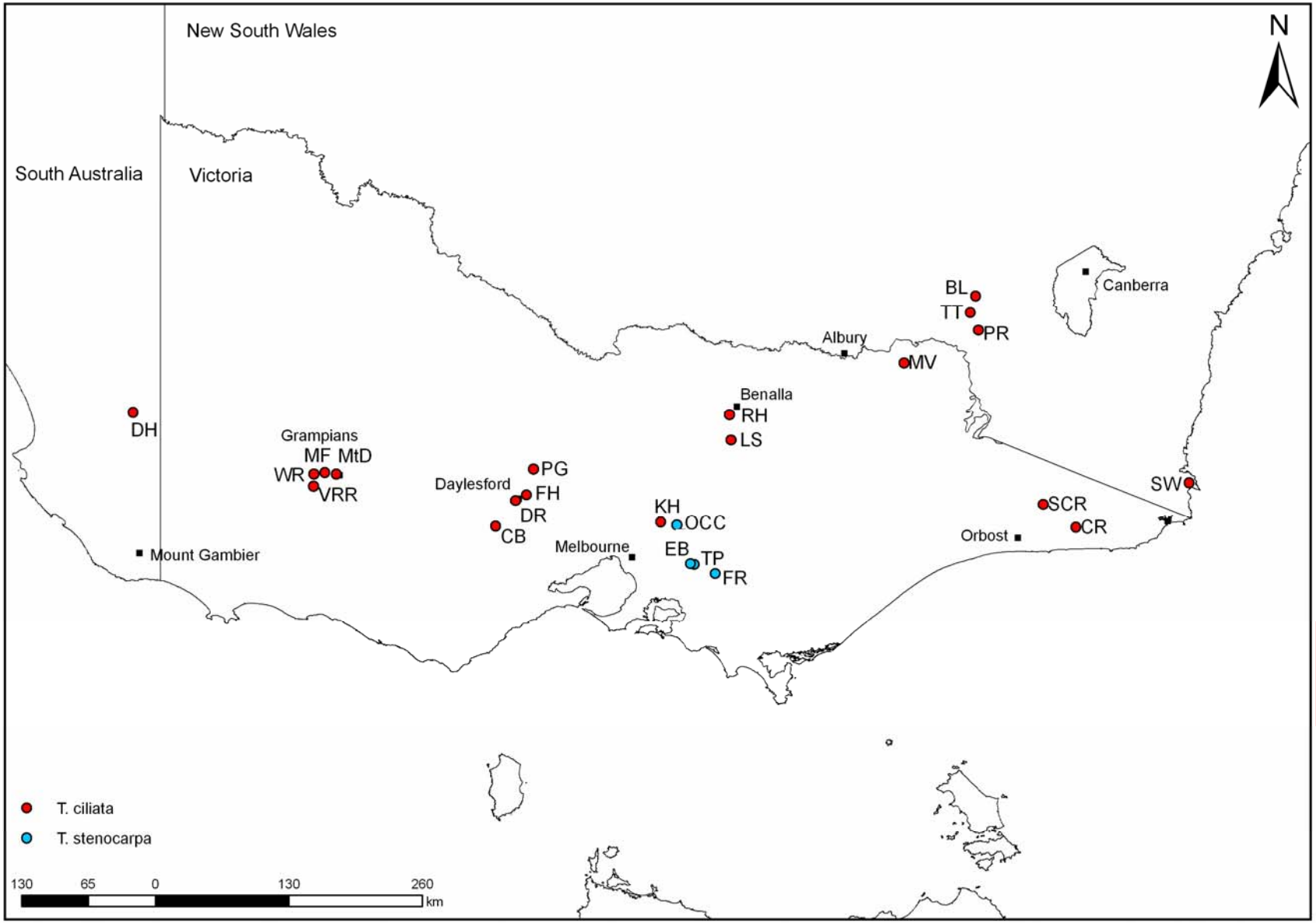



**Figure 3.11 Locations of populations sampled for the chloroplast microsatellite study of *Tetratheca ciliata* (indicated by red circles and population codes) and *Tetratheca stenocarpa* indicated by blue circles and population codes). Sampling information and interpretation of population codes is presented in Table 3.1**

### 3.4.2 Sampling

Twenty-three populations (three individuals from each) were collected from the ranges of *Tetratheca ciliata* and *T. stenocarpa* as shown in Figure 3.11. Nineteen populations of *T. ciliata* and four of *T. stenocarpa* were sampled and populations were selected to cover as much of the extent of the geographical range, morphological and environmental gradients of each of the species as possible. Populations of *T. ciliata* from Tasmania could not be sampled since the species is listed as rare under the Threatened Species Protection Act 1995.

Collecting localities were determined using herbarium data and despite a range of herbarium records of *Tetratheca ciliata* from South Australia and targeted searching, only one population was found between Adelaide and Mount Gambier. The small flora reserves that remain between farmland and timber plantations are protected from fire and the undergrowth is thick. *Tetratheca* thrives in disturbed, open areas and grows prolifically after fire (personal observation, 2005, 2006). Absence from these areas may be attributed to human-induced changes to the environment, particularly land clearing and exclusion of fire. It should also be noted that most *Tetratheca* species are difficult to find when they are not in flower, so it is also possible that some populations were overlooked. A subset of the populations of *T. ciliata* and *T. stenocarpa* sampled for this study will also be used for a population study (Chapter 5). This will enable comparisons of chloroplast and nuclear microsatellite data for detecting taxonomic boundaries between closely related taxa, genetic variability and differentiation within and among populations, and to separate current processes from longer-term processes affecting speciation in *Tetratheca.*

### 3.4.3 Chloroplast microsatellite analysis

Sixty nine individuals were genotyped: three individuals from each of 19 populations of *Tetratheca ciliata* and four populations of *T. stenocarpa*. Data analyses were performed as outlined in the Materials and Methods section of the current chapter. Missing data points were interpolated as described previously.

### 3.4.4 Results

Genotyping results are presented in Appendix 5. All five loci were polymorphic across populations of *Tetratheca ciliata*, however no variation was detected at either ccmp10 or ccmp4 for *T. stenocarpa*. Twenty-eight alleles were identified across all loci. There were large disjunctions between lengths of alleles at ccmp2 and ccmp10. Locus ccmp2 varied from 144-188 and ccmp10 from 122-137. The remaining loci seemed to be mononucleotide length polymorphisms. The most variable locus was ccmp2 with nine alleles, and ccmp10 and ccmp3 the least variable loci with three alleles each. Six and seven alleles were amplified at loci ccmp4 and Tecp01 respectively. The distributions of alleles by population and loci are presented in Figure 3.12, Figure 3.13, Figure 3.14, Figure 3.15, Figure 3.16.

There are combinations of common and widely distributed alleles, and rare, narrowly distributed alleles among the 23 populations genotyped. The most common allele at ccmp3 occurs across the entire range of *Tetratheca ciliata* and *T. stenocarpa*. Loci ccmp2 and ccmp4 each have one allele that is widespread and common across the range of *T. ciliata* but absent from populations of *T. stenocarpa*. At ccmp10 there are only three alleles present, one common to all populations of *T. ciliata* and *T. stenocarpa*, and the others only appearing in two individuals of *T. ciliata*.

Several geographic patterns are evident in the distribution of alleles at some loci. Markers ccmp10 and ccmp2 have alleles unique to the South Australian population. There are six alleles (four unique) at ccmp2 and four alleles (one unique) at ccmp4 present in the Grampians. The most common allele in the Grampians at locus Tecp01 is present in South Australia but occurs nowhere else within the range of *Tetratheca ciliata*. There are two private alleles within populations of *T. stenocarpa* at loci ccmp2 and Tecp01, although the latter also shares some alleles with *T. ciliata*. The most common allele in *T. stenocarpa* populations at ccmp4 is rare in populations of *T. ciliata*.

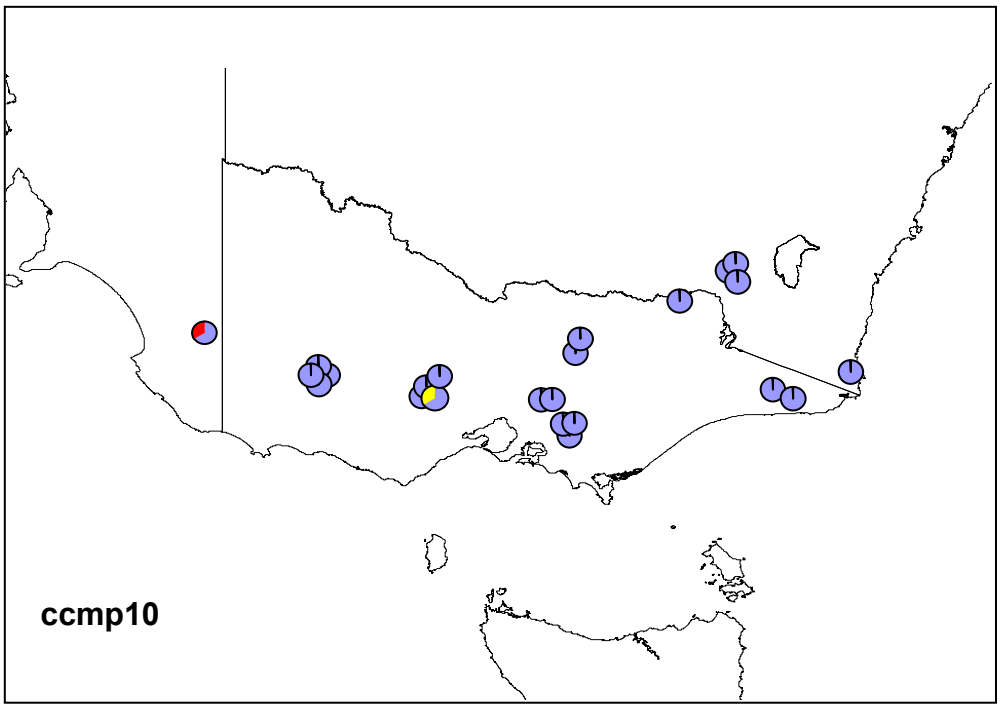



**Figure 3.12 Distribution of alleles at ccmp10.**

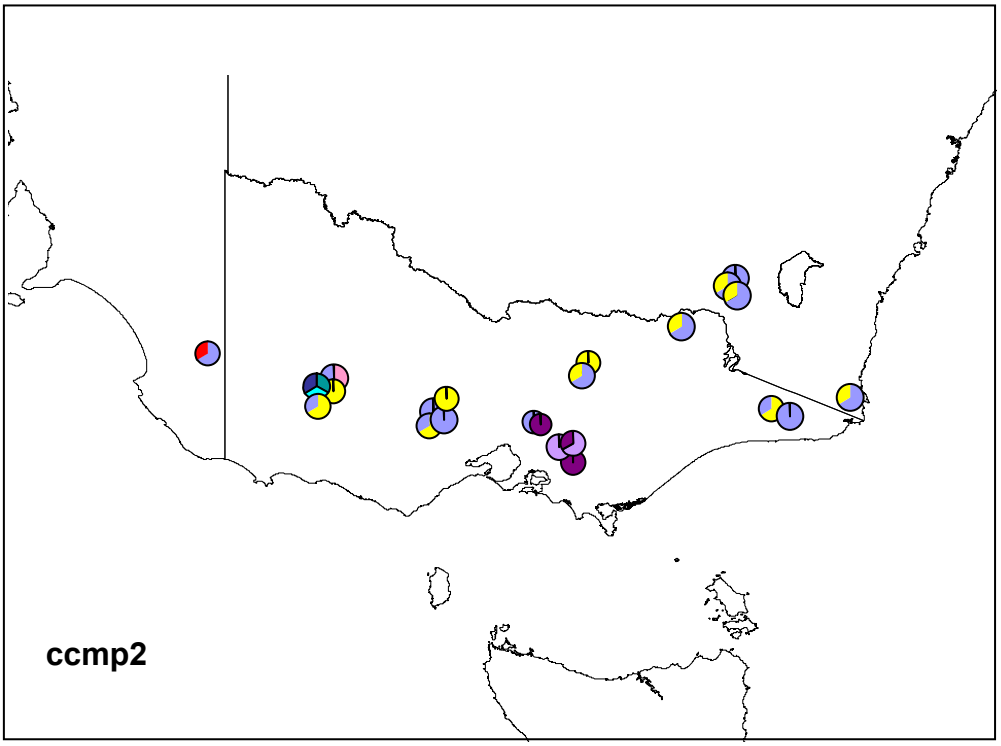



**Figure 3.13 Distribution of alleles at locus ccmp2.**

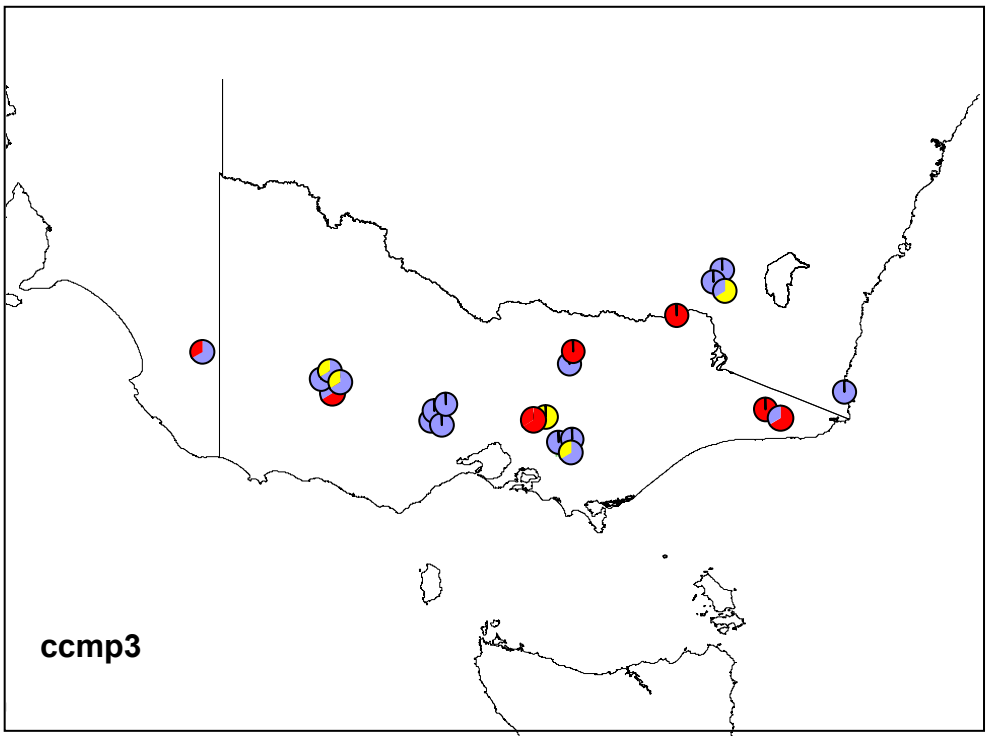



**Figure 3.14 Distribution of alleles at locus ccmp3.**

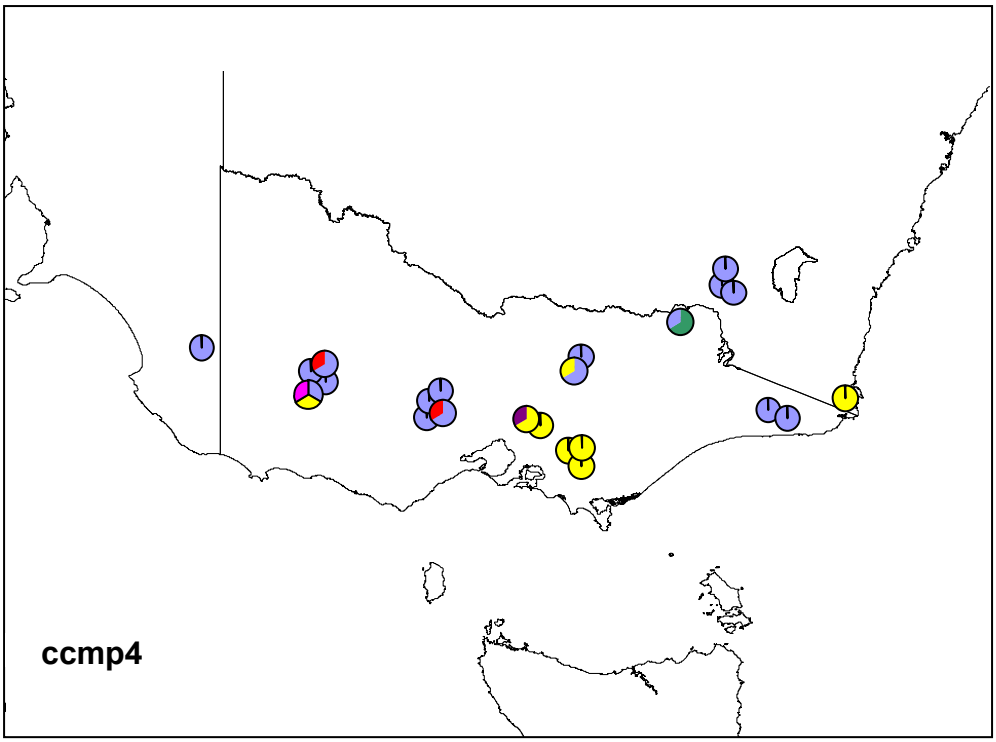



**Figure 3.15 Distribution of alleles at locus ccmp4.**

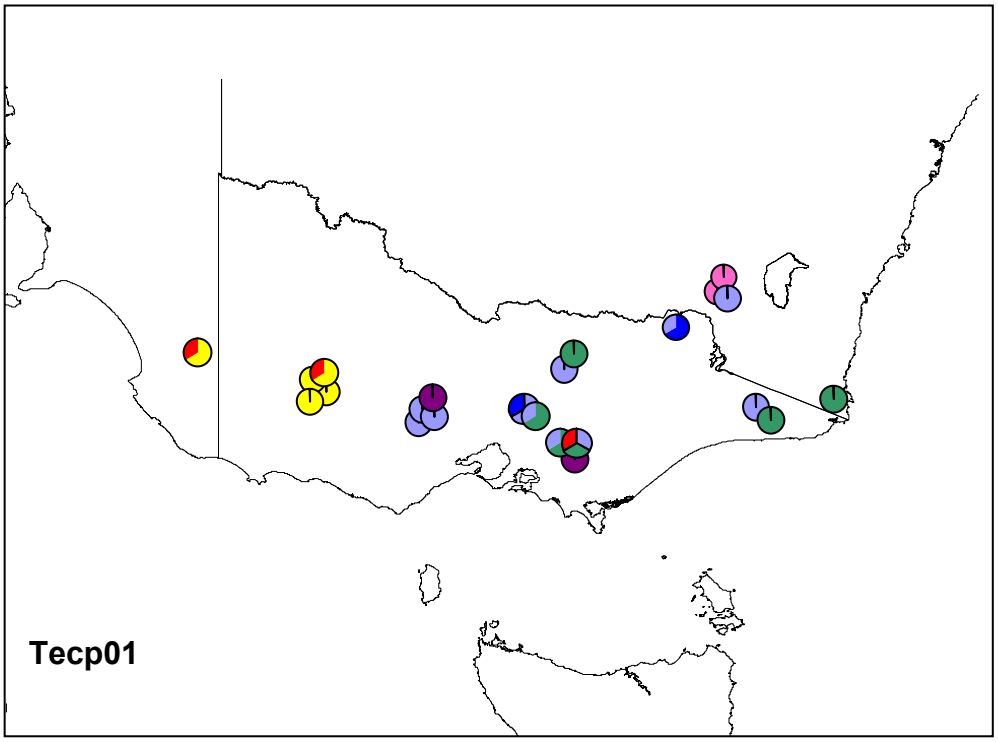



**Figure 3.16 Distribution of alleles at locus Tecp01**

The combination of 28 alleles among the five loci defined 38 haplotypes (see Appendix 6 for haplotype descriptions and Table 3.7 for distribution and frequency of haplotypes within and among populations and taxa). Haplotype 26 is the most common haplotype, occurring in three populations of *Tetratheca ciliata* in the Ballarat area and the population (LS) near Benalla (see Figure 3.11 for population localities). No haplotypes are shared among the two species. There are two haplotypes shared among two populations of *T. stenocarpa* and two haplotypes shared among individuals within populations. Seven haplotypes are shared among populations of *T. ciliata* and eleven are shared among individuals within one or more populations of *T. ciliata.*

Diversity statistics by population are shown in Table 3.8. The number of haplotypes varied from 1-3 with an average of 2 for *T. ciliata* and 2.3 for *T. stenocarpa*. Most populations contained private haplotypes: 16 of 19 populations of *T. ciliata* and all but one population of *T. stenocarpa*. The average numbers of private alleles were 1.4 and 1.3 for *T. ciliata* and *T. stenocarpa* respectively. Unbiased diversity ranged from 0-1. The highest diversity occurred in three of the four populations of *T. ciliata* from the Grampians, one population to the west of Albury, and two populations of *T. stenocarpa*. Four populations of *T. ciliata* and one of *T. stenocarpa* had zero haplotype diversity. Average diversity ($H_E$) was slightly higher in *T. stenocarpa* (0.667) than *T. ciliata* (0.596).

**Table 3.7 Number and distribution of haplotypes within and among populations and taxa**

| | *T. ciliata* | | | | | | | | | | | | | | | | | | *T. stenocarpa* | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **H** | **BL** | **CB** | **CR** | **DH** | **DR** | **FH** | **KH** | **LS** | **MF** | **MtD** | **MV** | **PG** | **PR** | **RH** | **SCR** | **SW** | **TT** | **VRR** | **WR** | **EB** | **FRV** | **OCC** | **TP** |
| 1 | | | | | | | | | | | | | | | | | | | | 1 | | | |
| 2 | | | | | | | | | | | | | | | | | | | | | | 1 | |
| 3 | | | | | | | | | | | | | | | | | | | | | | 1 | |
| 4 | | | | | | | | | | | | | | | | | | | | | 3 | | |
| 5 | | | | | | | | | | | | | | | | | | | | | | 1 | |
| 6 | | | | | | | | | | | | | | | | | | | | 1 | | | 2 |
| 7 | | | | | | | | | | | | | | | | | | | | 1 | | | 1 |
| 8 | | | | | | | | | | | | | | | | | | 1 | | | | | |
| 9 | | | | | | | | | | | | | | | | 1 | | | | | | | |
| 10 | | | | | | | | 1 | | | | | | | | | | | | | | | |
| 11 | | | | | | | | | | | | 3 | | | | | | | | | | | |
| 12 | | | | | | | | | | | | | | | | | 1 | | | | | | |
| 13 | | | | | | | | | | 1 | | | | | | | | 1 | | | | | |
| 14 | | 2 | | | | | | | | | | | 1 | | | | | | | | | | |
| 15 | | | | | | | | | | | 1 | | | | | | | | | | | | |
| 16 | | | | | | | | | | 2 | | | | | | | | | | | | | |
| 17 | | | | | | | | | | | | | | 3 | | | | | | | | | |
| 18 | | | | | | | | | | | | | | | 2 | 1 | | | | | | | |
| 19 | | | | | | | | | | | | | | | | | | 1 | | | | | |
| 20 | | | | | | | | | | | | | 2 | | | | | | | | | | |
| 21 | | | | | | | | | | | | | | | | 1 | | | | | | | |
| 22 | | | | | | | | | | | | | | | | | | 1 | 1 | | | | |
| 23 | 3 | | | | | | | | | | | | | | | | 2 | | | | | | |
| 24 | | | | 2 | | | | | | | | | | | | | | | | | | | |
| 25 | | | 1 | | | | | | | | | | | | | | | | | | | | |
| 26 | | 1 | | | 3 | 2 | | 2 | | | | | | | | | | | | | | | |

| | *T. ciliata* | | | | | | | | | | | | | | | | | | *T. stenocarpa* | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **H** | **BL** | **CB** | **CR** | **DH** | **DR** | **FH** | **KH** | **LS** | **MF** | **MtD** | **MV** | **PG** | **PR** | **RH** | **SCR** | **SW** | **TT** | **VRR** | **WR** | **EB** | **FRV** | **OCC** | **TP** |
| 27 | | | | | | | | | | | 1 | | | | | | | | | | | | |
| 28 | | | | | | | 1 | | | | | | | | | | | | | | | | |
| 29 | | | | | | | 2 | | | | | | | | | | | | | | | | |
| 30 | | | 2 | | | | | | | | | | | | | | | | | | | | |
| 31 | | | | | | | | | | | 1 | | | | 1 | | | | | | | | |
| 32 | | | | | | | | | | | | | | | | | | | 1 | | | | |
| 33 | | | | | | | | | | | | | | | | | | | 1 | | | | |
| 34 | | | | | | | | | 1 | | | | | | | | | | | | | | |
| 35 | | | | | | | | | 1 | | | | | | | | | | | | | | |
| 36 | | | | | | | | | 1 | | | | | | | | | | | | | | |
| 37 | | | | | | 1 | | | | | | | | | | | | | | | | | |
| 38 | | | | 1 | | | | | | | | | | | | | | | | | | | |

**Table 3.8 Diversity indices by population: sample size ($N$); number of haplotypes ($N_H$); mean number of private haplotypes ($N_P$); and Nei's (1987) unbiased diversity (uh).**

| Species and population | $N$ | $N_H$ | $N_P$ | uh |
|---|---|---|---|---|
| *T. ciliata* | 3 | 2 | 1.4 | 0.596 |
| cBL | 3 | 1 | 0 | 0.000 |
| cCB | 3 | 2 | 0 | 0.667 |
| cCR | 3 | 2 | 2 | 0.667 |
| cDH | 3 | 2 | 2 | 0.667 |
| cDR | 3 | 1 | 0 | 0.000 |
| cFH | 3 | 2 | 1 | 0.667 |
| cKH | 3 | 2 | 2 | 0.667 |
| cLS | 3 | 2 | 1 | 0.667 |
| cMF | 3 | 3 | 3 | 1.000 |
| cMtD | 3 | 2 | 1 | 0.667 |
| cMV | 3 | 3 | 2 | 1.000 |
| cPG | 3 | 1 | 1 | 0.000 |
| cPR | 3 | 2 | 1 | 0.667 |
| cRH | 3 | 1 | 1 | 0.000 |
| cSCR | 3 | 2 | 1 | 0.667 |
| cSW | 3 | 2 | 2 | 0.667 |
| cTT | 3 | 2 | 1 | 0.667 |
| cVRR | 3 | 3 | 2 | 1.000 |
| cWR | 3 | 3 | 3 | 1.000 |
| *T. stenocarpa* | 3 | 2.3 | 1.3 | 0.667 |
| sEB | 3 | 3 | 1 | 1.000 |
| sFRV | 3 | 1 | 1 | 0.000 |
| sOCC | 3 | 3 | 3 | 1.000 |
| sTP | 3 | 2 | 0 | 0.667 |

## Geographical structure of genetic variation

The results of the CPSSR and PERMUT analyses are presented in Table 3.9. Genetic differentiation (measured as $G_{ST}$) was 0.294 for *Tetratheca stenocarpa* and slightly higher (0.390) for *T. ciliata*. In both cases $R_{ST}$ and $N_{ST}$ were higher than $G_{ST}$, however, only for *T. stenocarpa* was the difference significant. Combining the pair into a single species, genetic differentiation was 0.382 and permutations of $R_{ST}$ and $N_{ST}$ were significant, indicating geographical structure in the dataset.

**Table 3.9 Comparison of genetic differentiation statistics for *Tetratheca ciliata* and *T. stenocarpa***

| Taxa | No. of pops | No. indivs | $N_A$ | $G_{ST}$ | $N_{ST}$ | $R_{ST}$ |
|---|---|---|---|---|---|---|
| *T. ciliata* | 19 | 57 | 31 | 0.390 | 0.561* | 0.558 |
| *T. stenocarpa* | 4 | 12 | 7 | 0.294 | 0.409 | 0.801* |
| combined | 23 | 69 | 38 | 0.382 | 0.599* | 0.975* |

## Geographic and taxonomic patterns

Median spanning networks of haplotypes in *Tetratheca ciliata* with *T. stenocarpa* are presented in Figure 3.17 and Figure 3.18. There were seven equally parsimonious trees used to construct the network, one of which is presented as an inset in Figure 3.18. The remaining trees can be found in Appendix 7. Figure 3.17 is coloured according to current taxonomy: *T. ciliata* and *T. stenocarpa*. The species are clearly separated into two main clusters of haplotypes with *T. ciliata* bottom left and *T. stenocarpa* top right. The species are separated by 34 evolutionary steps. This divergence is probably overstated due to the large jumps in allele lengths at ccmp2 and ccmp10, which may indicate that these loci are not evolving in a step-wise manner. When the large jumps at each locus were scored as single events and the dataset reanalysed, the separation of the two species was still evident. The results of the analysis with jumps at ccmp2 and ccmp10 scored as single events are not presented here since the structure of networks remained unchanged. There are no haplotypes shared between the two species so the hypothesis that conflict between nuclear and plastid data is the result of hybridisation is not supported.

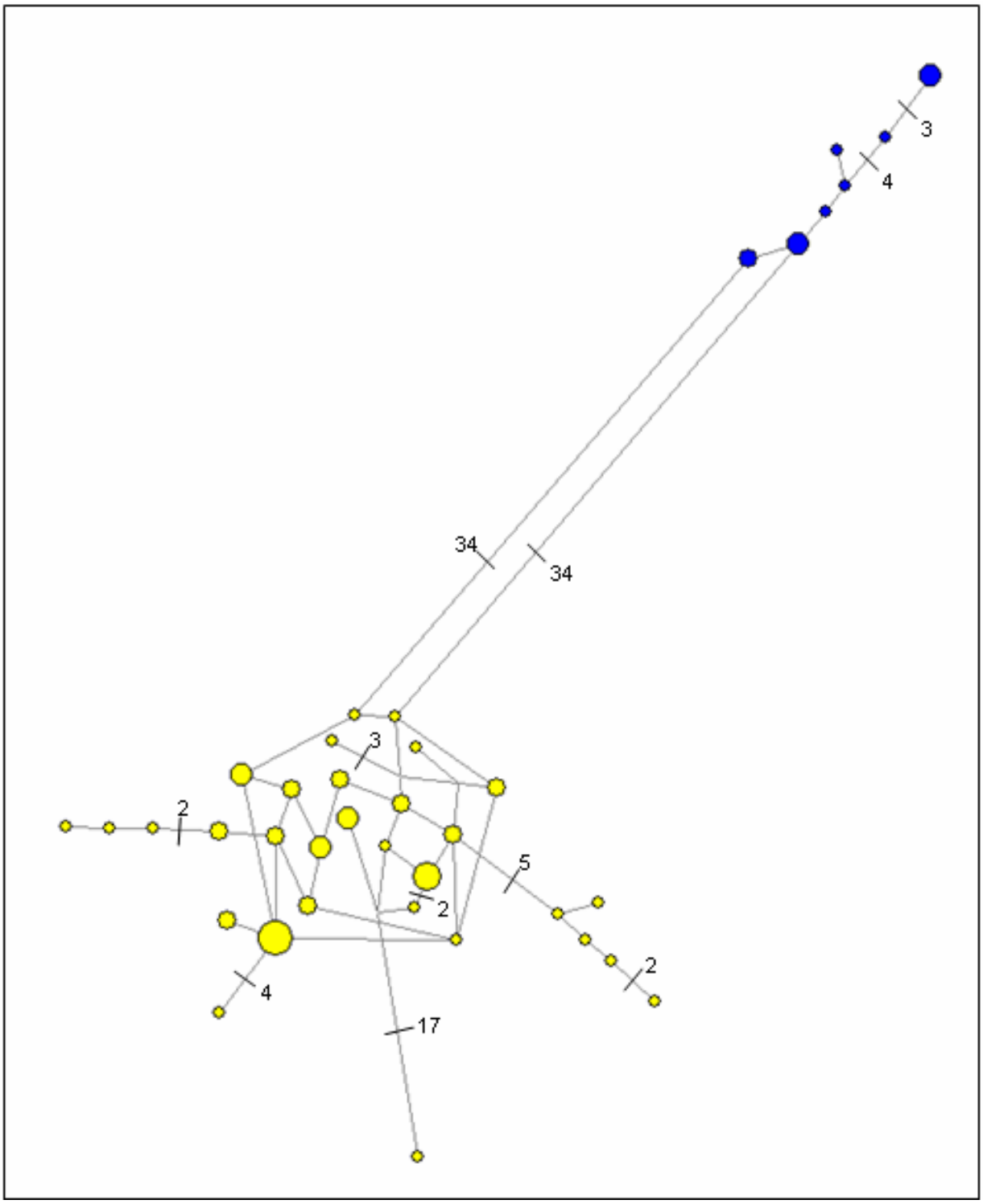



**Figure 3.17 Median-joining network coloured according to current taxonomy: *Tetratheca ciliata* - yellow; *T. stenocarpa* – blue.**

Most haplotypes in *Tetratheca ciliata* are joined to two or three others, and one haplotype to five others, forming a complex network. A large number of closed loops in the network may indicate homoplasy. There are several individuals or groups of individuals that diverge in a tree-like manner from the main group. Figure 3.18 is the same network with individuals of *T. ciliata* coloured according to geography. The boundaries of the western (red) and eastern (yellow) taxa were determined on the basis of the nuclear microsatellite study in Chapter 4 which identified two distinct genetic clusters within *T. ciliata.* The eastern cluster includes population KH to the north-east of Melbourne and all populations east of KH. This cluster corresponds with the eastern cluster of Downing's (2005) phenetic analysis based on the morphology of *T. ciliata.* The western cluster, detected using nuclear microsatellite markers, spans from the populations near Daylesford westward into South Australia and corresponds for the most part with the distributions of Downing's two western Victorian clusters. One of her morphological clusters overlaps geographically with each of the other two while the nuclear microsatellite data is clearly geographically separated.

The results retrieved by the comparison of $R_{ST,}$ $N_{ST}$ and $G_{ST}$ are evident in the network in Figure 3.18. The scattered distribution of east and west haplotypes indicates that individuals with similar haplotypes are not necessarily occurring in the same areas. There are however two branches diverging from the main cluster of the network. The red lineage (to the right of Figure 3.18) consists of some individuals from the Grampians and the yellow lineage consists of individuals from population MV east of Albury and population KH to the northwest of Melbourne. Although for the most part east and west haplotypes are scattered throughout the network in Figure 3.18, and the origins of each are not clear, there are only two haplotypes of the total 31 that are shared among eastern and western individuals.

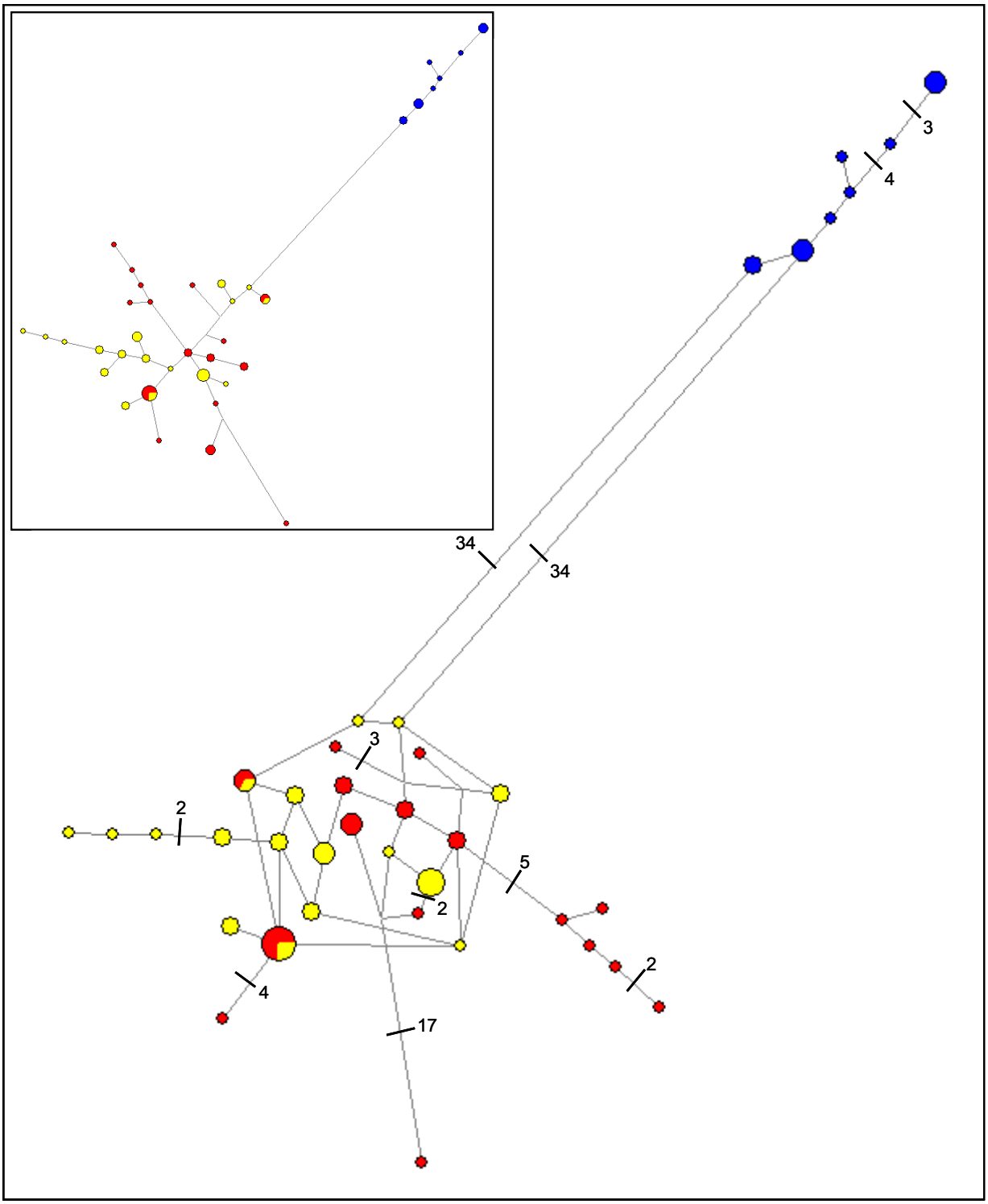


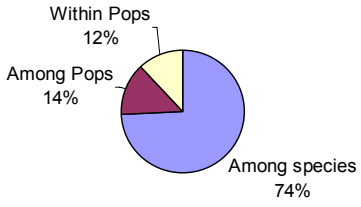

**Figure 3.18 Median-joining network with *Tetratheca ciliata* coloured according to geography: west - red; east - yellow. Inset: one of seven equally parsimonious trees.**

## Analysis of molecular variance

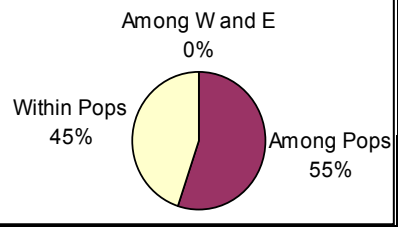
The results of the AMOVA tests are presented in Table 3.10. Current species limits and the portioning of variance among western and eastern populations of *Tetratheca ciliata* (as previously outlined) were tested. Variance among species was high (74%) and highly significant. No variance was detected among eastern and western populations of *T. ciliata.*

**Table 3.10 Partitioning of variance among *Tetratheca ciliata* and *T. stenocarpa* and among regions within *T. ciliata***

| **Source of variance** | **$\Phi_{PT}$** |
|---|---|
| *Tetratheca ciliata* and *T. stenocarpa* | |
| Among species | 0.881*** |
| Among populations | |
| Within populations | |




| **Source of variance** | **$\Phi_{PT}$** |
|---|---|
| *T. ciliata* W and E | |
| Among regions | 0.518*** |
| Among populations | |
| Within populations | |




***$p \leq 0.001$

## Spatial AMOVA (SAMOVA)

For *Tetratheca ciliata* and *T. stenocarpa* pooled as a single group, the highest $F_{CT}$ value ($F_{CT}$ = 0.98394) resolved seven groups, however each of the groupings K=2-8 had high $F_{CT}$ values and all were significant. The seven groups were:

1. *Tetratheca stenocarpa* FRV
2. *Tetratheca stenocarpa* OCC, TP, EB
3. *Tetratheca ciliata* DH
4. *Tetratheca ciliata* MtD
5. *Tetratheca ciliata* KH
6. *Tetratheca ciliata* PR
7. *Tetratheca ciliata* VRR, MF, WR, PG, FH, DR, CB, LS, RH, MV, BL, TT, SCR, SW, CR

Five groups maximised the proportion of total genetic variance ($F_{CT}$ = 0.73436) when *Tetratheca ciliata* was analysed alone. These groups corresponded to groups 3-7 shown above. There was no obvious geographic pattern since most populations formed a single group and other groups consisted of single populations. This result supports the Network analyses presented in Figures 3.17 and 3.18 which showed *Tetratheca ciliata* as a complex network with no obvious geographic structure.

**Fu's F test of selective neutrality**

There was no evidence for population demographic expansion in *Tetratheca ciliata* or *Tetratheca stenocarpa.* A negative value for Fu's $F_S$ was detected only in the *Tetratheca ciliata* population MtD and the *T. stenocarpa* population OCC but the results were not significant.

## 3.4.5 Discussion

**Testing current taxonomic concepts**

Haplotypes in *Tetratheca ciliata* and *T. stenocarpa* are clearly separated, and the current species concepts are supported by Downing's (2005) analyses based on morphology and the present cpSSR data. Nuclear microsatellites will be used in Chapter 4 to investigate current processes affecting these two species in order to further investigate the cause of the conflicting phylogenetic results (Chapter 2) which paired *T. ciliata* with *T. ericifolia* for ITS and *T. ciliata* with *T. stenocarpa* for *trn*L-*trn*F. Future studies of cpSSR variation in the eastern species of *Tetratheca,* including *T. ericifolia*, may also help to elucidate the reasons for phylogenetic conflict.

**Phylogeographic patterns in *Tetratheca ciliata* and *T. stenocarpa***

*Tetratheca stenocarpa* is narrowly distributed to the east of Melbourne and there are no geographic patterns evident among the four populations sampled. *Tetratheca ciliata*, on the

other hand, is widespread throughout Victoria, extending into New South Wales to the north-east of its range and South Australia at the western limits, and is also present (though rare) in Tasmania.

On the mainland, neither the geographic patterns found in Downing's (2005) phenetic analysis of morphology of *Tetratheca ciliata*, nor the similar pattern retrieved by the nuclear microsatellite analyses of the present study, were supported by cpSSR data. Eastern and western clusters of *T. ciliata,* as defined by the nuclear microsatellite study in Chapter 4, are mostly represented by unique haplotypes: only two are shared among regions. The most common haplotype is shared by the western populations at Canadian Ballarat, Daylesford Road and Frog Hollow (with less than 50 km between them), and the eastern population at Lima South approximately 200 km away. The other shared haplotype unites the population at Canadian Ballarat with Paddy River Falls approximately 400 km away. It may be that these haplotypes are more widespread and that, given the high diversity of haplotypes overall, three individuals per population was too small a number to capture the full extent of diversity in each population. It is also possible that the homoplasy indicated by the large number of loops in the network accounts for this similarity. This could also be assessed with a greater sample and/or more cpSSR loci.

**Evidence for historic hybridisation events or incomplete lineage sorting**

There is no evidence of hybridisation in the chloroplast data since no haplotypes are shared among species. No populations of *Tetratheca ciliata* and *T. stenocarpa* were found growing together despite targeted searches; the closest populations of each species were approximately 40 km apart. Either, there are no shared haplotypes among the species, or the limited number of populations of *T. stenocarpa* did not capture any, or three individuals per population were not sufficient to capture all haplotypes given the high diversity detected. Haplotypic diversity in both *T. ciliata and T. stenocarpa* is high, supporting the hypothesis of incomplete lineage sorting and despite clear delimitation of the two species this is further supported by conflicting patterns between cpSSR data and other data sources (morphological and nrSSR) across *T. ciliata.* No evidence of population demographic expansion was detected with Fu's F statistic which is consistent with

population numbers remaining high over a long period of time and therefore maintenance of high haplotypic diversity.

## 3.5 General conclusions

This study has shown that despite the high variability detected with cpSSR markers, these tools appear to be adequate for detecting species boundaries in *Tetratheca*. The following species concepts are supported: *Tetratheca pilosa, T. insularis, T. ciliata* and *T. stenocarpa.* The proportion of variance attributable to differences among *T. pilosa* and *T. insularis* was high and similar to that detected among *T. ciliata* and *T. stenocarpa* when tested by AMOVA. These results were also supported by SAMOVA analysis. That no variation was detected between subspecies of *T. pilosa* supports morphological evidence that the subspecies are artificial. The taxonomic status of *T. procumbens* remains unclear although it appears somewhat divergent from *T. pilosa.* The variance apportioned among the two species by AMOVA was significant but much lower than among the species pairs previously discussed and could possibly indicate that *T. procumbens* is a subspecies of *T. pilosa*. SAMOVA grouped the *Tetratheca procumbens* populations together with other populations of *Tetratheca pilosa*, thereby reiterating the results from the Network analysis. A comparison with Victorian specimens in the future may help to clarify the position of this species with respect to *T. pilosa.*

Chloroplast DNA has been widely used in studies assessing the effects of historical processes on the population structure of plants (Schaal *et al.* 1998, Avise 2000, Byrne *et al.* 2002). Many European studies have focussed on glacial refugia and post-glacial recolonisation routes (e.g. Petit *et al.* 1997, Taberlet *et al.* 1998, Heuertz *et al.* 2004). Malm and Prentice (2005) point out that most large-scale European plant studies using chloroplast or mitochondrial DNA markers to assess geographic variation and migration histories, predominantly focussed on trees. Their (2005) study of chloroplast DNA haplotypes in Nordic *Silene dioica* represents one of very few phylogeographic studies of a group of herbaceous plants. Chloroplast studies of Australian plants have increased over the past ten years and again the main focus has been on trees: including many studies of *Eucalyptus* (Steane et al. 1998, Jackson et al. 1999, McKinnon et al. 1999, Vaillancourt and Jackson 2000, McKinnon et al. 2001, McKinnon et al. 2004a, Rathbone et al. 2007).

The present study of the phylogeography of *Tetratheca* is the first broad-scale study of an herbaceous-shrubby native Australian plant.

Several studies of Australian plant groups have revealed a high level of chloroplast diversity within species (Steane *et al.* 1998, McKinnon *et al.* 1999, Byrne *et al.* 2002). The present cpSSR study of two species complexes within *Tetratheca* has also revealed high haplotypic diversity, particularly compared with tests of the same primer pairs on the related groups such as *Elaeocarpus* (M. Rossetto pers. comm. 2008) and species from the closely related family Cunoniaceae (M. Heslewood, pers. comm. 2008). The difference may partly be due to the short generation times of *Tetratheca* compared with the long-lived rainforest trees of the other groups.

Given that the chloroplast genome evolves much more slowly than the nuclear genome, and since *Tetratheca* are dispersed by ants, seeds are not likely to travel far from the parent, and one might expect chloroplast data to be strongly geographically structured. This was not the case in either study of the *Tetratheca pilosa* group or of *T. ciliata* and *T. stenocarpa.* There were very few obvious geographic patterns retrieved by the cpSSR analyses. Even where groups of populations were geographically isolated from one another (e.g. the three main areas of distribution of *T. pilosa* – South Australia, eastern Victoria and Tasmania) there was no significant variation detected between geographic areas. It would be interesting to include individuals of *T. ciliata* from Tasmania in future studies to investigate whether lack of geographic structure across such a barrier is similar to that found in the *Tetratheca pilosa* group.

The molecular dating analyses of Crayn *et al.* (2006) indicate *Tetratheca* is a recently evolved genus (radiating during the Oligo-Miocene) with most diversification occurring within the past 6-7 million years. The evolutionary rates in *Tetratheca* are also much faster than other genera within Elaeocarpaceae probably due to comparatively short generation times. Comes and Abbot (2001) highlight the importance of considering reticulation or incomplete lineage sorting when studying relationships between recently diverged plant groups, particularly where phylogenetic inferences are conflicting. Several studies have highlighted the difficulties in determining which of these processes is responsible for conflict between phylogenies, particularly in young, rapidly diverging groups (Comes and Abbott 2001, Byrne *et al.* 2002, Jakob and Blattner 2006). Jakob and Blattner (2006)

conducted a brief survey of recent literature and found 90% of chloroplast phylogenetic studies that reported branch lengths indicated the presence of zero-length branches. They suggest that retention of ancestral polymorphisms may be affecting phylogenetic inference more often than previously thought. These data together with Jakob and Blattner's chloroplast genealogy of the genus *Hordeum* L. (Poaceae) highlight the importance of broad sampling (taxonomically and geographically) for interpreting chloroplast data in recently diverged species groups.

The two concurrent studies of species complexes within *Tetratheca* presented here indicate that incomplete lineage sorting is probably affecting both resolution in the phylogenetic analyses (Chapter 2) and conflict between plastid and nuclear datasets. Although species in each group are closely related and morphologically similar over most of their ranges there was no extensive haplotype sharing among geographically close populations of either of the species complexes. Hybridisation does not appear to have been a significant factor influencing speciation of *Tetratheca* within either of these groups.

These studies highlight the value of analysing data from several species in order to thoroughly assess distribution patterns of haplotypes and therefore resolve that incomplete lineage sorting rather than reticulation better explains evolutionary patterns in *Tetratheca.* The importance of combining data from several loci in studies at the population level, particularly given the complexity detected at each locus and within each population is also highlighted here. Had results from any of the five loci been considered individually completely different patterns would have been revealed for each species complex. Combining individual chloroplast alleles into haplotypes also enables cladistic analyses of the data and therefore investigation of phylogenetic relationships within and between species.

# *Chapter 4* Population genetic diversity and structure within and among populations of *Tetratheca ciliata* and *T. stenocarpa*

## 4.1 Introduction

Levels and distribution of genetic variation within species are central to their potential for long-term survival (Avise 1994). Genetic variation contributes to reproductive success as well as the capacity for adaptation, particularly in response to environmental change or habitat fragmentation. Population size is a key factor affecting genetic variation and differentiation (Lowe *et al.* 2004). A genetic bottleneck is a reduction in effective population size via mortality within a population, or small numbers of individuals establishing a new population via colonisation (founder effect). Potential loss of allelic diversity or inbreeding may be increased in small post-bottleneck populations, which are more vulnerable to random drift. A severe reduction in effective population size can be detected as a loss of alleles and decline in average heterozygosity, since populations rely on mutation to bring the diversity of alleles back to previous levels. This process lags behind population size, which can return to pre-bottleneck levels relatively quickly.

The spatial distribution of alleles and genotypes is referred to as genetic structure. Population genetic structure may be shaped by geographic and ecological factors, along with long-term evolutionary history. In plants, reproductive biology (including factors such as pollen movement, seed dispersal or asexual reproduction) can have a marked effect on spatial patterns of genetic variation by impacting on gene flow. Historical events such as genetic bottlenecks and founder effects can also leave their signature in the present day structure of natural populations. The study of species complexes, containing closely related species with similar histories and breeding systems but with different distribution patterns, can therefore provide important insights into speciation processes and long-term survival potential of populations.

Interspecific gene flow (or hybridisation) can also affect the genetic composition of species and lead to extinction of populations or species. Hybridisation can therefore pose a particularly serious threat for rare species coming into contact with widespread, common species (Lorenzen and Siegismund 2004). *Tetratheca ciliata* and *T. stenocarpa* constitute a pair of closely related species within the genus. They are morphologically similar and have been resolved by cladistic analysis of morphological traits and molecular data (*trn*L-*trnF*) as sister species (Downing 2005, Crayn *et al.* 2006, and Chapter 2 of the present study). This result conflicted with the ITS strict consensus tree which showed the position of *T. stenocarpa* as uncertain, and support for *T. ciliata* with *T. ericifolia* and *T. juncea* (69 % bs, 0.96 pp) to the exclusion of the rest of the eastern Australian taxa. Possible causes of conflict between uniparentally and biparentally inherited data are hybridisation or introgression and/or incomplete lineage sorting (Jakob and Blattner 2006). Thompson (1976) postulated hybridisation as a factor influencing morphology and consequently the phenotypic similarity between these taxa. An investigation of population dynamics and phylogeography using chloroplast microsatellites was presented in Chapter 3. The patterns did not indicate large scale chloroplast sharing that might be expected if hybridisation had occurred to a significant extent in the past.

This chapter presents a comparative study genetic of patterns within *Tetratheca ciliata*, one of the most widespread and common species, and *T. stenocarpa,* a closely related, narrowly distributed species. *Tetratheca ciliata* occurs from southern NSW, through most of Victoria and into South Australia as well as northern Tasmania, where it is listed as rare under the Threatened Species Protection Act, 1995. *Tetratheca stenocarpa* is restricted to a small area north-west of Melbourne in Victoria (Thompson 1976, Gardner and Murray 1992, Australia's Virtual Herbarium). *Tetratheca stenocarpa* is listed as rare by the Department of Sustainability and Environment (2005).

Nuclear microsatellites can provide insights into current processes that determine distribution of populations, but longer term patterns often become obscured by recombination. Investigations of chloroplast and nuclear data together allow an assessment of relative contributions of seed and pollen to current distribution patterns. They can therefore provide a greater understanding of long- and short-term evolutionary patterns than either data source alone (Ennos 1994). Since the two sources of data also reflect patterns at different scales, comparisons of patterns detected from differentially inherited

data will facilitate more accurate interpretations of genetic diversity and structure. Studies utilising both data sources have the potential to enable more informed conservation management decisions (Heuertz *et al.* 2004).

The present chapter will utilise novel microsatellites developed to investigate genetic diversity, and the direction and strength of inter-population gene flow. An understanding of population genetic structure or homogeneity will help differentiate between species and populations, and provide insights into current processes shaping the distribution of these species. The specific aims of this chapter are to characterise highly polymorphic nrSSR markers and investigate population variation within and among populations of *Tetratheca ciliata* and *T. stenocarpa* in order to:

1. investigate whether nrSSR data can provide evidence for species delimitation
2. determine whether hybridisation is likely to occur between *Tetratheca ciliata* and *T. stenocarpa*.
3. detect whether genetic variation is structured geographically across the landscape
4. explore possible fluctuations in effective population sizes (e.g. bottlenecks) for both a widespread, common species and a rare, localised species

## 4.2 Materials and Methods

### 4.2.1 Development and characterisation of a nuclear SSR library for *Tetratheca*

A nuclear SSR (AG)-enriched library based on *Tetratheca ericifolia* from Hawkesbury sandstone areas around Sydney was developed and tested for variability at the population level and across a range of *Tetratheca* species. The library was based on *T. ericifolia* because this species is common around Sydney and is one of the only species that flowers throughout most of the year (J. Thompson, pers. comm. 2005) so collections were readily obtainable. The circumscription of *T. ericifolia* has been controversial. Smith (1804) circumscribed *T. ericifolia* narrowly, however, it was expanded significantly by subsequent

authors. Mueller (1860-1862), for example, included in *T. ericifolia* all other eastern Australian species except *T. ciliata*. Thompson described several new species in her 1976 revision of *Tetratheca*, splitting the expanded *T. ericifolia* and reinstating the original concept. Nevertheless, relationships with other species in the genus remain unresolved (see Chapters 2 and 3). Initial enrichment of the SSR library was conducted before the commencement of this project with a view to investigating population dynamics of *T. ericifolia*, however, the aims of the present study focus on other species within *Tetratheca*.

The remainder of the library development, testing and optimisation was carried out by the author. Twenty-three potentially useful SSR loci, with sizes from 154–423 bp, were identified and primer pairs designed using PRIMER 3 (Rozen and Skaletsky 2000). Primer pairs were analysed for compatibility using NETPRIMER (PREMIER Biosoft International). An M13 universal sequence (5'-TGTAAAACGACGGCCAGT-3') was appended to each forward primer to facilitate fluorescent labelling using the method of Schuelke (2000) as outlined in the previous chapter. Eleven loci were selected based on amplification quality as well as suitability of annealing temperatures and sizes for efficient multiplexing of PCR and genotyping. An additional six loci amplified for *Tetratheca ericifolia* but were not optimised for this species. The development and characterisation of the SSR library presented here has been published in *Molecular Ecology Resources* (McPherson *et al.* 2008, see Appendix 10). Details of the seventeen microsatellite loci characterised and used in the present population study are shown in Table 4.1.

**Table 4.1 Details of 17 microsatellite loci tested in the present study (size is based on *Tetratheca ericifolia*).**

| Locus | Repeat | *Primer sequences (5'-3') | **Size (bp) | Genbank Accession no. |
|---|---|---|---|---|
| Te01BGT | AAG(7) | Fwd: GCCTTTGTTGTTGAACGAGAG<br>Rev: TGGAGGCAAGACATGAGAATA | 325 | EU087915 |
| Te02BGT | GGT(6) | Fwd: CCAAGGAAAAAGCACAAGGA<br>Rev: GCCAGTCCCCATTGTAGAAG | 221 | EU087916 |
| Te03BGT | GA(8)N(49)GA(8) | Fwd: GCAAACAAACAACTCTCAGC<br>Rev: ACCGCAACAGAAGTAGTGAAAC | 423 | EU087917 |
| Te04BGT | CTT(4)N(18)AG(9)N(11)AG(3)N(108)GT(4) | Fwd: TCGGTGGAGAAGAAGGTGAC<br>Rev: CTTGGTGGCAGTTTTGGTTT | 391 | N/A |
| Te05TBGT | GA(8) | Fwd: GGGGCTGAATGAAAGTTTTG<br>Rev: TGTAACCCGAGAATCCACGA | 270 | EU087918 |
| Te06BGT | AG(9) | Fwd: CAACACGAAATCTTCAACATC<br>Rev: CATTAACACAAGGTCACCATTA | 173 | N/A |
| Te08BGT | GA(8) | Fwd: CAACCTACCGATGCTCTG<br>Rev: AAGGCAAAGTCCAAGAGTAT | 298 | N/A |
| Te09BGT | GA(10) | Fwd: CAAAACAGCAGGCATCTCAG<br>Rev: AGTCTGAAAGGAACGTGAAAAG | 139 | EU087920 |
| Te11BGT | GA(31) N(2) GA(8) | Fwd: GCACGAAACTGCCATATAAGG<br>Rev: CTGGGGATGGAAATTGTCAG | 176 | EU087921 |
| Te12BGT | AG(15) N(13) AG(8) | Fwd: TAAGTATGTGGTGGTGTAAT<br>Rev: AAGATAATGAAGATGATGGA | 266 | N/A |
| Te13BGT | AGG(8) | Fwd: AGAGGCGTTGCTGTTGTTG<br>Rev: TTTCAGTTTACCCTTGGGCT | 242 | N/A |
| Te14BGT | AG(2) N(4) AG(9) | Fwd: CGGATCAAGGAATCTCTGTCA<br>Rev: ACCCATCTTCACCGATTGTC | 372 | EU087922 |
| Te15BGT | CT(20) GA(3) N(8) GT(8) | Fwd: GCATTCTTGGGAGTGTTGGT<br>Rev: CAGTGCTTCCGATAATTGGTG | 349 | EU087923 |

| Locus | Repeat | *Primer sequences (5'-3') | **Size (bp) | Genbank Accession no. |
|---|---|---|---|---|
| Te16BGT | AG(9) | Fwd: TTCTTCTGCCCAAAATGTCC<br>Rev: AAAACCCTAGCAACAATACCTG | 284 | EU087924 |
| Te17BGT | GA(5) N(2) GA(11) | Fwd: TGTTGATGGTGGGCAGTTTA<br>Rev: CCAGTTGGTAGGATAGGTAGAAA | 180 | EU087925 |
| Te18BGT | GA(30) | Fwd: TGCCCCAAACAACTATCTTCA<br>Rev: CAAGGACTTCCCCAATCAAA | 223 | EU087926 |
| Te19BGT | AG(9) AC(7) | Fwd: AAATTCTCACTGTCTGCCAAT<br>Rev: TGATGAGCCGCTTTATGTTG | 289 | N/A |

* Forward primers were constructed with an M13 universal sequence (5'-TGTAAAACGACGGCCAGT-3') to facilitate fluorescent labelling (Schuelke 2000).

**expected size excluding M13 universal sequence

Primers labelled N/A will be lodged in Genbank as they are published.

### 4.2.2 Sampling

Using a simulation approach, Cavers *et al.* (2005) found that the optimal sampling strategy for microsatellite studies of spatial genetic structure in tree populations was between 100 and 200 individuals using 5-10 loci. Eleven populations of *Tetratheca ciliata* (158 individuals) and four populations (58 individuals) of *T. stenocarpa* were sampled in order to capture as much of the distributional ranges as well as the variety of environmental and ecological conditions for each species as possible. The lower numbers sampled from the rare species, *T. stenocarpa*, were due to targeted searches only locating four populations. Where possible, 15 individuals were sampled from approximately equal intervals along transects through each population. Transects varied in length according to apparent boundaries of each population. Some plants were reproducing asexually (suckering from roots) so, to avoid sampling clones, plants closer together than one metre were not collected. In five populations it was not possible to collect 15 plants so 9 and 10 samples each were sampled in these cases. Approximate sampling localities are indicated on the map in Figure 4.1 and sampling site details are presented in Table 4.2.

### 4.2.3 Plant material and DNA isolation

Total genomic DNA was isolated from silica-dried or fresh leaf tissue using the DNeasy plant mini kit or the Qiagen 96DNeasy Plant kit (Qiagen) following the manufacturer's instructions. Tissue was disrupted in AP1 lysis buffer (Qiagen) using the Qiagen Tissue Lyser.

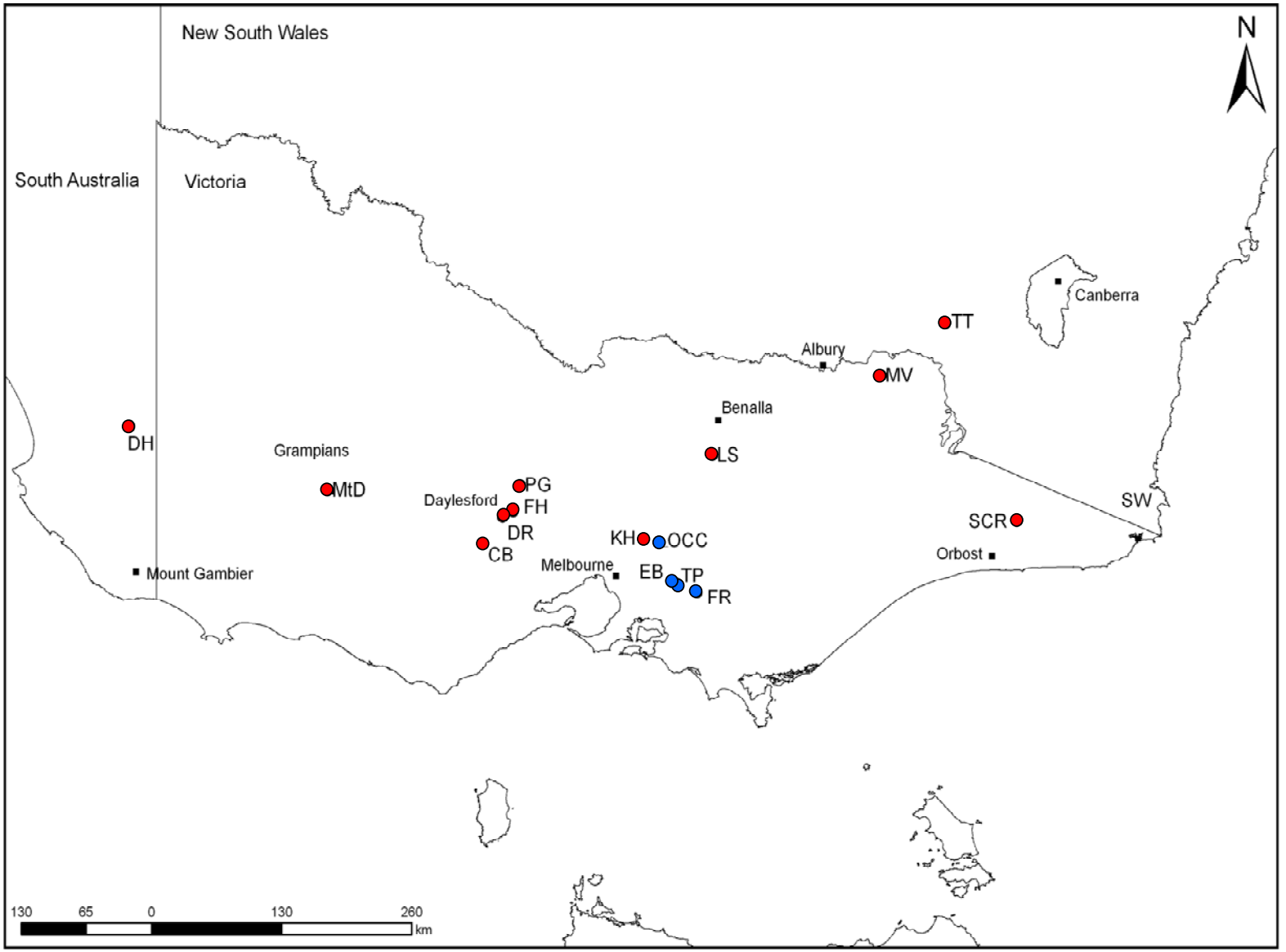



**Figure 4.1 Locations of populations sampled for the nuclear microsatellite study of *Tetratheca ciliata* (indicated by red circles and population codes) and *T. stenocarpa* (indicated by blue circles and population codes). Sampling information and interpretation of population codes is presented in Table 4.2.**

**Table 4.2 Sampling site details for the nuclear microsatellite analysis of *Tetratheca ciliata* and *T. stenocarpa***

| Taxon | N | Code | Locality | State | Latitude | Longitude |
|---|---|---|---|---|---|---|
| *T. ciliata* | 15 | CB | Canadian, Ballarat | VIC | -37.58083333 | 143.89472222 |
| *T. ciliata* | 15 | DH | Duke's Highway | SA | -36.58555556 | 140.72333333 |
| *T. ciliata* | 15 | DR | Daylesford Road | VIC | -37.36027778 | 144.06611111 |
| *T. ciliata* | 15 | FH | Frog Hollow, near Daylesford | VIC | -37.30722222 | 144.16305556 |
| *T. ciliata* | 15 | KH | Kinglake to Healesville Road | VIC | -37.54691667 | 145.33613889 |
| *T. ciliata* | 9 | LS | Lima South | VIC | -36.83000000 | 145.94888889 |
| *T. ciliata.* | 15 | MtD | Mount Difficult, Grampians | VIC | -37.12638889 | 142.49944444 |
| *T. ciliata* | 15 | MV | Murray Valley Highway | VIC | -36.15083333 | 147.46194444 |
| *T. ciliata* | 15 | PG | Poverty Gully | VIC | -37.08277778 | 144.22388889 |
| *T. ciliata.* | 15 | SCR | Sardine Creek Road | VIC | -37.39083333 | 148.67861111 |
| *T. ciliata* | 15 | TT | Tumut-Tumbarumba road | NSW | -35.71194444 | 148.04250000 |
| *T. stenocarpa* | 15 | EB | East Beenak Road | VIC | -37.91936111 | 145.62980556 |
| *T. stenocarpa* | 15 | FRV | Forest Road | VIC | -37.99863889 | 145.81169444 |
| *T. stenocarpa* | 15 | OCC | Old Chum Creek Road | VIC | -37.57061111 | 145.47622222 |
| *T. stenocarpa* | 10 | TP | Tentpole Road | VIC | -37.91094444 | 145.59791667 |

### 4.2.4 Locus selection

Nine loci were selected (see Table 4.3) from the total characterised. Primer sequences and details of repeat types are presented in Table 4.1. Te08BGT had not amplified well for *Tetratheca ericifolia* but was tested for amplification in *T. ciliata* and *T. stenocarpa* and included here. Loci were chosen on the basis of amplification success (determined by initial screening and cross-transferability tests), and appropriate sizes and temperatures for multiplexing.

**Table 4.3 Details of multiplexes used for PCR reaction for the nuclear microsatellite analysis**

| Locus | *Size (bp) | Ta (°C) | Dye | |
|---|---|---|---|---|
| Te02BGT | 221 | 63 | VIC | Multiplex I |
| Te11BGT | 176 | 63 | VIC | |
| Te15BGT | 349 | 63 | VIC | |
| Te03BGT | 423 | 57 | NED | Multiplex II |
| Te08BGT | 298 | 57 | NED | |
| Te18BGT | 223 | 57 | NED | |
| Te05BGT | 270 | 63 | FAM | Multiplex III |
| Te09BGT | 139 | 63 | FAM | |
| Te17BGT | 180 | 63 | FAM | |

*expected size excluding 5'M13 universal sequence

### 4.2.5 Locus screening I: amplification using PCR

Initial PCR amplification used a CP2-03 Thermal Cycler (Corbett Research, Mortlake, Australia) and a protocol modified from Schuelke (2000), in order to append a fluorescently dye-labelled M13 primer. PCR conditions were optimised for nine microsatellite loci using DNA from 2 individuals of *Tetratheca ciliata*.

All PCR reactions were conducted with fluorescent labels. Each 15 µL reaction contained 1.5 µL 10x NH4 buffer (Bioline, Luckenwalde, Germany), 1.5–2.0 mmol MgCl2, 0.2 mM of each dNTP and 0.06 units BIOTAQ™ DNA polymerase (Bioline, Luckenwalde, Germany), 0.05µM forward primer and 0.2µM of each of the reverse primer and fluorescent M13 primer (labelled with FAM, NED or VIC)

The PCR profile used was: one cycle of 94ºC incubation for 5 min; 30 cycles of denaturation at 94ºC for 30 s; annealing at 50, 54, 56 or 58ºC for 45 s; and extension at 72ºC for 45 s; eight cycles of denaturation at 94ºC for 30 s; annealing at 48 or 53ºC for 45 s; and extension at 72ºC for 45 s. A final extension was performed at 72ºC for 5 min. PCR products were multiplexed according to colour and sizes and amplification products were detected on an ABI 3730 Sequencer (Applied Biosystems, Foster City, California, USA) at the Automated DNA Sequencing Facility of the University of New South Wales.

### 4.2.6 Locus screening II: multiplexing PCR reactions

Multiplexing can help to reduce genotyping costs, so a second phase of locus screening was carried out using Qiagen Multiplex mix to test whether both PCRs and PCR products could be multiplexed. This would reduce the number of genotyping plates required for the study. PCRs were conducted following a method modified from Schuelke (2004) and the manufacturer's protocol. Conditions were optimised for the nine microsatellite loci using DNA from the same two individuals screened in the initial tests. A gradient of annealing temperatures (in three degree increments) from 57 - 63ºC were tested for all loci. Loci were multiplexed in several different combinations and with a range of dyes.

Each 10µL multiplex reaction contained 5µL Multiplex PCR mix (Qiagen), 0.05µM of each forward primer, 0.2µM of each reverse primer, and fluorescent M13 primer (FAM, NED or VIC dyes) at half the total concentration of the reverse primers. The cycling profile was: 15min incubation at 94ºC; 38 cycles of denaturation (30s at 94ºC), annealing (1min 30s at 57 or 63ºC for 30 cycles and 53ºC for the last eight cycles) and extension (45s at 72ºC); final 30min incubation at 60ºC. Products were electrophoresed on 2% agarose gels at 130V for 45 min, stained with ethidium bromide and visualised on an ultra-violet transilluminator.

All PCR reactions were conducted using a CP2-03 Thermal Cycler (Corbett Research, Mortlake, Australia) or Veriti (Applied Biosystems, Foster City, California, USA). PCR products were diluted 1 in 80 and multiplexed according to colour and size. Amplification products were detected on an ABI 3730 Sequencer as outlined above.

### 4.2.7 Nuclear microsatellite analysis

PCR amplification of 9-15 individuals from each population of *Tetratheca ciliata* and *T. stenocarpa* (213 individuals in total) used the multiplexing profiles outlined in Table 4.3 at either 57 C or 63 C. The products from the three multiplex PCRs were then combined in a single reaction mix and diluted 1in 80 for genotyping. Amplification products were detected on an ABI 3730 Sequencer as outlined above, and microsatellite profiles were

sized and scored manually using Genemapper (v3.7, Applied Biosystems, Foster City, California, USA). Most loci produced a stutter pattern so the largest peak in the expected size range was scored for each locus. Heterozygotes that were close together could be scored because of the pattern of stutter overlap. Products less than 200 scans in signal strength were ignored unless they were more than 100% higher than background levels.

In some cases a third or fourth peak was visualised. Mostly these were significantly smaller than the others (<10% peak height) and were assumed to be an artefact of the PCR. Where genotyping peaks were weak (approximately 5% of samples with extra peaks), it was difficult to separate the real peak from the artefact. In these cases peaks that were more common in the population were selected over the others. If none of the peaks corresponded to alleles in the rest of the population the data was recorded as missing. For individuals that failed to amplify, data was not interpolated but treated as missing in all the data analyses.

### 4.2.8 Error checking

Genotyping errors can occur as a result of sequence variation (producing null alleles), low quantity or quality of DNA, biochemical artefacts (causing stutter), or human error (Pompanon *et al.* 2005). Any one of these factors can affect the biological interpretations of a molecular study. It is important to recognise, test and report errors in genotyping studies so that real biological phenomena can be separated from experimental artefacts. Pompanon *et al.* (2005) recommend conducting blind replicates of at least 5-10% of the samples or systematic replication of some samples, depending on the nature of the study and the sensitivity to error of biological statistics investigated.

Genotyping of an average of 30% of individuals was repeated in order to determine reliability of the results of the population analyses. The presence of null alleles, short allele dominance and stutter were investigated using Microchecker 2.2 (Van Oosterhout *et al.*, 2004). Microchecker identifies the likelihood of errors based on detection of significant excess of homozygotes. Where multi-locus genotypes are used Microchecker can discriminate between deviations from Hardy-Weinberg Equilibrium (HWE) due to inbreeding, Wahlund effects and null alleles (Van Oosterhout *et al.*, 2004). When some

loci are in HWE and others are not Microchecker assumes this is a locus-specific problem (scoring an error or null allele) rather than a biological phenomenon.

### 4.2.9 Data analysis

#### Diversity statistics and HWE

In order to assess diversity levels for each locus, values for $N_A$ (number of alleles), $H_O$ (observed heterozygosity) and $H_E$ (expected heterozygosity) were calculated using GenAlEx 6.1 (Peakall and Smouse 2006). Hardy-Weinberg proportions and linkage disequilibrium were tested in GENEPOP 3.3 (Raymond and Rousset 1995) using the exact test, with significance levels determined after 500 batches of 5000 iterations each. Bonferroni corrections were applied to significant data to determine adjusted significance levels following Rice (1989).

$F_{IS}$ is known as the inbreeding coefficient but more accurately represents a test for non-random mating. $F_{IS}$ was calculated for each population of each species using GENEPOP 3.3 (Raymond and Rousset 1995). $F_{IS}$ measures deviation of observed heterozygosity of an individual relative to heterozygosity expected under the assumption of random mating/HWE (Avise 1994). Significance of $F_{IS}$ was tested for departures from HWE for each population using exact tests in the same program. Five hundred batches of 5000 iterations each were run and Bonferroni corrections were applied (Rice 1989).

#### Evidence for Bottlenecks

To detect recent genetic bottlenecks a test was carried out for each population, and the combined populations for each species using Bottleneck v1.2.02 (Piry *et al.* 1999). Bottleneck tests for an excess or deficit of observed heterozygotes with respect to proportions expected under HWE. Populations that have experienced a recent reduction of effective population size will have a lower heterozygosity than allelic diversity at polymorphic loci. Bottleneck calculates $H_{EQ}$ (heterozygosity expected under mutation-drift

equilibrium) by using three different models: Infinite Allele Model (IAM); Step-wise Mutation Model (SMM); and the Two-phased Model of Mutation (TPM) (Cornuet and Luikart 1996). The TPM is recommended by Cornuet and Luikart (1996) as the most appropriate model for analysis of microsatellite data because it assumes mostly step-wise mutation but a proportion of multi-step changes. Once calculated $H_{EQ}$ is compared with $H_E$ (assuming HWE) and if $H_E$ is significantly higher a recent bottleneck is indicated.

One thousand iterations of the TPM model were performed with the program defaults of 30% multi-step changes and 70% step-wise changes. Bottleneck was also run assuming IAM and SMM to see if results differed. There are three tests outlined by Cornuet and Luikart (1996) and Luikart et al (1998): the sign test, a standardised differences test and a Wilcoxon sign-rank test. The standardised test was not performed because it is not recommended by the authors for studies utilising fewer than 20 polymorphic loci.

**Population differentiation, gene flow and admixture**

$F_{ST}$ is a measure of genetic variation between subpopulations as a proportion of the total population variation. FSTAT was also used to calculate F-statistics for each locus across all populations. Estimates of $F_{ST}$ and $F_{IS}$ were tested for significance at the 95% confidence interval (divergence from panmixia) by 5000 permutations of genotypes among populations. Weir and Cockerham's (1984) values for $F_{ST}$ and $F_{IS}$ were calculated since these estimators avoid assumptions about numbers of populations, heterozygote frequencies and sample sizes.

$R_{ST}$ is an alternative statistic to $F_{ST.}$ While $F_{ST}$ is assessed under the infinite alleles model (IAM), $R_{ST}$ follows a step-wise mutation model (SMM) which usually approximates more closely the mode of evolution of microsatellites. $R_{ST}$ values were calculated using FSTAT as ρ, an estimator of $R_{ST}$ that allows for unequal sample sizes (Michalakis and Excoffier 1996). Due to the small number of loci (five) $F_{ST}$ may be a more reliable estimate, therefore $F_{ST}$ has been used in remaining calculations that require measures of differentiation (e.g. Principal Co-ordinate Analyses and Analysis of Molecular Variance).

Principal co-ordinate analyses (PCoA) were performed using GenAlEx 6.1 (Peakall and Smouse 2006) to investigate the relationship between pair-wise differentiation indices ($F_{ST}$) within and among populations of *Tetratheca ciliata* and *T. stenocarpa*.

A Bayesian assignment analysis was implemented in Structure 2.2 (Pritchard *et al.* 2000) to detect genetically differentiated groups of populations among *Tetratheca ciliata* and *T. stenocarpa.* The number of populations (*K*=1-n, where n is the total number of populations) was tested without *a priori* assumptions. The test was performed to investigate whether current species concepts correspond with genetically distinct entities, whether any further genetic structure is evident and whether admixture is occurring between entities. Significant admixture indicates gene flow between genetic populations and, if detected among populations of *T. ciliata* and *T. stenocarpa*, would indicate that hybridisation is occurring.

After initial exploration of the data the burn-in was set to 300,000 and a run length of 500,000 iterations was applied using the model of admixture. Five independent runs of *K* were performed to test the consistency of results. The optimal *K*-value was determined using the $\Delta K$ statistical approach of Evanno *et al.* (2005) to calculate mean log-likelihood values. Each of the two species was analysed individually to test for genetic structure, and together, to determine whether there is evidence of gene flow between them.

Analyses of Molecular Variance (AMOVA) were carried out using GenAlEx 6.1 (Peakall and Smouse 2006) to investigate the partitioning of variance components within and among populations and given regions, and the differentiated groups identified by PCoA and Bayesian assignment analysis.

**Isolation by Distance**

The influence of geographic distances between populations on patterns of genetic differentiation, the isolation by distance model (IBD), was investigated using Mantel tests applied in GenAlEx 6.1 (Peakall and Smouse 2006). To test the significance of the results 999 random permutations were performed between pair-wise geographic distance matrices (calculated from decimal latitude and longitude values recorded using GPS in the field)

and genetic distance matrices based on $F_{ST.}$ Evaluations of IBD were tested on each species individually and the two together.

## 4.3 Results

### 4.3.1 A nuclear SSR library for *Tetratheca*

Details of isolation and characterisation of the new microsatellite library are available in McPherson *et al.* (2008) (Appendix 10).

### 4.3.2 Locus screening and error checking

Initial screening identified that Te09BGT did not amplify reliably in *Tetratheca ciliata* or *T. stenocarpa*. Peaks were weak and difficult to read, therefore this locus was discarded. Te02BGT, Te03BGT and Te05BGT failed to amplify with either method for most individuals of *T. ciliata* despite producing clear reliable products for *T. stenocarpa*. These three loci were also discarded so that the same analyses could be applied to both *T. ciliata* and *T. stenocarpa*. For the remaining five loci the genotyping peaks resulting from both PCR methods produced the same shapes and allele lengths. Peak heights retrieved by the multiplex reactions, however, were much higher than those genotyped using the initial protocol. The number of individuals failing to amplify at each locus was greatly reduced using the multiplex mix.

At all loci except for Te15BGT there were several peaks that were difficult to interpret for *Tetratheca ciliata*. In 18% and 7% of samples, a third or fourth peak was visualised respectively. No extra peaks were found in individuals of *T. stenocarpa*. From 86 to 100% of all individuals amplified across the remaining five loci for populations of *T. ciliata* and *T. stenocarpa*. Of the 30% of individuals re-amplified, an error rate of 7% was calculated for *T. ciliata* and approximately 1.6% for *T. stenocarpa*. In most cases this was due to weak amplifications resulting in a homozygote being read instead of a heterozygote. The results from Microchecker 2.2 (Van Oosterhout *et al.* 2004) indicated a significant excess of homozygotes as a result of the presence of null alleles at Te15BGT for two populations

of *T. ciliata* (MtD and FH), and at loci Te08BGT and Te18BGT in the *T. ciliata* population LS. Homozygote excess due to null alleles was indicated at Te17BGT for two populations of *T. stenocarpa* (OCC and FR). There was no evidence of error due to stutter or short allele dominance. All analyses included all populations and loci. Tests were also performed excluding populations with null alleles but since the results were the same, only the analyses with all populations included are discussed here.

### 4.3.3 Diversity statistics

Allele length polymorphisms for each individual at each locus are presented in Appendices 11 (*Tetratheca ciliata*) and 12 (*T. stenocarpa*). Genotyping of 213 individuals (158 among 11 populations of *T. ciliata* and 55 among four populations of *T. stenocarpa*) produced a total of 139 alleles across five loci. The average numbers of alleles detected across the five loci for populations of each species are presented in Table 4.4. For *Tetratheca ciliata* they ranged from 5-12.2 and for *T. stenocarpa* 6.2-9.4. Allele size ranges varied from 56 to 96 base pairs between smallest and largest alleles in *T. ciliata* and 24 to 136 in *T. stenocarpa*. All alleles were dinucleotide repeats however there are mixtures of odd and even lengths at each locus and for each species. Te11BGT, Te15BGT and Te18BGT have complex repeat patterns and contain the largest number of mixed values.

Of the total of 74 alleles found among populations of *T. stenocarpa*, 50% were private alleles. There were 45 private alleles from a total of 133 detected among populations of *T. ciliata* (34%). The average number of private alleles in each population of each species is presented in Table 4.4. When the datasets were combined the number of alleles unique to *T. stenocarpa* was 15 (11% of the total) and to *T. ciliata* was 45 (32% of the total). Many rare and common alleles were shared among species.

### 4.3.4 Hardy-Weinberg and genotypic linkage equilibria

There was no significant genotypic disequilibrium among any pairs of loci for *Tetratheca ciliata* or *T. stenocarpa*. Results of tests for departures from HWE are shown in Table 4.4 and Table 4.5. The *T. ciliata* populations MtD and LS diverged from HWE, although only the MtD result remained significant after Bonferroni corrections were applied. The inbreeding coefficient ($F_{IS}$) showed heterozygote deficiency across all loci in the following populations of *T. ciliata*: DH, CB, FH, KH and LS. The population LS was the only one to retain significance after Bonferroni correction. One population (MtD) showed heterozygote excess but was not significant after correction.

All four populations of *T. stenocarpa* diverged from HWE, and after Bonferroni correction three (OCC, EB and FR) retained significance. Heterozygote deficit was detected in all populations of *T. stenocarpa* across all loci. Only populations EB and OCC retained significance after Bonferroni correction.

**Table 4.4 Diversity indices by population among five loci: number of individuals sampled ($N$); average number of alleles ($N_A$); average number of private alleles ($N_P$); expected ($H_E$) and observed ($H_O$) heterozygosities; inbreeding coefficient ($F_{IS}$).**

| Population | code | $N$ | $N_A$ | $N_P$ | $H_E$ | $H_O$ | $F_{IS}$ |
|---|---|---|---|---|---|---|---|
| *T. ciliata* | all | 14.36 | 7.95 | 0.82 | 0.763 | 0.717 | 0.061 |
| Dukes Highway, SA | DH | 15 | 5 | 0.2 | 0.566 | 0.497 | 0.129 |
| Mount Difficult, Grampians | MtD | 15 | 8.4 | 1.2 | 0.811 | 0.834 | -0.030**+ |
| Canadian, Ballarat | CB | 15 | 7.8 | 0.4 | 0.742 | 0.830 | -0.122 |
| Poverty Gully | PG | 14 | 7 | 0.4 | 0.796 | 0.751 | 0.059 |
| Frog Hollow | FH | 15 | 6.8 | 0 | 0.760 | 0.674 | 0.122 |
| Daylesford Road | DR | 15 | 8.8 | 0.4 | 0.776 | 0.751 | 0.035 |
| Kinglake-Healesville Road | KH | 15 | 12.2 | 2.2 | 0.877 | 0.771 | 0.124 |
| Lima South | LS | 9 | 9.4 | 2.2 | 0.894 | 0.711 | 0.215** |
| Tumbarumba-Tumut Road | TT | 15 | 7.4 | 0.2 | 0.728 | 0.678 | 0.071 |
| Murray Valley Highway | MV | 15 | 8.8 | 0.8 | 0.789 | 0.761 | 0.037 |
| Sardine Creek Road | SCR | 15 | 5.8 | 1 | 0.651 | 0.634 | 0.027 |
| *T. stenocarpa* | all | 13.75 | 7.45 | 1.85 | 0.728 | 0.624 | 0.146 |
| Old Chum Creek | OCC | 15 | 6.2 | 1.2 | 0.711 | 0.622 | 0.129**+ |
| Tentpole Road | TP | 10 | 6.4 | 1.2 | 0.766 | 0.644 | 0.168* |
| East Beenak | EB | 15 | 9.4 | 3 | 0.778 | 0.633 | 0.192**+ |
| Forest Road | FRV | 15 | 7.8 | 2 | 0.657 | 0.597 | 0.096**+ |

Levels of significance are shown as *p<0.05, **p<0.01, +p<0.05 after Bonferroni correction

Average observed heterozygosity ($H_O$) across all populations of *Tetratheca ciliata* was 0.717 whereas average $H_O$ for *T. stenocarpa* was 0.624. The lowest $H_O$ for *T. ciliata* was in

population DH (0.497) from South Australia and the highest at MtD in the Grampians (0.834). Generally, heterozygosity was high for populations of *T. ciliata*, however, at both the western and eastern extremes of the distribution sampled, heterozygosity was much lower.

For *Tetratheca stenocarpa* the range of $H_O$ values was much smaller: from 0.539 at OCC to 0.635 at FR. Average expected heterozygosity ($H_E$) was 0.763 for *T. ciliata* and 0.728 for *T. stenocarpa*. The *T. ciliata* population DH had the lowest $H_E$ (0.566) and the highest was at LS (0.894). The lowest $H_E$ for the *T. stenocarpa* populations was at FR (0.657) and the highest at EB (0.778).

### 4.3.5 Bottlenecks

There was no evidence of recent bottleneck events in *Tetratheca ciliata* or *T. stenocarpa* under either test or any of the models applied using BOTTLENECK v1.2.02 (Piry *et al.* 1999).

**Table 4.5 Diversity indices and differentiation statistics by locus for *Tetrathecaciliata and T. stenocarpa* : average number of alleles ($N_A$); expected ($H_E$) and observed ($H_O$) heterozygosities; inbreeding coefficient ($F_{IS}$); Weir and Cockerham's $F_{ST}$; $R_{ST}$ estimator (ρ).**

| ***T. ciliata*** | | | | | | |
|---|---|---|---|---|---|---|
| **Locus** | $N_A$ | $H_E$ | $H_O$ | $F_{IS}$ | $F_{ST}$ | $\rho$ |
| Te11BGT | 6.2 | 0.664 | 0.628 | 0.053 | 0.137 | 0.357 |
| Te15BGT | 7.6 | 0.761 | 0.558 | 0.249**+ | 0.187 | 0.672 |
| Te08BGT | 8.7 | 0.816 | 0.784 | 0.035 | 0.089 | 0.099 |
| Te18BGT | 7.6 | 0.747 | 0.734 | 0 | 0.165 | 0.17 |
| Te17BGT | 9.5 | 0.826 | 0.883 | -0.079 | 0.109 | 0.308 |
| mean | 7.9 | 0.763 | 0.717 | 0.049 | 0.138 | 0.321 |
| ***T. stenocarpa*** | | | | | | |
| **Locus** | $N_A$ | $H_E$ | $H_O$ | $F_{IS}$ | $F_{ST}$ | $\rho$ |
| Te11BGT | 5 | 0.548 | 0.465 | 0.139* | 0.164 | 0.226 |
| Te15BGT | 12 | 0.906 | 0.816 | 0.091 | 0.052 | 0.205 |
| Te08BGT | 6.25 | 0.642 | 0.606 | 0.043 | 0.049 | -0.024 |
| Te18BGT | 5.25 | 0.67 | 0.653 | 0.133 | -0.007 | -0.012 |
| Te17BGT | 8.75 | 0.875 | 0.581 | 0.294**+ | 0.027 | -0.022 |
| mean | 7.45 | 0.728 | 0.624 | 0.147 | 0.053 | 0.075 |

Levels of significance are shown as *p<0.05, **p<0.01, +p<0.05 after Bonferroni correction

### 4.3.6 Population differentiation

Weir and Cockerham's (1984) estimator of population differentiation ($F_{ST}$) at each locus, ranged from 0.109-0.187 for *Tetratheca ciliata* and from -0.007-0.164 for *T. stenocarpa* with averages of 0.138 and 0.053 respectively. Pair-wise comparisons of $F_{ST}$ values for populations are shown in Table 4.6 and Table 4.7, with pair-wise geographic distances (to the nearest kilometre) in the upper triangles. The geographic distances between populations of *Tetratheca stenocarpa* range from 3 to 56 km and 10 to 712 km between populations of *T. ciliata*. There is greater differentiation between *T. ciliata* and *T. stenocarpa* than between populations within either species. In a comparison of both species most pair-wise $F_{ST}$ values were significant (not indicated here). There is significant differentiation between all *T. stenocarpa* populations except for the comparison between EB and TP. Pair-wise $F_{ST}$ values range from 0.0700-0.0108 between populations of *T. stenocarpa* with an average of 0.051. The range of values for *T. ciliata* populations is much higher: from 0.010 to 0.366 (average 0.134). In both cases the smallest pair-wise $F_{ST}$ value is between the two populations that are geographically closest and the highest value is between the populations furthest apart. None of the comparisons between populations of *T. ciliata* was significant. The average pair-wise $F_{ST}$ value among western populations of *T. ciliata* is 0.066 compared with 0.098 among eastern populations. Average differentiation between eastern and western populations was much higher (0.173) than within each region. Western populations included: DH, MtD, CB, PG, FH and DR and eastern populations were: KH, LS, TT, MV and SCR. These groupings were determined by the Bayesian analysis and PCoA in the following sections.

**Table 4.6 Pair-wise $F_{ST}$ values among populations of *Tetratheca ciliata* (lower triangle) and distance in km among populations (upper triangle).**

| | DH | MtD | CB | PG | FH | DR | KH | LS | TT | MV | SCR |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **DH** | | 169 | 302 | 316 | 316 | 309 | 423 | 467 | 664 | 605 | 712 |
| **MtD** | 0.133 | | 133 | 153 | 149 | 141 | 255 | 308 | 520 | 456 | 548 |
| **CB** | 0.180 | 0.042 | | 63 | 39 | 29 | 127 | 200 | 424 | 355 | 423 |
| **PG** | 0.155 | 0.060 | 0.102 | | 26 | 34 | 111 | 156 | 374 | 307 | 396 |
| **FH** | 0.182 | 0.018 | 0.061 | 0.066 | | 10 | 107 | 167 | 389 | 321 | 399 |
| **DR** | 0.127 | 0.010 | 0.047 | 0.060 | 0.010 | | 114 | 177 | 400 | 331 | 408 |
| **KH** | 0.216 | 0.084 | 0.134 | 0.094 | 0.107 | 0.092 | | 96 | 316 | 245 | 295 |
| **LS** | 0.261 | 0.104 | 0.155 | 0.111 | 0.114 | 0.116 | 0.027 | | 225 | 155 | 250 |
| **TT** | 0.232 | 0.155 | 0.183 | 0.134 | 0.175 | 0.138 | 0.089 | 0.128 | | 72 | 195 |
| **MV** | 0.261 | 0.136 | 0.173 | 0.138 | 0.151 | 0.130 | 0.030 | 0.075 | 0.064 | | 175 |
| **SCR** | 0.366 | 0.239 | 0.267 | 0.234 | 0.247 | 0.232 | 0.156 | 0.168 | 0.125 | 0.099 | |

**Table 4.7 Pair-wise $F_{ST}$ values among populations of *Tetratheca stenocarpa* (lower triangle) and distance in km among populations (upper triangle).**

| | OCC | TP | EB | FRV |
|---|---|---|---|---|
| **OCC** | | 39 | 41 | 56 |
| **TP** | 0.035* | | 3 | 21 |
| **EB** | 0.067* | 0.011 | | 18 |
| **FRV** | 0.071* | 0.068* | 0.056* | |

The Principal Co-ordinate Analysis retrieved three groups among 15 populations and are shown in Figure 4.2. *Tetratheca ciliata* populations from western Victoria (circled in red) are genetically distinct from the remaining five populations of *T. ciliata* from eastern Victoria (in green). The four populations of *T. stenocarpa* (indicated in blue) are genetically distinct from either of the *T. ciliata* groups.

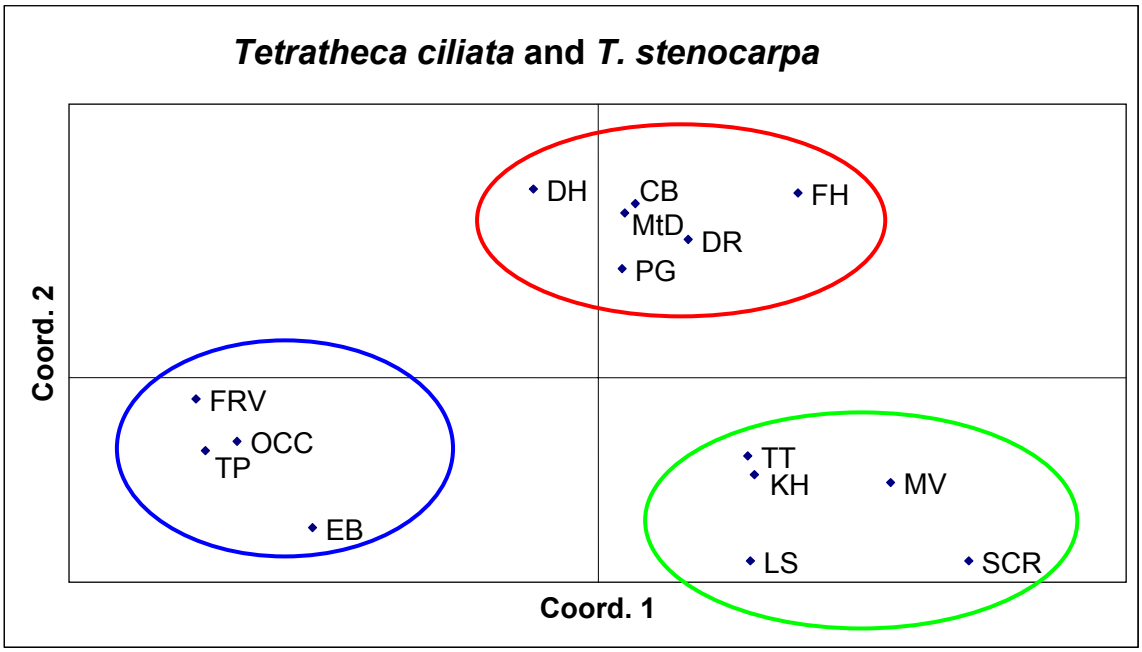



**Figure 4.2 Results of the principal co-ordinate analysis of *Tetratheca ciliata* and *T. stenocarpa* populations (coloured circles correspond with genetic clusters found by the Bayesian assignment analysis - see Figure 4.3)**

The results of the Bayesian assignment analysis of the two species confirm the separation of *Tetratheca stenocarpa*, and western and eastern genetic clusters of *T. ciliata* as retrieved by PCoA (see Figure 4.3). Some individuals are admixed as indicated in Figure 4.3, however the boundaries between the populations are distinct and there is no substantial gene flow between these groups.

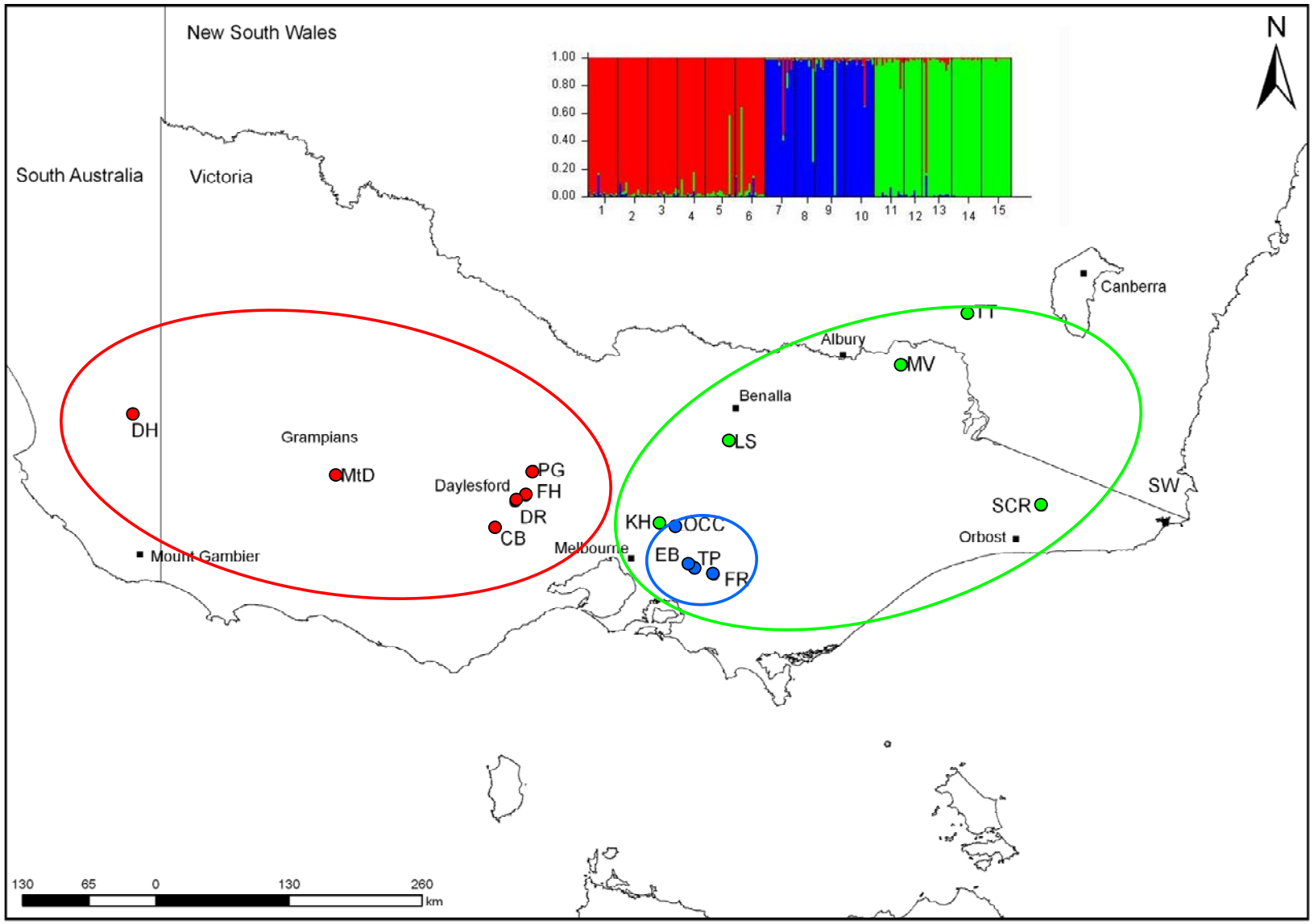


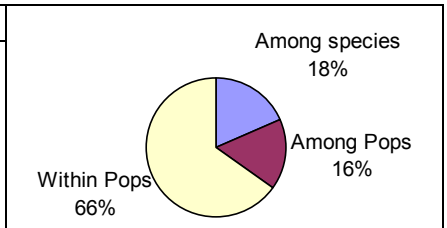

**Figure 4.3 Results of the Bayesian assignment analysis of *Tetratheca ciliata* and *T. stenocarpa.* Structure plot indicating three clusters above and clusters represented geographically in circles of corresponding colours below.**

Bayesian assignment analyses of each species individually (not presented here) resulted in *Tetratheca ciliata* forming two significant clusters (the same western and eastern populations as retrieved by the combined analyses). There was no significant structure within *T. stenocarpa*.

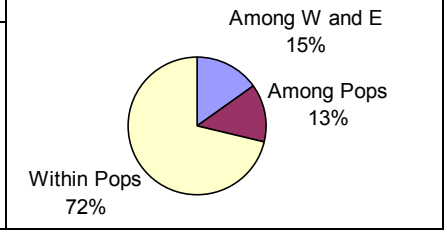
AMOVA results (see Table 4.8) on the combined data (*Tetratheca ciliata* and *T. stenocarpa*) showed that 66% of the variance occurred within populations, 16% among populations and 18% among species. When the western and eastern clusters of *T. ciliata* from the PCoA and Structure analysis were tested by AMOVA, a similar proportion of variance occurred within populations (72%). Variance among regions was 15% and 13% among populations.

**Table 4.8 Partitioning of variance among *Tetratheca ciliata* and *T. stenocarpa* and among regions within *T. ciliata***

| **Source of variance** | **% total** | **$\Phi_{PT}$** |
|---|---|---|
| *Tetratheca ciliata* and *T. stenocarpa* | | |
| Among species | 18 | 0.346*** |
| Among populations | 16 | |
| Within populations | 66 | |




| **Source of variance** | **% total** | **$\Phi_{PT}$** |
|---|---|---|
| *T. ciliata* W and E | | |
| Among regions | 15 | 0.285*** |
| Among populations | 13 | |
| Within populations | 72 | |




***$p \leq 0.001$

Mantel tests of isolation by distance confirmed patterns found by other analyses. There is a positive and significant correlation between genetic and geographic distance among populations across the entire geographic range of *Tetratheca ciliata* (Figure 4.4). The results of Mantel tests on the western and eastern clusters of *T. ciliata* individually, however, were not significant (Figure 4.5 and Figure 4.6). No correlation was found between genetic and geographic distance for *T. stenocarpa* (Figure 4.7).

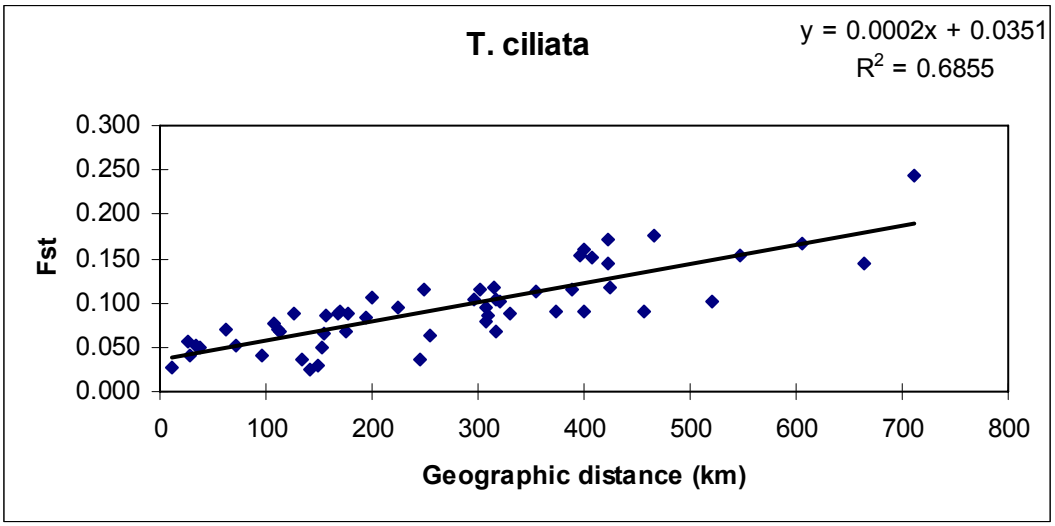



**Figure 4.4 Analysis of correlation between genetic and geographic distance calculated by Mantel test for *Tetratheca ciliata* (p=0.001)**

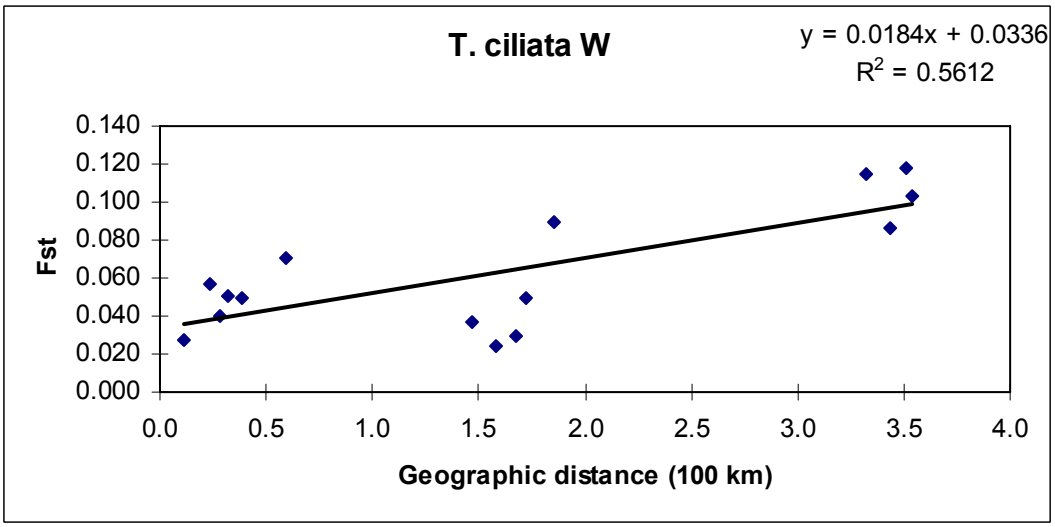



**Figure 4.5 Analysis of correlation between genetic and geographic distance calculated by Mantel test for the western cluster of *Tetratheca ciliata* (p=0.091)**

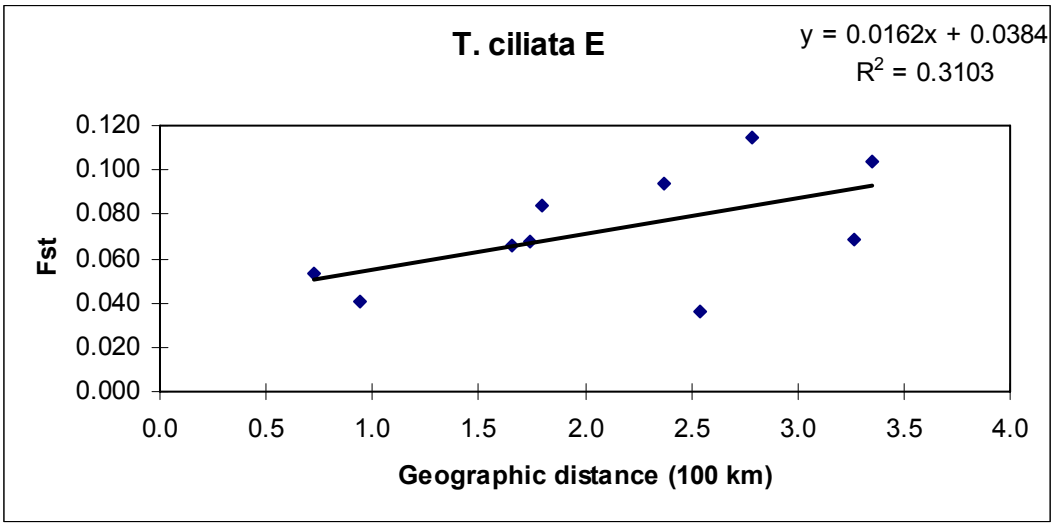



**Figure 4.6 Analysis of correlation between genetic and geographic distance calculated by Mantel test for the eastern cluster of *Tetratheca ciliata* (p=0.091)**

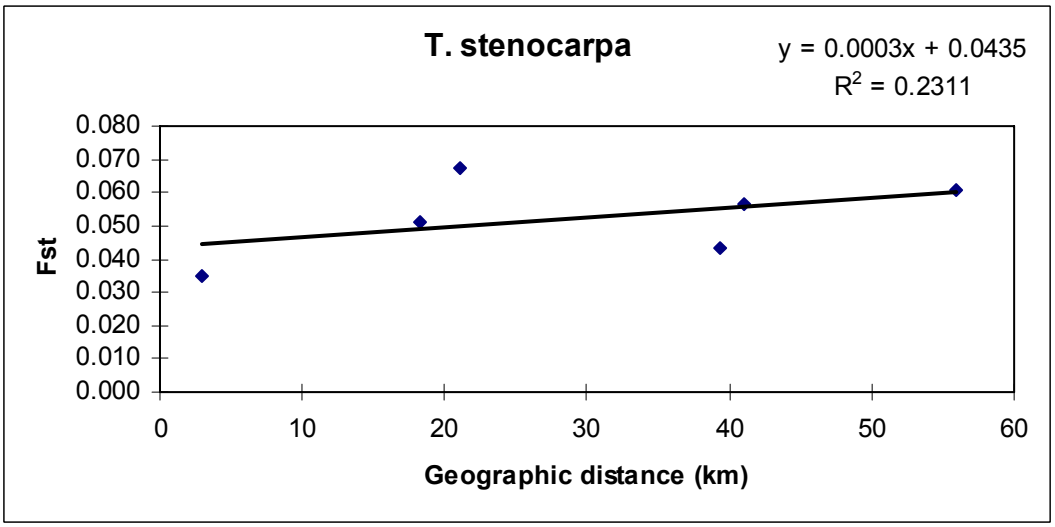



**Figure 4.7 Analysis of correlation between genetic and geographic distance calculated by Mantel test for the eastern cluster of *Tetratheca stenocarpa* (p=0.244)**

## 4.4 Discussion

The present study has shown that a library of polymorphic SSR markers characterised for *Tetratheca ericifolia* is applicable for broader studies of population variation in *Tetratheca.* Products successfully amplified in twelve species and a detailed study of variation in *T. ciliata* and *T. stenocarpa* is presented here. This comparison of *T. ciliata,* a widespread and common species in eastern Australia, with *T. stenocarpa*, narrowly distributed and rare, indicates that the latter is only slightly less diverse than the former (mean $H_E = 0.76$ compared with 0.73 respectively). Pair-wise comparisons of $F_{ST}$ showed higher divergence between populations of *T. ciliata* than *T. stenocarpa*, however, none was significant. Pair-wise comparisons of *T. stenocarpa* populations were significant in all but one.

For both species heterozygosity levels were similar to that found in *Tetratheca ericifolia* ($H_E = 0.73$), a relatively widespread species, continuously distributed around the Sydney region of New South Wales. Heterozygosity was high compared with microsatellite studies of species of *Elaeocarpus*: for example mean $H_E = 0.41$ *Elaeocarpus sedentarius* D.J.Maynard & Crayn (Rossetto *et al.* 2008); mean $H_E = 0.54$ *E. largiflorens* C.T.White and mean $H_E = 0.61$ *Elaeocarpus angustifolius* Blume (Rossetto *et al.* 2007). It should be noted, however, that although closely related to *Tetratheca, Elaeocarpus* are large trees with different life history traits. *Tetratheca* species are relatively short-lived plants with comparatively fast generation times and this may contribute to the different heterozygosities.

Slightly lower levels of heterozygosity in *Tetratheca stenocarpa* may indicate a founder effect or bottleneck event in the past however no recent bottleneck was detected whether loci with null alleles were included in the analyses or not. Modelling approaches have shown that a bottleneck event that reduces effective population size to 50 individuals may only be detected for up to 250 generations (Cornuet and Luikart 1996). Crayn *et al.* (2006) noted some species of *Tetratheca* resprouting and flowering only nine months after fire. For *Tetratheca,* 250 generations represents a small time in the evolutionary history of the genus. At all four sites sampled plants were abundant over at least 1-2 km and sites were adjacent to or within a large reserve. There is no strong evidence for large reductions in effective population size for *T. stenocarpa* despite its narrow distribution and rare status.

Although heterozygosity was high for most populations of *Tetratheca ciliata*, populations at the western (DH) and eastern (SCR) extremes of the distribution had much lower heterozygosity values. These populations could represent the edge of suitable habitat and therefore the limit of the distribution or a front of expansion: in either case lower heterozygosity is expected. Pujol and Pannell (2008) showed that populations at the margin of expanding geographic distributions of species have reduced genetic variation due to colonisation bottlenecks and therefore have a higher potential for extinction in changing environments. The Dukes Highway population was the only place in South Australia that *Tetratheca ciliata* was found despite extensive searching along the coast and inland between Adelaide and the Victorian Border. Herbarium data shows that *T. ciliata* has been collected from several localities in South Australia in the past but most of the native bushland in this area is now restricted to small reserves. Each is surrounded by agricultural land and in many cases the exclusion of fire has left vegetation dense. It may be that there is little suitable, open habitat for *T. ciliata* in South Australia now, and genetic diversity has been lost.

### 4.4.1 Species boundaries and hybridisation

Discerning the boundaries between populations and species can be difficult, particularly in recently diverged taxa. Microsatellites can provide evidence for delimitation of species via comparisons of levels of differentiation (e.g. Drummond and Hamilton 2007). Since present levels of divergence, measured as $F_{ST}$, are the result of both historical divergence and isolation of lineages, and population dynamics within lineages, it is necessary to be cautious in interpreting relative divergence levels between species and populations. To assume that species should have higher levels of divergence than populations or subpopulations may not be appropriate for taxa with high retention of ancestral polymorphism (Drummond and Hamilton 2007). Consideration of a variety of data at a range of hierarchical levels may help with interpretation of divergence patterns. The benefit of the present study is that patterns detected with nuclear microsatellites can be interpreted in the light of those found with cpSSRs and phylogenetic analyses.

Nuclear microsatellite analyses revealed that existing species (*Tetratheca ciliata* and *T. stenocarpa*) are supported by significant genetic differentiation and that *T. ciliata* is divided into two distinct genetic clusters. There is a marked genetic disjunction between *T. ciliata* and *T. stenocarpa* in the Bayesian assignment analysis suggesting that little if any gene flow occurs between them despite the close geographic proximity of some populations. There is negligible admixture between the two species as shown by the Bayesian assignment analysis, and there is no overlap of populations in the PCoA. It is therefore unlikely that hybridisation occurs between *T. ciliata* and *T. stenocarpa*. No sympatric populations were found during searches but whether the two species are entirely geographically separated or not has not been ascertained. Nevertheless, the geographic extents of the two significant genetic clusters of *T. ciliata* were very broad (each approximately 300 km wide). If admixture was occurring to any great extent it would have likely been detected by the Bayesian analysis. Perhaps a more surprising result is that little to no admixture is occurring between the two genetic clusters of *T. ciliata.* This raises the question of whether *Tetratheca ciliata* is a single species or whether it should be considered either as two species or two subspecies. This will be discussed in further detail in subsequent sections.

### 4.4.2 Geographic structure of genetic variation

Phenetic analyses by Downing (2005) showed high morphological variability within *Tetratheca ciliata,* detecting three main clusters across the range of the species. Nuclear microsatellite data have shown that *T. ciliata* is divided into two distinct genetic clusters according to geography. The eastern and western clusters found within *T. ciliata* correspond roughly to two of the morphological clusters detected by Downing (2005). A third cluster that overlaps with each of the eastern and western clusters was also identified on the basis of morphology however this result is not supported by nuclear microsatellite data.

AMOVA results show significant and similar levels of variance among species as they do among the two genetic clusters of *Tetratheca ciliata.* The cpSSR analysis in Chapter 3 resolved *T. stenocarpa* and *T. ciliata* as separate species and the separation of eastern and western clusters of *T. ciliata* was indicated by 93% of all *T. ciliata* haplotypes being

unique. The origins of each cluster however were not obvious due to the high diversity of haplotypes. Widespread species often display a broad range of genetic and morphological diversity since they are influenced by a wide variety of environmental factors. Distribution of genetic diversity will also be influenced by pollination and dispersal mechanisms. Given that *T. ciliata* is such a widespread species, the high diversity detected is not surprising.

Differentiation between western and eastern clusters of *Tetratheca ciliata* was much higher than within either cluster, but pair-wise $F_{ST}$ values were not significant between the eastern and western clusters. A significant positive correlation was found by the Mantel test of isolation by distance for *T. ciliata* across the entire species distribution but not within either eastern or western clusters nor within *T. stenocarpa*. Gross *et al*. (2003) demonstrated that the favoured reproductive strategy of *T. juncea* was outcrossing with heavy reliance on pollinators. The present data suggests that *T. ciliata* is outcrossing within each genetic cluster although no gene flow occurs between them. Each cluster is homogeneous over a long distance (approximately 300 km each) given that *Tetratheca* species require buzz pollination and the seeds are dispersed by ants.

There are several possible explanations for the reproductive isolation of different parts of a species distribution: e.g. vicariance of a formerly widespread species and subsequent evolution of the two halves in isolation; a slight change in flowering times between the two areas initiating a reproductive barrier; habitat specificity of two different pollinators or localised adaptation with selection preventing gene flow. Herbarium data (Australia's Virtual Herbarium) showed no obvious disjunction in flowering times between western and eastern collections of *Tetratheca ciliata* although only approximately 100 specimens reported presence of flowers. Collections for the present study were made in October, November and January (over two years, 2005 and 2006) and do not add any further information about flowering times between west and east since nine of the eleven populations had very few to no flowers.

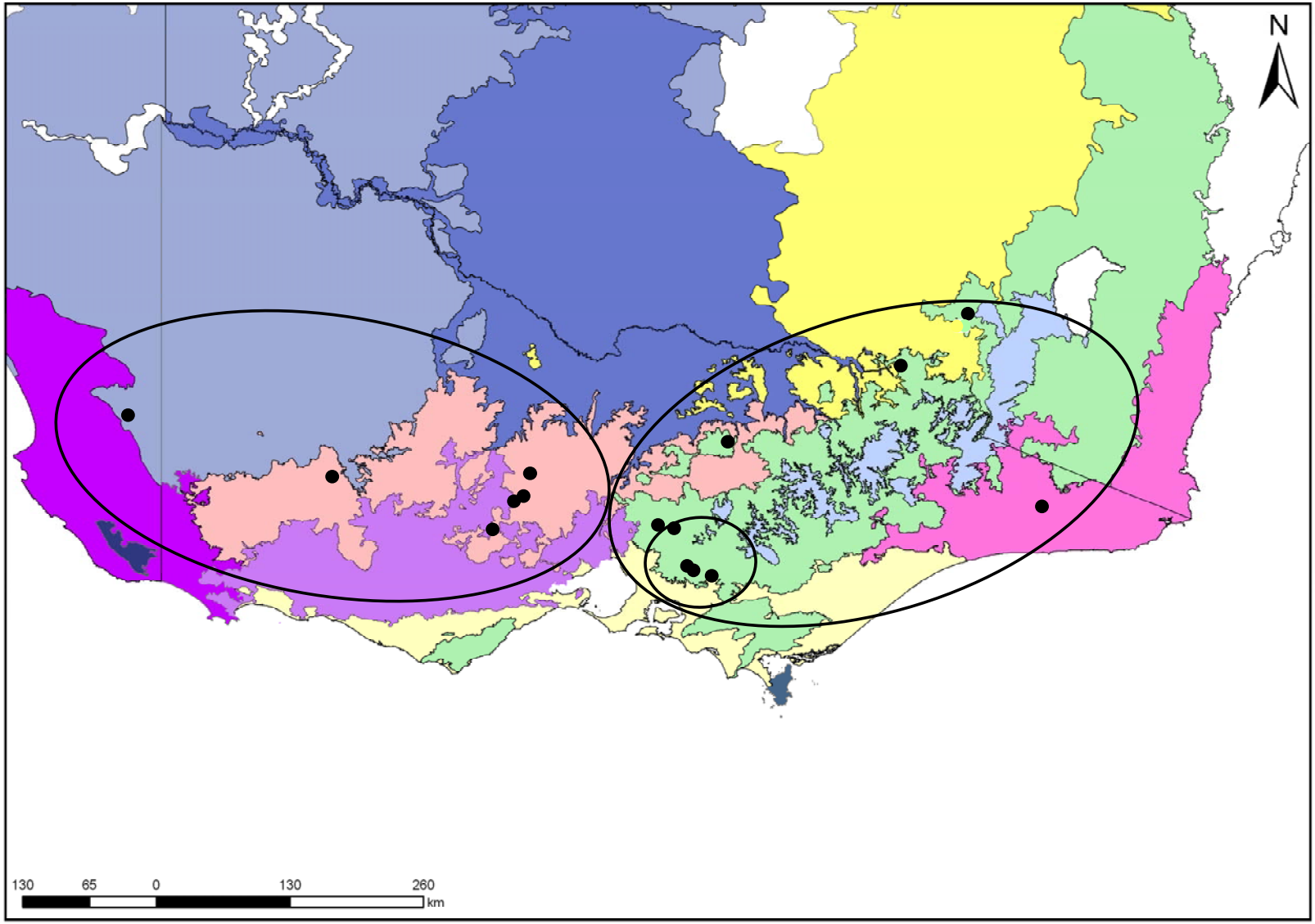



**Figure 4.8 IBRA bioregions with sampling localities for *Tetratheca ciliata* and *T. stenocarpa*. Genetic clusters circled as per Figure 4.3. Pale pink region represents the Victorian Midlands and pale green is the South Eastern Highlands**

The *Interim Biogeographic Regionalisation for Australia* project (Thackaway and Cresswell 1995, IBRA 2005) described 85 bioregions across the continent using a combination of data including ecological information, climate, geomorphology, landform, lithology, and characteristic flora and fauna (Thackaway and Cresswell 1995). The two genetic clusters of *Tetratheca ciliata* occur within different bioregions. Populations of the western cluster fall within the Victorian Midlands bioregion with the exception of DH which occurs in the Murray Darling Depression. The eastern cluster occurs in the South Eastern Highlands bioregion except for SCR which is on the South East Coastal Plain. The Victorian Midlands and the South Eastern Highlands Bioregions meet at their eastern and western limits just near Melbourne, corresponding with the point where the two clusters of *T. ciliata* also meet. *Tetratheca gunnii* is thought to be restricted to serpentinite rocks (Brown *et al.* 1986), and several species of *Tetratheca* in Western Australia are endemic to isolated ironstone ranges (Bull 2007) so it is possible that these two clusters are adapted to different substrates.

Investigation of life history and environmental traits along with genetic data would help to elucidate the cause of this distinctive pattern but this was beyond the scope of the present study. The alignment of the two clusters with different IBRA bioregions suggests that *Tetratheca ciliata* is divided into clusters with different habitat specificities. There is no evidence of IBD, and the distance between populations PG (western cluster) and KH (eastern cluster) is much smaller than between many populations within each cluster, supporting the hypothesis of local adaptation resulting in two reproductively isolated clusters is supported. The different levels of heterozygosity and diversity detected in the extreme west (DH) and extreme east (SCR) populations, could be due to environmental factors since these two populations occur in different bioregions from the remaining populations studied here.

The present study has shown strong population differentiation within *Tetratheca ciliata* and between *T. ciliata* and *T. stenocarpa* supporting previous morphological studies, recognising them as distinct species. The results of the nuclear SSR study indicate similar amounts of divergence between each of the clusters of *T. ciliata* and *T. stenocarpa*. Since $F_{ST}$ values do not distinguish between current processes and historic effects (Schaal *et al.* 1998, Zhang and Hewitt 2003), comparison with phylogeographic results is necessary to

determine whether divergence between *T. ciliata* clusters is representative of a specific or subspecific boundary or neither. The results of the cpSSR analyses did not indicate such a clear distinction between eastern and western clusters of *T. ciliata* and AMOVA results (in the cpSSR study) found no variance attributable to the east and west regions. The genetic clusters representing western and eastern distributions of *T. ciliata* therefore do not appear to constitute separate species. They may represent an intermediate stage in the processes leading to the pattern of localised endemic species seen across the genus. In other words, genotypic divergence and reproductive isolation shown by the present study indicate that *T. ciliata* is undergoing species diversification.

# *Chapter 5* Discussion and conclusions

## Summary

The main aim of the present study has been to investigate speciation processes in the Australian endemic genus *Tetratheca*, in order to better understand the levels and structure of genetic diversity, and the evolutionary processes that have shaped current distribution patterns. A hierarchy of molecular approaches has been employed to investigate the processes driving speciation in *Tetratheca* at a variety of taxonomic, geographic and temporal scales.

The first approach was to construct a detailed molecular phylogeny for *Tetratheca* to determine relationships between species, and investigate cladistic and biogeographic patterns, as well as possible origins of radiations of lineages. Both plastid and nuclear phylogenies were poorly resolved. For the most part, patterns retrieved by the two methods were congruent and some clades were well supported, however there were some points of conflict and many clades with poor support. Approximately 85% of the known species of *Tetratheca* were sequenced for each region, and most species were represented by a single individual. Multiple samples of *Tetratheca ciliata, T. hirsuta*, *T. pilosa, T. labillardierei, T. procumbens* and *T. stenocarpa* were sequenced. These taxa had been identified in previous (Thompson 1976, Downing 2005) and ongoing (R. Butcher, pers. comm. 2008) morphological studies as complex, and species boundaries in some cases were not clear.

The present study confirms that the genus is monophyletic and that the eastern Australian taxa (excluding *Tetratheca halmaturina*) form a clade arising from a grade of western Australian taxa. The two endemic species on Kangaroo Island do not form a clade, supporting Thompson's (1976) conclusion that *T. halmaturina* is more closely related to Western Australian species and *T. insularis* to species in the east. These results concur with the hypotheses of Downing *et al.* (2008) that several lineages diverged first in Western Australia and that the two lineages present in the east are more recent. Vicariant events that have influenced the southern Australian flora such as the establishment of the Nullarbor

Plain and subsequent marine incursions (Crisp and Cook 2007) have probably influenced the present distribution of *Tetratheca.*

Most of the morphological groups proposed by Thompson (1976) are not supported by molecular data. Phylogenetic resolution was poor and several clades in the *trn*L-*trn*F strict consensus tree contained individuals with no sequence divergence between them (zero substitutions). This indicates the presence of multiple derived lineages evolved from a single ancestral lineage, and also persistence of ancestral haplotypes along with their descendants (Posada and Crandall 2001). There were several points of conflict between the ITS and *trn*L-*trn*F strict consensus trees (e.g. *Tetratheca ciliata* was resolved as sister to *T. stenocarpa* with *trn*L-*trn*F data but sister to *T. ericifolia* with ITS).

Two hypotheses for low resolution and phylogenetic conflict are hybridisation or introgression, and incomplete lineage sorting (Comes and Abbott 2001). Hybridisation has often been suggested as the primary cause for conflict between plastid and nuclear phylogenies but Jakob and Blattner (2006) suggest that incomplete lineage sorting possibly accounts for more instances of poor resolution and incongruence between phylogenetic analyses than previously thought. It is often difficult to distinguish between these two evolutionary processes, particularly in recently derived lineages where species may not be fully reproductively isolated. To determine which process is affecting phylogenetic inference requires broad sampling of molecular variation within and among species across their geographic ranges (Comes and Abbott 2001).

The second approach of the present study was to use chloroplast microsatellites to investigate phylogeographic patterns within two species complexes with uncertain taxonomy, a range of widespread and narrowly distributed taxa, and a broad environmental gradient. The specific aims (presented in Chapter 3) were to clarify taxonomic boundaries, investigate phylogeographic patterns and to identify possible hybridisation or incomplete lineage sorting in *Tetratheca*. For both species complexes haplotypic patterns retrieved were characterised by high diversity and limited geographic structure. This high haplotypic diversity indicates that perhaps sequencing *trn*L-*trn*F for a single individual for each species would have captured only a small and potentially arbitrary portion of the total chloroplast variation, thereby resulting in an incorrect assessment of phylogenetic

relationships. In *Tetratheca pilosa* alone, up to 24 haplotypes were detected among 26 populations using cpSSRs. The haplotype network for the *Tetratheca pilosa* group supports *T. insularis* as a separate species probably derived from *T. pilosa*. Phenetic analyses by Downing (2005) showed that the two existing subspecies of *T. pilosa* were artificial: a result confirmed by the present study. Despite being widely distributed on the Australian mainland and Tasmania there was no evidence to support the division of the species. *Tetratheca procumbens* should be regarded as a subspecies of *T. pilosa* since it shows significant but incomplete divergence from *T. pilosa. Tetratheca ciliata* and *T. stenocarpa* were confirmed as distinct species, sharing no haplotypes between them. This study has shown that chloroplast microsatellites can help to resolve species boundaries where the phylogenetic analyses could not achieve sufficient resolution.

Haplotypic distribution does not always correspond with taxonomic boundaries, particularly in recently derived groups where hybridisation or incomplete lineage sorting are occurring. Crayn *et al.* (2006) showed, using molecular dating analyses, that *Tetratheca* is a recently derived and rapidly evolving element of the Australian flora. Analyses of the distribution of chloroplast haplotypes presented in Chapter 3 have helped to determine the cause of low resolution and conflict in phylogenies (presented in Chapter 2). Spatial analyses of shared haplotypes in *Eucalyptus* has indicated that introgression and hybridisation have played a major role in shaping current distribution patterns of populations and species (McKinnon *et al.* 2001, McKinnon *et al.* 2004b). These studies found extensive haplotype sharing between populations and/or species from the same geographic area, irrespective of taxonomic boundaries. The present study found no indication of hybridisation, since shared haplotypes were not detected in geographically proximate populations. There were, however, very few haplotypes shared among species or populations, and it may be that sampling three individuals per population was inadequate to address this question in light of the unexpectedly high haplotypic diversity detected.

In a study of the *Acacia acuminata* Benth. complex, Byrne *et al.* (2002) found similarly high chloroplast diversity and concluded that the results were due to incomplete lineage sorting, since all taxa contained shared haplotypes in the central (ancestral) positions of the network and unique haplotypes towards the tips (recently derived). The unique haplotypes in their study were a mixture of derived and divergent haplotypes indicating incomplete

lineage sorting prior to divergence of both the nuclear genome and morphological traits into separate entities. The distribution of haplotypes found across the *Tetratheca pilosa* group shows the same patterns of ancestral shared haplotypes, with more recently derived or divergent lineages towards the tips of the networks (indicating incomplete lineage sorting). *Tetratheca ciliata* and *T. stenocarpa* also show evidence of incomplete lineage sorting since haplotypic diversity is high although no haplotypes are shared among taxa. There are only two haplotypes shared among the eastern and western distributions of *T. ciliata*, however high homoplasy indicated by the large number of closed loops in the network make interpretation of the relationships among populations difficult.

Persistence of ancestral haplotypes can be due to increasing effective population size and, provided the effective population size is large enough, diversity can be maintained even in isolated populations (Nei and Takahata 1993, Jakob and Blattner 2006). Isolated populations can buffer against lineage extinctions and therefore contribute to high diversity across a species. Both of these points may be relevant to understanding chloroplast patterns in *Tetratheca*. In the case of *Tetratheca stenocarpa* which is narrowly distributed and rare, for example, haplotypic diversity remains high and may indicate increasing effective population size. The high haplotypic diversity and the distribution patterns of diversity across all taxa studied here suggest that effective population size has remained high throughout the evolutionary history of the genus.

Bermingham and Moritz (1998) and Moritz and Faith (1998) outlined the benefits of comparative phylogeography for describing the evolution of landscapes, and analysing the effects of historical and geographical processes on the distribution of organisms at a range of spatial scales. Identification of congruent distribution patterns across a range of species can help to detect common historical influences and thereby lead to a better understanding of the contribution of environmental effects on the evolution of organisms at a variety of scales.

Jakob and Blattner (2006) established the a chloroplast genealogy for the genus, *Hordeum*, and demonstrated the benefits of a comparative study across a large number of species. They showed that restricting a phylogeographic study to a single species would have resulted in the wrong interpretation of the relative ages of haplotypes throughout their

network. In the present study, species complexes were chosen on the basis of morphological and phylogenetic studies that indicated close relationships between species. In order to ascertain the relative ages of species and the likely migration routes with respect to past climate changes, a more appropriate approach might be to expand sampling to include all eastern Australian species. Since so many species of *Tetratheca* are restricted in distribution, and are considered rare or threatened, a broader comparative approach would allow for a better assessment of historical landscape processes that have shaped distribution patterns and therefore better inform management decisions in planning for future climate change.

The third approach used novel microsatellites to examine the population genetics of two closely related species of *Tetratheca* to see if current distributions reflect longer term (phylogeographic) patterns. Novel microsatellites developed for *Tetratheca ericifolia* proved useful for population level studies across a variety of species of *Tetratheca.* A comparison of patterns of diversity within and among a widespread common species (*T. ciliata*) and an endangered narrow endemic (*T. stenocarpa*) indicated that hybridisation is not occurring between the two species. The cpSSR study showed that *T. ciliata* and *T. stenocarpa* are distinct entities with no evidence of historical hybridisation and nrSSR results confirm that the two species remain reproductively isolated. *Tetratheca stenocarpa* was only slightly less diverse than *T. ciliata* and no evidence of bottleneck events was found in either species, supporting cpSSR results that effective population sizes have remained large throughout the evolutionary history of the eastern Australian species. This is further supported by tests of Fu's test of selective neutrality which detected no significant population demographic expansion in either of the species complexes.

Drummond and Hamilton (2007) used microsatellite data to determine limits between species of *Lupinus* L. and highlight the importance of investigating divergence data at a variety of hierarchical levels. Present levels of divergence (measured as $F_{ST}$) are the result of historical processes, and isolation of lineages as well as population dynamics within lineages, therefore species may not necessarily have higher levels of divergence than populations or subpopulations. Instead congruence of patterns at a variety of hierarchical levels can provide evidence for identification of species boundaries.

The present study detected significant pair-wise $F_{ST}$ values between *Tetratheca ciliata* and *T. stenocarpa* and also among populations of *T. stenocarpa.* Despite clear separation of *T. ciliata* into two genetic clusters, pair-wise $F_{ST}$ values between the two regions were not significant. *Tetratheca ciliata* had an identical *trn*L-*trn*F sequence to *T. stenocarpa* but the two species were distinct in Downing's (2005) phenetic analysis of species concepts, and when analysed using cpSSR data. The nrSSR results concur with the concept that they are separate species. Downing (2005) found three morphological clusters within *T. ciliata* and two of these were retrieved by the nrSSR analysis in Chapter 4, indicating that genetic variation parallels morphology to some extent. There were only two chloroplast haplotypes shared between western and eastern clusters however, no other analyses of chloroplast data resolved the clusters as distinct. Therefore, although the level of divergence between the two genetic clusters was similar to that detected between species (*T. ciliata* and *T. stenocarpa*), splitting *T. ciliata* into two separate taxa is not supported. Rather, the nrSSR patterns indicate that the two clusters of *T. ciliata* represent entities undergoing speciation.

The correspondence of western and eastern clusters of *T. ciliata* with IBRA bioregions suggests that each cluster has adapted over time to specific habitat and/or environmental conditions, eventually reproductively isolating the clusters. The distinct morphological clusters found by Downing's (2005) analysis in other widespread species may also parallel underlying genetic structure. On a landscape scale, the patterns of narrow endemism and disjunctly distributed populations in *Tetratheca* may be due to locally adapted populations (within formerly widespread distributions) and subsequent reproductive isolation, driving speciation.

Hughes and Hollingworth (2008) found in microsatellite study of *Begonia* L. that macro-evolutionary patterns were also retrieved at the micro-evolutionary scale. They determined that within *Begonia* there is high population differentiation and that geographically close populations are more genetically similar than those further apart. The high number of endemic taxa and disjunct populations in *Tetratheca,* indicate dispersal limitations to many species ranges. The few widespread species tend to be highly morphologically variable across their ranges (e.g. *Tetratheca ciliata, T. pilosa, T. labillardierei*) as shown by Downing's (2005) phenetic analyses of morphology within the genus and the present study has shown that some of this morphological variation parallels genetic variation. Even a

seemingly continuously distributed species such as *T. ciliata* has reproductively isolated populations within its range.

Since *Tetratheca* is a recently derived and rapidly evolving genus, an understanding of micro-evolutionary processes is essential for accurate interpretation of macro-evolutionary processes such as speciation. The present study indicates that incomplete lineage sorting is affecting phylogenetic inference due to high diversity of retained ancestral haplotypes. Nevertheless, contemporary patterns of gene flow detected using nuclear microsatellites have informed interpretations of phylogeographic and phylogenetic data.

# Conclusions

Speciation can be detected at the population level, however genetic diversity occurs as a hierarchy, as do the processes influencing speciation. In the present study therefore, I have approached the question of which factors are driving speciation in *Tetratheca* at a variety of taxonomic, spatial and temporal scales, using three molecular approaches. Each approach yielded different information about evolutionary dynamics in *Tetratheca.* Since *Tetratheca* is a recent and rapidly evolving genus, the poorly resolved, conflicting phylogenies were not surprising. Studies at the population level have helped to tease out which processes are more likely to be causing problems with phylogenetic inference. Assessing the distribution patterns of genetic diversity across a range of closely related species confirmed that hybridisation is not occurring and neither has it played an important role in the evolutionary histories of the study species. The hypothesis of incomplete lineage sorting, however, is supported at macro- and micro-evolutionary scales.

At the macro-evolutionary scale vicariant events have contributed to the current distinctiveness of the *Tetratheca* floras of western and eastern Australia. Nevertheless, haplotypic diversity is high and resolution of relationships between species is sometimes poor due to incomplete lineage sorting. Distribution patterns within species of *Tetratheca* from eastern Australia appear to reflect patterns of genetic diversity at the micro-evolutionary scale. Even isolated, endemic species such as *Tetratheca stenocarpa* and *T.*

*insularis*, have maintained high haplotypic diversity indicating that effective population sizes have probably remained large: a result supported by both cpSSR and nrSSR data.

Local adaptation plays an important role in speciation within *Tetratheca*. The distinct genetic clusters detected within *Tetratheca ciliata,* along with morphological patterns across the distributional range of this widespread species, correspond with different IBRA bioregions. Since the bioregions are modelled using ecological, climatic, geological and geomorphological data, the genetic clusters of *T. ciliata* have different habitat specificities. The patterns of clustering of morphological data found in several widespread species by Downing (2005) may be a result of local adaptation and subsequent reproductive isolation to form genetically distinct subunits within species. This, along with the morphological similarity between species of *Tetratheca*, may indicate that the large numbers of narrow endemic species and disjunctly distributed populations are the result of local adaptation and diversifications of previously more widespread species. The present study confirms that comparative approaches at a variety of hierarchical levels are useful for understanding evolutionary processes and the genetic diversity of any single species.

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# Appendices

**Appendix 1 Description of unique indels scored for ITS data**

| Indel no. | Indel type | No. of bases | Taxa |
|---|---|---|---|
| 1 | I | 1 | *T. aphylla* ssp. *aphylla, T. aphylla* ssp. *megacarpa* |
| 2 | I | 1 | *T. bauerifolia, T. decora, T. glandulosa, T. gunnii, T. insularis, T. labillardierei (sll), T. neglacta, T. pilosa* ssp. *pilosa* (all) *T. pilosa* ssp. *latifolia* (all), *T. procumbens* (all), *T. rubioides, T. rupicola, T. shiressii, T. stenocarpa* (all), *T. thymifolia, T. retrorsa* |
| 3 | I | 1 | *T.* sp. Boonanarring, *T.* sp. Granite, *T. hirsuta* (pink), *T. hirsuta* (white) |
| 4 | I | 8 | *T.* sp. Boonanarring, *T.* sp. Granite, *T. hirsuta* (pink), *T. hirsuta* (white), *T.* ?*hirsuta, T.* ?*setigera, T. pilifera, T. similis, T.* aff. *Hirsuta* |
| 5 | I | 2 | *T. ciliata* (all), *T. halmaturina, T. filiformis* |
| 6 | D | 1 | *T. decora, T. labillardierei (NSW)* |
| 7 | I | 2 | *T. efoliata, T. erubescens, T. confertifolia* |
| 8 | I | 2 | *T. ericifolia, T. juncea* |
| 9 | D | 3 | *T. ericifolia, T. juncea* |
| 10 | D | 3 | *T. ericifolia, T. juncea* |
| 11 | I | 6 | *T. ericifolia, T. juncea* |
| 12 | I | 1 | *Tr. diffusa, T. filiformis, T. affinis* |
| 13 | D | 4 | *T. filiformis, T. affinis* |
| 14 | I | 4 | *T. nuda, T. halmaturina, T. paynterae* ssp. *paynterae, T. paynterae* ssp. *cremnobata* |
| 15 | D | 2 | *T. paynterae* ssp. *paynterae, T. paynterae* ssp. *cremnobata* |
| 16 | I* | 3 | *T. paynterae* ssp. *paynterae, T. paynterae* ssp. *cremnobata* |
| 17 | D | 18 | *T. paynterae* ssp. *paynterae, T. paynterae* ssp. *cremnobata* |
| 18 | D | 1 | *T. paynterae* ssp. *paynterae, T. paynterae* ssp. *cremnobata, T. nephelioides, T. hispidissima, T. applanata, T. parvifolia* |
| 19 | D | 1 | *P. juniperina, P. galioides* |
| 20 | I | 1 | *P. juniperina, P. galioides* |
| 21 | I | 1 | *P. juniperina, P. galioides* |
| 22 | I | 2 | *P. juniperina, P. galioides* |
| 23 | I* | 3 | *P. juniperina, P. galioides* |
| 24 | D | 3 | *P. juniperina, P. galioides* |
| 25 | D | 3 | *P. juniperina, P. galioides* |
| 26 | I* | 4 | *P. juniperina, P. galioides* |
| 27 | D | 4 | *P. juniperina, P. galioides* |
| 28 | D | 4 | *P. juniperina, P. galioides* |
| 29 | I | 8 | *P. juniperina, P. galioides* |
| 30 | I | 8 | *P. juniperina, P. galioides* |
| 31 | D | 12 | *P. juniperina, P. galioides* |

| Indel no. | Indel type | No. of bases | Taxa |
|---|---|---|---|
| 32 | I | 2 | *P. juniperina, P. galioides* |
| 33 | D | 1 | *P. juniperina, P. galioides, Tr. diffusa* |
| 34 | D | 4 | *T.* ?*hirsuta, T.* ?*setigera, T. pilifera, T. similis, T.* aff. *hirsuta* |
| 35 | I | 1 | *T.* ?*hirsuta, T.* aff. *hirsuta* |
| 36 | D | 10 | *T. retrorsa, T.* ?*setigera, T. pilifera, T. similis, T.* aff. *hirsuta, T.* ?*hirsuta, T. hirsuta* (pink)*, T. hirsuta* (white)*, T.* sp. Granite*, T.* sp. Boonanarring*, T. harperi, T. angulata, T. exasperata* |
| 37 | I | 2 | *T. retrorsa, T.* ?*setigera, T. pilifera, T. similis, T.* aff. *hirsuta, T.* ?*hirsuta, T. hirsuta* (pink)*, T. hirsuta* (white)*, T.* sp. Granite*, T.* sp. Boonanarring*, T. harperi, T. angulata, T. exasperata, T. aphylla* ssp. *aphylla, T. aphylla* ssp. *megacarpa, T. erubescens* |
| 38 | I | 3 | *T. similis, T. pilifera* |

(shaded rows represent clades present in the strict consensus; * indicates an insertion that is a copy of adjacent sequence)

**Appendix 2 Description of unique indels scored for *trn*L-*trn*F data**

| Indel no. | Indel type | No. of bases | Taxa |
|---|---|---|---|
| 1 | D | 10 | *T. affinis, Tr. diffusa* |
| 2 | I* | 5 | *T. bauerifolia, T. juncea, T. labillardierei 959, T. subaphylla* |
| 3 | I* | 18 | *T. ciliata, T. stenocarpa* |
| 4 | I* | 22 | *T.* sp. *Mundaring, T. retrorsa, T. virgata* |
| 5 | I* | 5 | *T. nuda, T. ?setigera* |
| 6 | I* | 8 | *T. nuda, T. ?setigera* |
| 7 | I* | 12 | *T. paynterae* ssp. *paynterae, T. paynterae* ssp. *cremnobata* |
| 8 | I | 28 | *T. paynterae* ssp. *paynterae, T. paynterae* ssp. *cremnobata* |
| 9 | D | 2 | *T. paynterae* ssp. *paynterae, T. paynterae* ssp. *cremnobata, T. efoliata* |
| 10 | D | 2 | *T. phoenix, T. exasperata, T. thymifolia, T. rubioides, T. rupicola, T. shiressii, T. neglecta, T. pilosa* ssp. *latifolia 7116, T. labillardierei TAS, T. gunnii, T. insularis, T. ericifolia, T. pilosa ssp latifolia FI, T. bauerifolia* |
| 11 | D | 1 | *P. juniperina, P. galioides* |
| 12 | I | 2 | *P. juniperina, P. galioides* |
| 13 | I* | 14 | *P. juniperina, P. galioides* |
| 14 | D | 6 | *P. juniperina, P. galioides, T. filiformis, T. affinis* |
| 15 | D | 1 | *P. juniperina, P. galioides, Tr. diffusa, Tr. stelligera* |
| 16 | D | 1 | *P. juniperina, P. galioides, Tr. diffusa, Tr. stelligera, T. filiformis* |
| 17 | D | 1 | *Tr. diffusa, Tr. stelligera* |
| 18 | D | 1 | *Tr. diffusa, Tr. stelligera* |
| 19 | D | 5 | *Tr. diffusa, Tr. stelligera* |
| 20 | D | 6 | *Tr. diffusa, Tr. stelligera* |
| 21 | D | 6 | *Tr. diffusa, Tr. stelligera* |
| 22 | D | 7 | *Tr. diffusa, Tr. stelligera* |
| 23 | I* | 11 | *Tr. diffusa, Tr. stelligera* |
| 24 | I* | 12 | *Tr. diffusa, Tr. stelligera* |

(shaded rows represent clades present in the strict consensus; * indicates an insertion that is a copy of adjacent sequence)

**Appendix 3 Chloroplast microsatellite data for the *Tetratheca pilosa* group**

| | | | Locus | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **Taxon** | **Pop** | **ccmp10** | **ccmp2** | **ccmp3** | **ccmp4** | **Tecp01** | **Sample number** |
| 1 | T.insularis | Pop1 | 122 | 147 | 141 | 134 | 222 | iBHR_10-hm288 |
| 2 | *T.insularis* | Pop1 | 122 | 148 | 141 | 134 | 222 | iBHR_1-hm287 |
| 3 | *T.insularis* | Pop1 | 122 | 147 | 141 | 134 | 222 | iBHR_6-hm289 |
| 4 | *T.insularis* | Pop2 | 122 | 147 | 140 | 134 | 222 | iK2_10-hm293 |
| 5 | *T.insularis* | Pop2 | 122 | 147 | 140 | 134 | 222 | iK2_2-hm294 |
| 6 | *T.insularis* | Pop2 | 122 | 146 | 140 | 134 | 222 | iK2_6-hm295 |
| 7 | *T.insularis* | Pop3 | 122 | 148 | 140 | 134 | 221 | iSB_10-hm297 |
| 8 | *T.insularis* | Pop3 | 122 | 148 | 140 | 134 | 221 | iSB_1-hm296 |
| 9 | *T.insularis* | Pop3 | 122 | 148 | 140 | 134 | 221 | iSB_6-hm298 |
| 10 | *T.insularis* | Pop4 | 122 | 146 | 141 | 134 | 222 | iSR1_10-hm300 |
| 11 | *T.insularis* | Pop4 | 109 | 154 | 141 | 134 | 218 | iSR1_1-hm299 |
| 12 | *T.insularis* | Pop4 | 122 | 146 | 141 | 134 | 222 | iSR1_6-hm301 |
| 13 | *T.insularis* | Pop5 | 122 | 147 | 140 | 134 | 222 | iSR3i_10-hm303 |
| 14 | *T.insularis* | Pop5 | 122 | 146 | 140 | 134 | 219 | iSR3i_1-hm302 |
| 15 | *T.insularis* | Pop5 | 122 | 147 | 140 | 134 | 222 | iSR3i_6-hm304 |
| 16 | T. pilosa ssp. latifolia | Pop6 | 122 | 180 | 141 | 137 | 222 | plBT_1-hm305 |
| 17 | *T. pilosa* ssp. *latifolia* | Pop6 | 122 | 180 | 141 | 137 | 222 | plBT_3-hm306 |
| 18 | *T. pilosa* ssp. *latifolia* | Pop6 | 122 | 180 | 141 | 137 | 222 | plBT_hm307 |
| 19 | *T. pilosa* ssp. *latifolia* | Pop7 | 122 | 180 | 141 | 137 | 222 | plCCJ_1-hm308 |
| 20 | *T. pilosa* ssp. *latifolia* | Pop7 | 122 | 180 | 141 | 137 | 222 | plCCJ_3-hm309 |
| 21 | *T. pilosa* ssp. *latifolia* | Pop7 | 122 | 180 | 141 | 137 | 222 | plCCJ_hm310 |
| 22 | *T. pilosa* ssp. *latifolia* | Pop8 | 122 | 182 | 141 | 137 | 222 | plCT_1-hm311 |
| 23 | *T. pilosa* ssp. *latifolia* | Pop8 | 122 | 182 | 141 | 137 | 222 | plCT_3-hm312 |
| 24 | *T. pilosa* ssp. *latifolia* | Pop8 | 122 | 182 | 141 | 137 | 222 | plCT_hm313 |
| 25 | *T. pilosa* ssp. *latifolia* | Pop9 | 122 | 179 | 141 | 134 | 222 | plGCT_1-hm314 |
| 26 | *T. pilosa* ssp. *latifolia* | Pop9 | 122 | 179 | 141 | 134 | 222 | plGCT_3-hm315 |
| 27 | *T. pilosa* ssp. *latifolia* | Pop9 | 122 | 179 | 141 | 134 | 222 | plGCT_hm316 |
| 28 | *T. pilosa* ssp. *latifolia* | Pop10 | 122 | 180 | 141 | 135 | 222 | plGR1_1-hm317 |
| 29 | *T. pilosa* ssp. *latifolia* | Pop10 | 122 | 180 | 142 | 135 | 222 | plGR1_3-hm318 |
| 30 | *T. pilosa* ssp. *latifolia* | Pop10 | 122 | 180 | 141 | 138 | 222 | plGR1l_hm319 |
| 31 | *T. pilosa* ssp. *latifolia* | Pop11 | 122 | 144 | 141 | 136 | 222 | plKR_10-hm321 |
| 32 | *T. pilosa* ssp. *latifolia* | Pop11 | 122 | 146 | 141 | 136 | 222 | plKR_1-hm320 |
| 33 | *T. pilosa* ssp. *latifolia* | Pop11 | 122 | 146 | 141 | 136 | 222 | plKR_6-hm322 |
| 34 | *T. pilosa* ssp. *latifolia* | Pop12 | 122 | 180 | 141 | 136 | 221 | plNL-hm410 |
| 35 | *T. pilosa* ssp. *latifolia* | Pop12 | 122 | 179 | 141 | 134 | 222 | plNL-hm411 |
| 36 | *T. pilosa* ssp. *latifolia* | Pop12 | 122 | 180 | 141 | 136 | 221 | plNL-hm412 |
| 37 | *T. pilosa* ssp. *latifolia* | Pop13 | 122 | 181 | 141 | 136 | 220 | plNR_12-hm407 |
| 38 | *T. pilosa* ssp. *latifolia* | Pop13 | 122 | 181 | 141 | 136 | 220 | plNR_6-hm408 |
| 39 | *T. pilosa* ssp. *latifolia* | Pop13 | 122 | 181 | 141 | 136 | 220 | plNR-hm409 |
| 40 | *T. pilosa* ssp. *latifolia* | Pop14 | 122 | 180 | 141 | 137 | 222 | plPL_1-hm404 |
| 41 | *T. pilosa* ssp. *latifolia* | Pop14 | 122 | 180 | 141 | 137 | 222 | plPL_9-hm406 |
| 42 | *T. pilosa* ssp. *latifolia* | Pop14 | 122 | 180 | 141 | 137 | 221 | plPL_hm405 |
| 43 | *T. pilosa* ssp. *latifolia* | Pop15 | 122 | 180 | 142 | 135 | 222 | plTR_1-hm401 |
| 44 | *T. pilosa* ssp. *latifolia* | Pop15 | 122 | 180 | 141 | 136 | 222 | plTR_6_-hm403 |
| 45 | *T. pilosa* ssp. *latifolia* | Pop15 | 122 | 180 | 141 | 136 | 222 | plTR_-hm402 |
| 46 | T. pilosa ssp. pilosa | Pop16 | 122 | 180 | 141 | 135 | 222 | ppBBR_1-hm371 |
| 47 | *T. pilosa* ssp. *pilosa* | Pop16 | 122 | 180 | 141 | 135 | 222 | ppBBR_2-hm372 |

| | | | Locus | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **Taxon** | **Pop** | **ccmp10** | **ccmp2** | **ccmp3** | **ccmp4** | **Tecp01** | **Sample number** |
| 48 | *T. pilosa* ssp. *pilosa* | Pop16 | 122 | 180 | 141 | 135 | 222 | ppBBR_3-hm373 |
| 49 | *T. pilosa* ssp. *pilosa* | Pop17 | 122 | 146 | 141 | 136 | 222 | ppBR_10-hm324 |
| 50 | *T. pilosa* ssp. *pilosa* | Pop17 | 122 | 146 | 141 | 136 | 222 | ppBR_1hm323 |
| 51 | *T. pilosa* ssp. *pilosa* | Pop17 | 122 | 146 | 141 | 136 | 222 | ppBR_6-hm325 |
| 52 | *T. pilosa* ssp. *pilosa* | Pop18 | 122 | 179 | 141 | 136 | 222 | ppCRT_10-hm327 |
| 53 | *T. pilosa* ssp. *pilosa* | Pop18 | 122 | 179 | 140 | 137 | 222 | ppCRT_1-hm326 |
| 54 | *T. pilosa* ssp. *pilosa* | Pop18 | 122 | 179 | 141 | 136 | 222 | ppCRT_6-hm328 |
| 55 | *T. pilosa* ssp. *pilosa* | Pop19 | 122 | 182 | 143 | 136 | 220 | ppCV_10-hm330 |
| 56 | *T. pilosa* ssp. *pilosa* | Pop19 | 122 | 149 | 143 | 136 | 220 | ppCV_1-hm329 |
| 57 | *T. pilosa* ssp. *pilosa* | Pop19 | 122 | 182 | 143 | 136 | 220 | ppCV_6-hm331 |
| 58 | *T. pilosa* ssp. *pilosa* | Pop20 | 122 | 180 | 142 | 135 | 222 | ppGR1_11-hm333 |
| 59 | *T. pilosa* ssp. *pilosa* | Pop20 | 122 | 180 | 142 | 135 | 222 | ppGR1_1-hm332 |
| 60 | *T. pilosa* ssp. *pilosa* | Pop20 | 122 | 180 | 142 | 135 | 222 | ppGR1_6-hm334 |
| 61 | *T. pilosa* ssp. *pilosa* | Pop21 | 122 | 182 | 142 | 134 | 220 | ppKD_1-hm335 |
| 62 | *T. pilosa* ssp. *pilosa* | Pop21 | 122 | 182 | 142 | 134 | 220 | ppKD_3-hm336 |
| 63 | *T. pilosa* ssp. *pilosa* | Pop21 | 122 | 182 | 142 | 134 | 220 | ppKD_hm337 |
| 64 | *T. pilosa* ssp. *pilosa* | Pop22 | 122 | 180 | 142 | 135 | 222 | ppMH_10-hm354 |
| 65 | *T. pilosa* ssp. *pilosa* | Pop22 | 122 | 180 | 142 | 135 | 222 | ppMH_1-hm353 |
| 66 | *T. pilosa* ssp. *pilosa* | Pop22 | 122 | 180 | 142 | 135 | 222 | ppMH_6-hm355 |
| 67 | *T. pilosa* ssp. *pilosa* | Pop23 | 122 | 180 | 142 | 135 | 223 | ppML_10-hm357 |
| 68 | *T. pilosa* ssp. *pilosa* | Pop23 | 122 | 180 | 142 | 135 | 222 | ppML_1hm356 |
| 69 | *T. pilosa* ssp. *pilosa* | Pop23 | 122 | 179 | 140 | 135 | 222 | ppML_6-hm358 |
| 70 | *T. pilosa* ssp. *pilosa* | Pop24 | 122 | 181 | 142 | 136 | 220 | ppOH_10-hm339 |
| 71 | *T. pilosa* ssp. *pilosa* | Pop24 | 122 | 178 | 141 | 134 | 222 | ppOH_1hm338 |
| 72 | *T. pilosa* ssp. *pilosa* | Pop24 | 122 | 182 | 141 | 136 | 220 | ppOH_6-hm340 |
| 73 | *T. pilosa* ssp. *pilosa* | Pop25 | 122 | 179 | 141 | 134 | 222 | ppPT_1-hm341 |
| 74 | *T. pilosa* ssp. *pilosa* | Pop25 | 122 | 182 | 141 | 136 | 220 | ppPT_3-hm342 |
| 75 | *T. pilosa* ssp. *pilosa* | Pop25 | 122 | 179 | 141 | 134 | 222 | ppPT_hm343 |
| 76 | *T. pilosa* ssp. *pilosa* | Pop26 | 122 | 146 | 141 | 136 | 223 | ppRB_10-hm344 |
| 77 | *T. pilosa* ssp. *pilosa* | Pop26 | 122 | 146 | 141 | 136 | 223 | ppRB_1hm345 |
| 78 | *T. pilosa* ssp. *pilosa* | Pop26 | 122 | 146 | 141 | 136 | 223 | ppRB_7-hm346 |
| 79 | *T. pilosa* ssp. *pilosa* | Pop27 | 122 | 179 | 142 | 135 | 222 | ppRR_10-hm360 |
| 80 | *T. pilosa* ssp. *pilosa* | Pop27 | 122 | 179 | 142 | 135 | 222 | ppRR_1-hm359 |
| 81 | *T. pilosa* ssp. *pilosa* | Pop27 | 122 | 179 | 142 | 135 | 222 | ppRR_6-hm361 |
| 82 | *T. pilosa* ssp. *pilosa* | Pop28 | 122 | 180 | 141 | 135 | 220 | ppSS_1-hm347 |
| 83 | *T. pilosa* ssp. *pilosa* | Pop28 | 122 | 181 | 140 | 136 | 220 | ppSS_7-hm349 |
| 84 | *T. pilosa* ssp. *pilosa* | Pop28 | 122 | 181 | 140 | 136 | 220 | ppSS_hm348 |
| 85 | *T. pilosa* ssp. *pilosa* | Pop29 | 122 | 179 | 143 | 134 | 222 | ppVH_1-hm365 |
| 86 | *T. pilosa* ssp. *pilosa* | Pop29 | 122 | 179 | 142 | 135 | 222 | ppVH_6-hm366 |
| 87 | *T. pilosa* ssp. *pilosa* | Pop29 | 122 | 179 | 142 | 135 | 222 | ppVH12-hm367 |
| 88 | *T. pilosa* ssp. *pilosa* | Pop30 | 122 | 180 | 142 | 135 | 222 | ppWP_1-hm368 |
| 89 | *T. pilosa* ssp. *pilosa* | Pop30 | 122 | 180 | 142 | 135 | 222 | ppWP_3-hm369 |
| 90 | *T. pilosa* ssp. *pilosa* | Pop30 | 122 | 180 | 142 | 135 | 222 | ppWP_hm370 |
| 91 | *T. pilosa* ssp. *pilosa* | Pop31 | 122 | 179 | 141 | 134 | 222 | ppWR_13-hm350 |
| 92 | *T. pilosa* ssp. *pilosa* | Pop31 | 122 | 179 | 141 | 134 | 222 | ppWR_6-hm351 |
| 93 | *T. pilosa* ssp. *pilosa* | Pop31 | 122 | 179 | 141 | 134 | 222 | ppWR_9-hm352 |
| 94 | *T. procumbens* | Pop32 | 122 | 179 | 141 | 134 | 222 | prBG_10-hm375 |
| 95 | *T. procumbens* | Pop32 | 122 | 179 | 141 | 134 | 222 | prBG_1-hm374 |
| 96 | *T. procumbens* | Pop32 | 122 | 179 | 141 | 134 | 222 | prBG_6-hm376 |
| 97 | *T. procumbens* | Pop33 | 122 | 179 | 141 | 134 | 222 | prBLT_10-hm378 |
| 98 | *T. procumbens* | Pop33 | 122 | 179 | 141 | 134 | 222 | prBLT_1hm377 |

| | | | Locus | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **Taxon** | **Pop** | **ccmp10** | **ccmp2** | **ccmp3** | **ccmp4** | **Tecp01** | **Sample number** |
| 99 | *T. procumbens* | Pop33 | 122 | 179 | 141 | 134 | 222 | prBLT_6-hm379 |
| 100 | *T. procumbens* | Pop34 | 122 | 179 | 141 | 134 | 222 | prFG_10-hm381 |
| 101 | *T. procumbens* | Pop34 | 122 | 179 | 141 | 134 | 222 | prFG_1-hm380 |
| 102 | *T. procumbens* | Pop34 | 122 | 179 | 141 | 134 | 222 | prFG_6-hm382 |
| 103 | *T. procumbens* | Pop35 | 122 | 179 | 141 | 134 | 222 | prIB_10-hm384 |
| 104 | *T. procumbens* | Pop35 | 122 | 179 | 141 | 134 | 222 | prIB_1-hm383 |
| 105 | *T. procumbens* | Pop35 | 122 | 179 | 141 | 134 | 222 | prIB_6-hm385 |
| 106 | *T. procumbens* | Pop36 | 122 | 179 | 141 | 134 | 222 | prLR_1-hm386 |
| 107 | *T. procumbens* | Pop36 | 122 | 179 | 141 | 134 | 222 | prLR_hm387 |
| 108 | *T. procumbens* | Pop36 | 122 | 179 | 141 | 134 | 228 | prLRB_hm388 |
| 109 | *T. halmaturina* | Pop37 | 122 | 178 | 142 | 134 | 218 | hBHR2_1-hm422 |
| 110 | *T. halmaturina* | Pop37 | 122 | 178 | 144 | 134 | 218 | hBHR2_3-hm423 |
| 111 | *T. halmaturina* | Pop37 | 122 | 178 | 142 | 134 | 220 | hBHR2_5-hm424 |
| 112 | *T. halmaturina* | Pop38 | 122 | 178 | 144 | 134 | 220 | hSR2_1-hm419 |
| 113 | *T. halmaturina* | Pop38 | 122 | 178 | 144 | 134 | 220 | hSR2_3-hm420 |
| 114 | *T. halmaturina* | Pop38 | 122 | 178 | 144 | 134 | 218 | hSR2_5-hm421 |
| 115 | *T. neglecta* | Pop39 | 123 | 177 | 141 | 134 | 222 | nR_1_-hm414 |
| 116 | *T. neglecta* | Pop39 | 123 | 177 | 141 | 134 | 222 | nR_12-hm415 |
| 117 | *T. neglecta* | Pop39 | 123 | 177 | 141 | 134 | 223 | nR_3-hm413 |
| 118 | *T. rubioides* | Pop40 | 122 | 148 | 141 | 134 | 216 | rMtB_10-hm418 |
| 119 | *T. rubioides* | Pop40 | 122 | 180 | 143 | 134 | 221 | rMtB_1-hm416 |
| 120 | *T. rubioides* | Pop40 | 122 | 177 | 141 | 134 | 223 | rMtB_5-hm417 |

**Appendix 4 Haplotypes within and among taxa from the *Tetratheca pilosa* group**

| Haplotype | No. individuals | ccmp10 | ccmp2 | ccmp3 | ccmp4 | Tecp01 |
|---|---|---|---|---|---|---|
| 1 | 1 | 109 | 154 | 141 | 134 | 218 |
| 2 | 1 | 122 | 144 | 141 | 136 | 222 |
| 3 | 1 | 122 | 146 | 140 | 134 | 219 |
| 4 | 1 | 122 | 146 | 140 | 134 | 222 |
| 5 | 2 | 122 | 146 | 141 | 134 | 222 |
| 6 | 5 | 122 | 146 | 141 | 136 | 222 |
| 7 | 3 | 122 | 146 | 141 | 136 | 223 |
| 8 | 4 | 122 | 147 | 140 | 134 | 222 |
| 9 | 2 | 122 | 147 | 141 | 134 | 222 |
| 10 | 3 | 122 | 148 | 140 | 134 | 221 |
| 11 | 1 | 122 | 148 | 141 | 134 | 222 |
| 12 | 1 | 122 | 149 | 143 | 136 | 220 |
| 13 | 1 | 122 | 178 | 141 | 134 | 222 |
| 14 | 1 | 122 | 179 | 140 | 135 | 222 |
| 15 | 1 | 122 | 179 | 140 | 137 | 222 |
| 16 | 23 | 122 | 179 | 141 | 134 | 222 |
| 17 | 1 | 122 | 179 | 141 | 134 | 228 |
| 18 | 2 | 122 | 179 | 141 | 136 | 222 |
| 19 | 5 | 122 | 179 | 142 | 135 | 222 |
| 20 | 1 | 122 | 179 | 143 | 134 | 222 |
| 21 | 1 | 122 | 180 | 141 | 135 | 220 |
| 22 | 4 | 122 | 180 | 141 | 135 | 222 |
| 23 | 2 | 122 | 180 | 141 | 136 | 221 |
| 24 | 2 | 122 | 180 | 141 | 136 | 222 |
| 25 | 1 | 122 | 180 | 141 | 137 | 221 |
| 26 | 8 | 122 | 180 | 141 | 137 | 222 |
| 27 | 1 | 122 | 180 | 141 | 138 | 222 |
| 28 | 12 | 122 | 180 | 142 | 135 | 222 |
| 29 | 1 | 122 | 180 | 142 | 135 | 223 |
| 30 | 2 | 122 | 181 | 140 | 136 | 220 |
| 31 | 3 | 122 | 181 | 141 | 136 | 220 |
| 32 | 1 | 122 | 181 | 142 | 136 | 220 |
| 33 | 2 | 122 | 182 | 141 | 136 | 220 |
| 34 | 3 | 122 | 182 | 141 | 137 | 222 |
| 35 | 3 | 122 | 182 | 142 | 134 | 220 |
| 36 | 2 | 122 | 182 | 143 | 136 | 220 |

**Appendix 5 Chloroplast microsatellite data from *Tetratheca ciliata* and *T. stenocarpa***

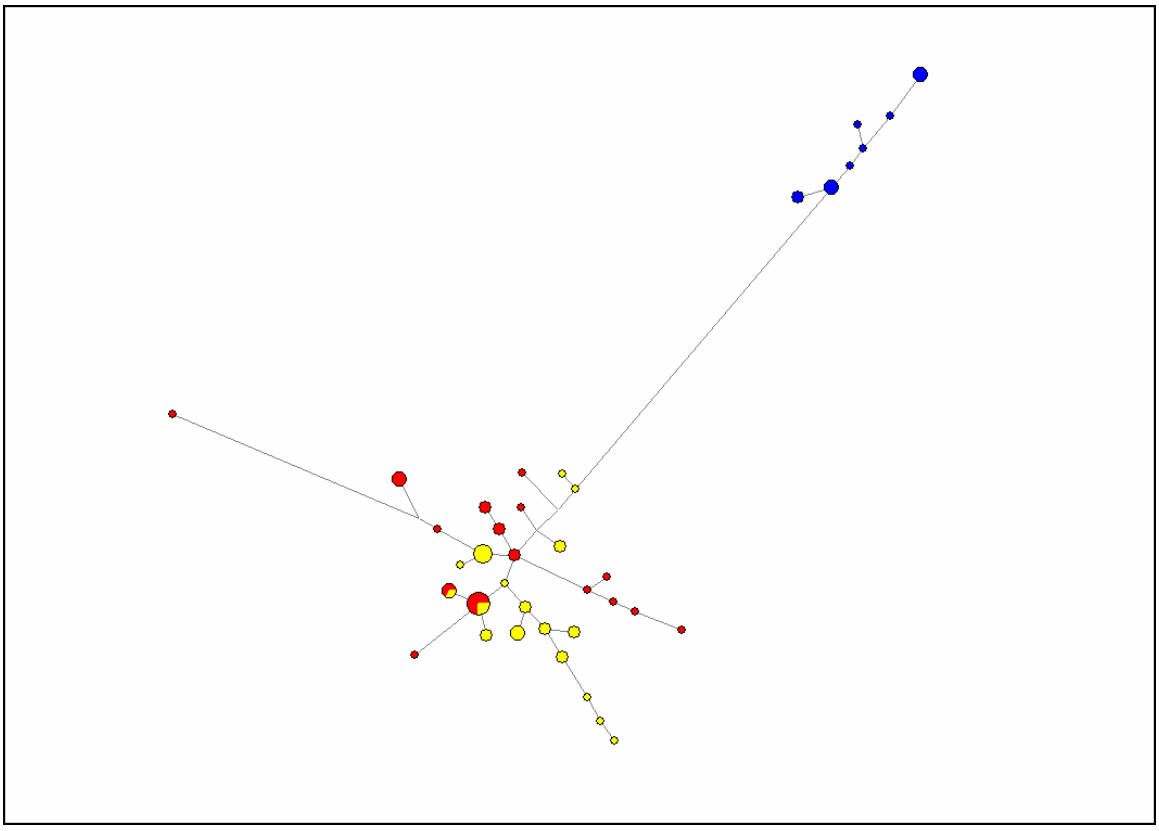
| | | | Locus | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **Taxon** | **Population** | **ccmp10** | **ccmp2** | **ccmp3** | **ccmp4** | **Tecp01** | **Sample number** |
| 1 | *T. ciliata* | Pop1 | 122 | 180 | 141 | 137 | 220 | cBL_01-hm233 |
| 2 | *T. ciliata* | Pop1 | 122 | 180 | 141 | 137 | 220 | cBL_10-hm234 |
| 3 | *T. ciliata* | Pop1 | 122 | 180 | 141 | 137 | 220 | cBL_6-hm235 |
| 4 | *T. ciliata* | Pop2 | 122 | 179 | 141 | 137 | 223 | cCB_10-hm237 |
| 5 | *T. ciliata* | Pop2 | 122 | 179 | 141 | 137 | 223 | cCB_1-hm236 |
| 6 | *T. ciliata* | Pop2 | 122 | 180 | 141 | 137 | 223 | cCB_7-hm238 |
| 7 | *T. ciliata* | Pop3 | 122 | 180 | 142 | 137 | 222 | cCR_1-hm239 |
| 8 | *T. ciliata* | Pop3 | 122 | 180 | 141 | 137 | 222 | cCR_3-hm240 |
| 9 | *T. ciliata* | Pop3 | 122 | 180 | 142 | 137 | 222 | cCR_hm241 |
| 10 | *T. ciliata* | Pop4 | 137 | 178 | 142 | 137 | 218 | cDH_10-hm243 |
| 11 | *T. ciliata* | Pop4 | 122 | 180 | 141 | 137 | 221 | cDH_1-hm242 |
| 12 | *T. ciliata* | Pop4 | 122 | 180 | 141 | 137 | 221 | cDH_6-hm244 |
| 13 | *T. ciliata* | Pop5 | 122 | 180 | 141 | 137 | 223 | cDR_10-hm246 |
| 14 | *T. ciliata* | Pop5 | 122 | 180 | 141 | 137 | 223 | cDR_1-hm245 |
| 15 | *T. ciliata* | Pop5 | 122 | 180 | 141 | 137 | 223 | cDR_6-hm247 |
| 16 | *T. ciliata* | Pop6 | 122 | 180 | 141 | 137 | 223 | cFH_10-hm249 |
| 17 | *T. ciliata* | Pop6 | 125 | 180 | 141 | 138 | 223 | cFH_1-hm248 |
| 18 | *T. ciliata* | Pop6 | 122 | 180 | 141 | 137 | 223 | cFH_6-hm250 |
| 19 | *T. ciliata* | Pop7 | 122 | 180 | 142 | 136 | 223 | cKH_10-hm396 |
| 20 | *T. ciliata* | Pop7 | 122 | 180 | 142 | 136 | 223 | cKH_1-hm395 |
| 21 | *T. ciliata* | Pop7 | 122 | 180 | 142 | 135 | 224 | cKH_6-hm397 |
| 22 | *T. ciliata* | Pop8 | 122 | 179 | 141 | 136 | 223 | cLS_10-hm252 |
| 23 | *T. ciliata* | Pop8 | 122 | 180 | 141 | 137 | 223 | cLS_1-hm251 |
| 24 | *T. ciliata* | Pop8 | 122 | 180 | 141 | 137 | 223 | cLS_6-hm253 |
| 25 | *T. ciliata* | Pop9 | 122 | 186 | 141 | 137 | 221 | cMF_10-hm255 |
| 26 | *T. ciliata* | Pop9 | 122 | 187 | 141 | 137 | 221 | cMF_1-hm254 |
| 27 | *T. ciliata* | Pop9 | 122 | 188 | 140 | 137 | 221 | cMF_6-hm256 |
| 28 | *T. ciliata* | Pop10 | 122 | 179 | 142 | 137 | 221 | cMtD_10-hm258 |
| 29 | *T. ciliata* | Pop10 | 122 | 179 | 141 | 137 | 221 | cMtD_1-hm257 |
| 30 | *T. ciliata* | Pop10 | 122 | 179 | 142 | 137 | 221 | cMtD_6-hm259 |
| 31 | *T. ciliata* | Pop11 | 122 | 180 | 142 | 137 | 223 | cMV_10-hm261 |
| 32 | *T. ciliata* | Pop11 | 122 | 180 | 142 | 134 | 224 | cMV_1-hm260 |
| 33 | *T. ciliata* | Pop11 | 122 | 179 | 142 | 134 | 224 | cMV_6-hm262 |
| 34 | *T. ciliata* | Pop12 | 122 | 179 | 141 | 137 | 216 | cPG_10-hm264 |
| 35 | *T. ciliata* | Pop12 | 122 | 179 | 141 | 137 | 216 | cPG_1-hm263 |
| 36 | *T. ciliata* | Pop12 | 122 | 179 | 141 | 137 | 216 | cPG_6-hm265 |
| 37 | *T. ciliata* | Pop13 | 122 | 180 | 140 | 137 | 223 | cPR_10-hm267 |
| 38 | *T. ciliata* | Pop13 | 122 | 180 | 140 | 137 | 223 | cPR_1-hm266 |
| 39 | *T. ciliata* | Pop13 | 122 | 179 | 141 | 137 | 223 | cPR_6-hm268 |
| 40 | *T. ciliata* | Pop14 | 122 | 179 | 142 | 137 | 222 | cRH_1-hm269 |
| 41 | *T. ciliata* | Pop14 | 122 | 179 | 142 | 137 | 222 | cRH_3-hm270 |
| 42 | *T. ciliata* | Pop14 | 122 | 179 | 142 | 137 | 222 | cRH_hm271 |
| 43 | *T. ciliata* | Pop15 | 122 | 179 | 142 | 137 | 223 | cSCR_10-hm273 |
| 44 | *T. ciliata* | Pop15 | 122 | 180 | 142 | 137 | 223 | cSCR_1-hm272 |
| 45 | *T. ciliata* | Pop15 | 122 | 179 | 142 | 137 | 223 | cSCR_6-hm274 |
| 46 | *T. ciliata* | Pop16 | 122 | 180 | 141 | 136 | 222 | cSW_1-hm275 |
| 47 | *T. ciliata* | Pop16 | 122 | 180 | 141 | 136 | 222 | cSW_3-hm276 |

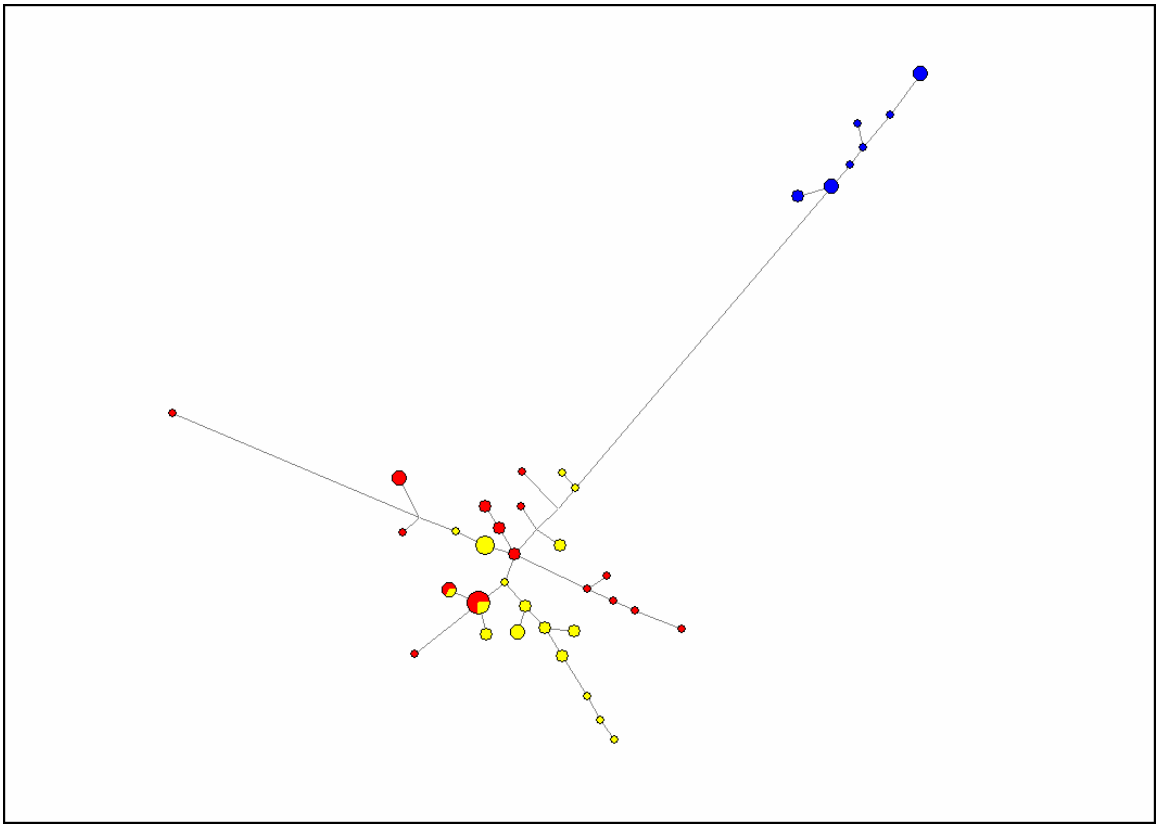
| | | | Locus | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **Taxon** | **Population** | **ccmp10** | **ccmp2** | **ccmp3** | **ccmp4** | **Tecp01** | **Sample number** |
| 48 | *T. ciliata* | Pop16 | 122 | 179 | 141 | 136 | 222 | cSW_hm277 |
| 49 | *T. ciliata* | Pop17 | 122 | 179 | 141 | 137 | 220 | cTT_10-hm279 |
| 50 | *T. ciliata* | Pop17 | 122 | 180 | 141 | 137 | 220 | cTT_1-hm278 |
| 51 | *T. ciliata* | Pop17 | 122 | 180 | 141 | 137 | 220 | cTT_6-hm280 |
| 52 | *T. ciliata* | Pop18 | 122 | 179 | 141 | 137 | 221 | cVRR_10-hm282 |
| 53 | *T. ciliata* | Pop18 | 122 | 180 | 140 | 136 | 221 | cVRR_1-hm281 |
| 54 | *T. ciliata* | Pop18 | 122 | 179 | 141 | 133 | 221 | cVRR_6-hm283 |
| 55 | *T. ciliata* | Pop19 | 122 | 180 | 141 | 137 | 218 | cWR_10-hm284 |
| 56 | *T. ciliata* | Pop19 | 122 | 185 | 141 | 137 | 221 | cWR_2-hm285 |
| 57 | *T. ciliata* | Pop19 | 122 | 185 | 141 | 138 | 221 | cWR_6-hm286 |
| 58 | *T. stenocarpa* | Pop20 | 122 | 145 | 141 | 136 | 223 | sEB_10-hm390 |
| 59 | *T. stenocarpa* | Pop20 | 122 | 144 | 140 | 136 | 218 | sEB_1-hm389 |
| 60 | *T. stenocarpa* | Pop20 | 122 | 145 | 141 | 136 | 222 | sEB_6-hm391 |
| 61 | *T. stenocarpa* | Pop21 | 122 | 144 | 141 | 136 | 216 | sFRV_10-hm393 |
| 62 | *T. stenocarpa* | Pop21 | 122 | 144 | 141 | 136 | 216 | sFRV_1-hm392 |
| 63 | *T. stenocarpa* | Pop21 | 122 | 144 | 141 | 136 | 216 | sFRV_6-hm394 |
| 64 | *T. stenocarpa* | Pop22 | 122 | 144 | 140 | 136 | 222 | sOCC_10-hm399 |
| 65 | *T. stenocarpa* | Pop22 | 122 | 144 | 141 | 136 | 222 | sOCC_1-hm398 |
| 66 | *T. stenocarpa* | Pop22 | 122 | 144 | 140 | 136 | 223 | sOCC_6-hm400 |
| 67 | *T. stenocarpa* | Pop23 | 122 | 145 | 141 | 136 | 222 | sTP_13-hm363 |
| 68 | *T. stenocarpa* | Pop23 | 122 | 145 | 141 | 136 | 222 | sTP_1-hm362 |
| 69 | *T. stenocarpa* | Pop23 | 122 | 145 | 141 | 136 | 223 | sTP_6-hm364 |

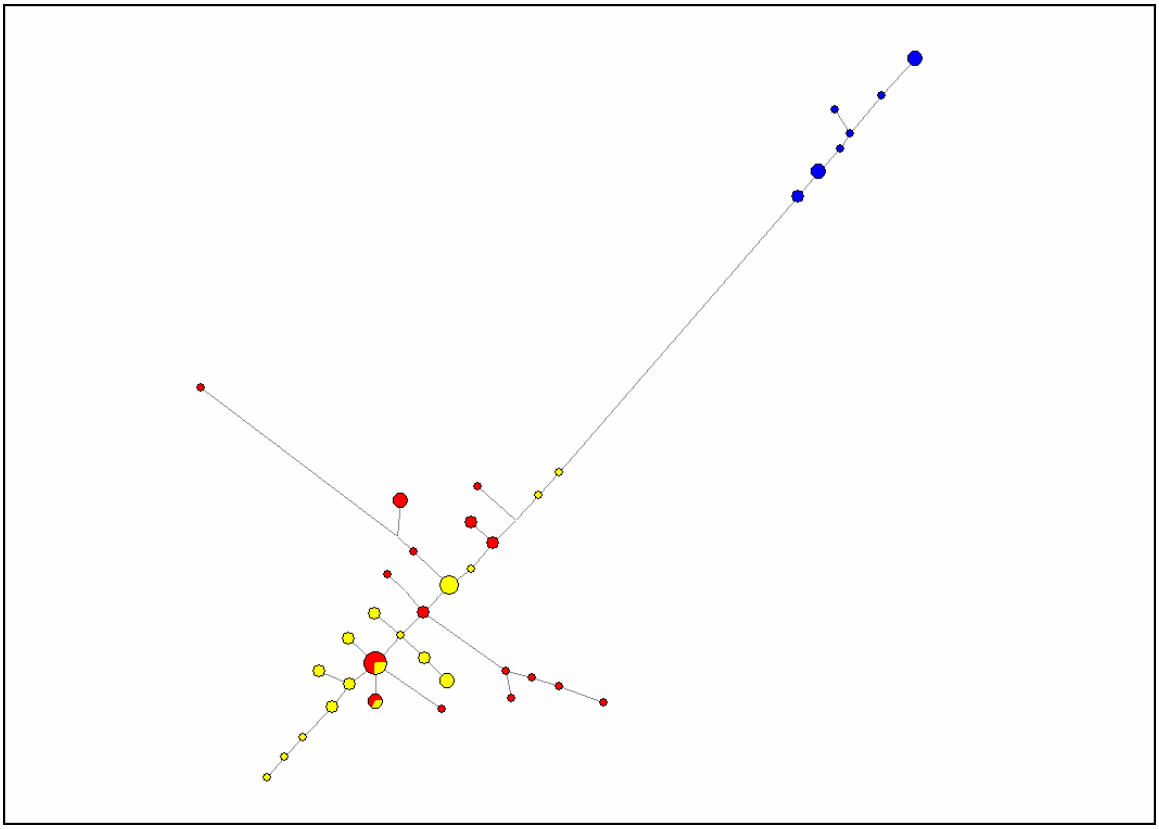
**Appendix 6 Haplotypes within and among *Tetratheca ciliata* and *T. stenocarpa***

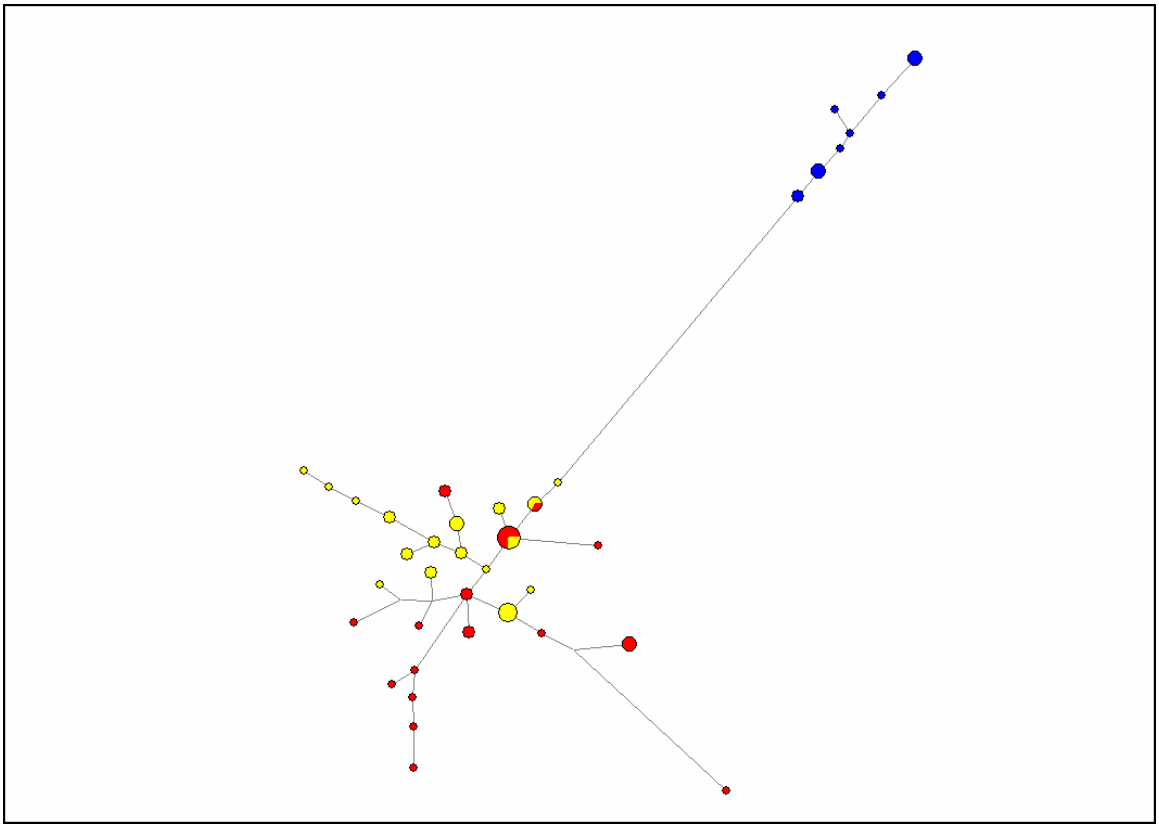
| Haplotype no. | No. individuals | ccmp10 | ccmp2 | ccmp3 | ccmp4 | Tecp01 |
|---|---|---|---|---|---|---|
| 1 | 1 | 122 | 144 | 140 | 136 | 218 |
| 2 | 1 | 122 | 144 | 140 | 136 | 222 |
| 3 | 1 | 122 | 144 | 140 | 136 | 223 |
| 4 | 3 | 122 | 144 | 141 | 136 | 216 |
| 5 | 1 | 122 | 144 | 141 | 136 | 222 |
| 6 | 3 | 122 | 145 | 141 | 136 | 222 |
| 7 | 2 | 122 | 145 | 141 | 136 | 223 |
| 8 | 1 | 122 | 179 | 141 | 133 | 221 |
| 9 | 1 | 122 | 179 | 141 | 136 | 222 |
| 10 | 1 | 122 | 179 | 141 | 136 | 223 |
| 11 | 3 | 122 | 179 | 141 | 137 | 216 |
| 12 | 1 | 122 | 179 | 141 | 137 | 220 |
| 13 | 2 | 122 | 179 | 141 | 137 | 221 |
| 14 | 3 | 122 | 179 | 141 | 137 | 223 |
| 15 | 1 | 122 | 179 | 142 | 134 | 224 |
| 16 | 2 | 122 | 179 | 142 | 137 | 221 |
| 17 | 3 | 122 | 179 | 142 | 137 | 222 |
| 18 | 2 | 122 | 179 | 142 | 137 | 223 |
| 19 | 1 | 122 | 180 | 140 | 136 | 221 |
| 20 | 2 | 122 | 180 | 140 | 137 | 223 |
| 21 | 2 | 122 | 180 | 141 | 136 | 222 |
| 22 | 1 | 122 | 180 | 141 | 137 | 218 |
| 23 | 5 | 122 | 180 | 141 | 137 | 220 |
| 24 | 2 | 122 | 180 | 141 | 137 | 221 |
| 25 | 1 | 122 | 180 | 141 | 137 | 222 |
| 26 | 8 | 122 | 180 | 141 | 137 | 223 |
| 27 | 1 | 122 | 180 | 142 | 134 | 224 |
| 28 | 1 | 122 | 180 | 142 | 135 | 224 |
| 29 | 2 | 122 | 180 | 142 | 136 | 223 |
| 30 | 2 | 122 | 180 | 142 | 137 | 222 |
| 31 | 2 | 122 | 180 | 142 | 137 | 223 |
| 32 | 1 | 122 | 185 | 141 | 137 | 221 |
| 33 | 1 | 122 | 185 | 141 | 138 | 221 |
| 34 | 1 | 122 | 186 | 141 | 137 | 221 |
| 35 | 1 | 122 | 187 | 141 | 137 | 221 |
| 36 | 1 | 122 | 188 | 140 | 137 | 221 |
| 37 | 1 | 125 | 180 | 141 | 138 | 223 |
| 38 | 1 | 137 | 178 | 142 | 137 | 218 |

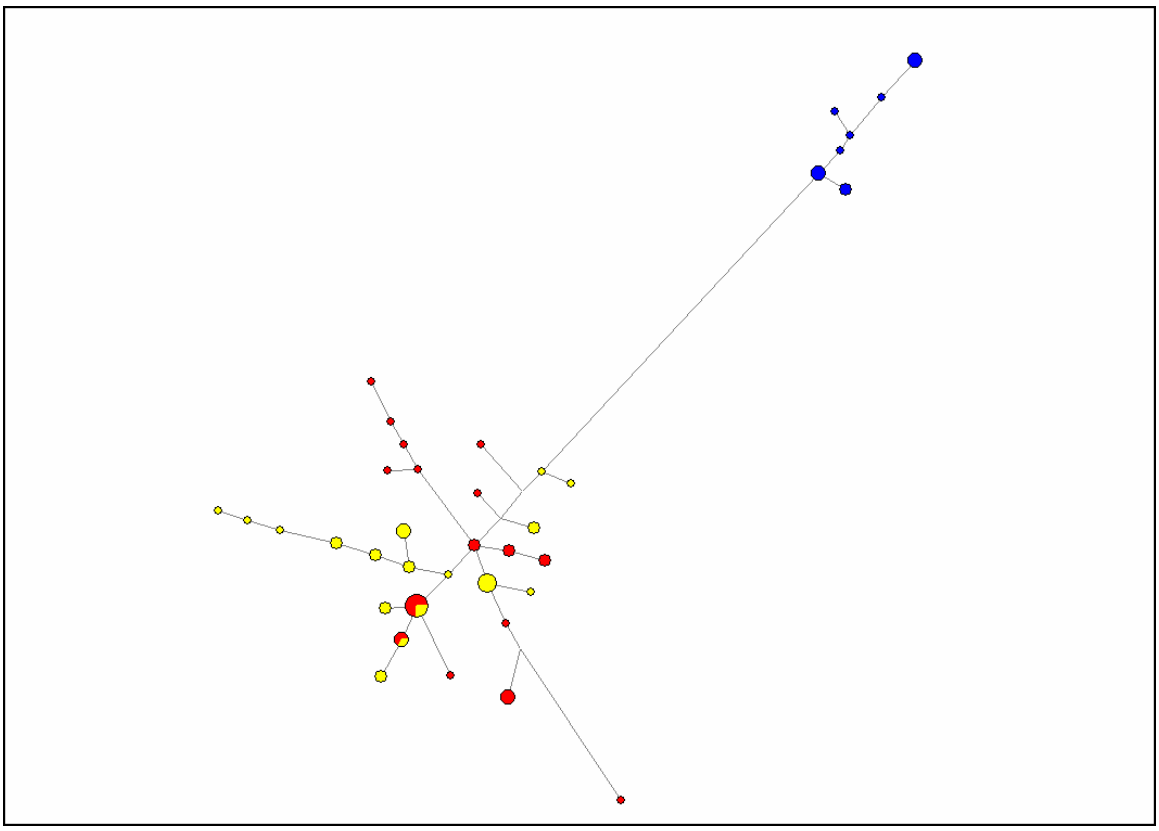
**Appendix 7 Seven equally parsimonious trees constructed using chloroplast microsatellites for *Tetratheca ciliata and T. stenocarpa***

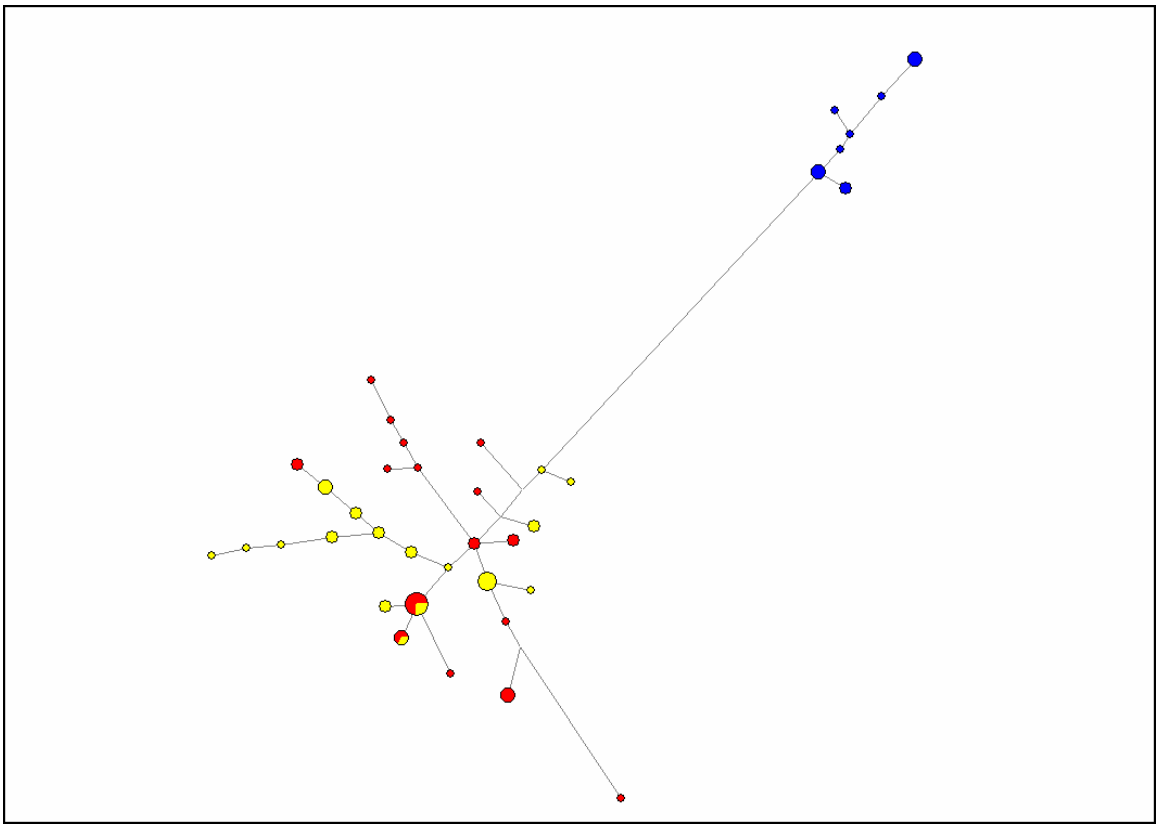












**Appendix 8 Nuclear microsatellite data for *Tetratheca ericifolia* from Brisbane Water National Park**

| | Microsatellite Locus | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Sample** | **Te01BGT** | | **Te02BGT** | | **Te03BGT** | | **Te05BGT** | | **Te09BGT** | | **Te11BGT** | | **Te14BGT** | | **Te15BGT** | | **Te16BGT** | | **Te17BGT** | | **Te18BGT** | |
| B01 | 335 | 362 | 239 | 239 | 445 | 451 | 278 | 278 | 161 | 161 | 139 | 149 | 391 | 391 | 345 | 349 | 293 | 300 | 196 | 198 | 223 | 227 |
| B02 | | | 239 | 239 | | | | | 161 | 161 | 139 | 149 | | | 347 | 347 | 298 | 300 | 184 | 196 | 207 | 227 |
| B03 | | | 239 | 239 | 445 | 445 | 278 | 278 | 161 | 161 | 143 | 149 | | | 327 | 351 | 298 | 298 | | | 215 | 215 |
| B04 | 335 | 338 | 239 | 239 | 451 | 451 | 278 | 280 | 161 | 161 | 139 | 145 | 355 | 385 | 355 | 355 | 302 | 302 | 192 | 192 | 203 | 211 |
| B05 | 335 | 362 | 239 | 239 | 447 | 447 | 278 | 278 | 161 | 161 | 139 | 149 | 383 | 389 | 351 | 359 | 302 | 302 | 196 | 198 | 205 | 235 |
| B06 | 335 | 362 | 239 | 239 | 459 | 459 | 278 | 284 | 161 | 161 | 143 | 149 | 385 | 385 | 359 | 369 | 300 | 300 | 196 | 196 | 205 | 209 |
| B07 | | | 239 | 239 | | | 280 | 280 | 161 | 161 | 139 | 145 | | | 351 | 351 | 298 | 307 | 190 | 192 | 207 | 215 |
| B08 | 335 | 362 | 239 | 239 | | | | | | | 141 | 153 | 395 | 395 | 341 | 357 | 298 | 302 | 192 | 198 | 205 | 239 |
| B09 | 327 | 335 | 239 | 242 | 449 | 460 | 278 | 282 | 161 | 161 | 147 | 147 | 347 | 347 | 353 | 355 | 300 | 300 | 194 | 198 | 209 | 221 |
| B10 | 332 | 335 | 239 | 239 | | | 278 | 278 | 161 | 161 | 139 | 139 | | | 349 | 357 | 300 | 300 | 192 | 198 | 209 | 227 |
| B11 | 335 | 341 | 239 | 245 | 447 | 451 | 278 | 284 | 161 | 161 | 139 | 139 | 403 | 403 | 353 | 357 | 300 | 304 | 194 | 196 | 205 | 215 |
| B12 | 335 | 350 | 239 | 239 | 451 | 459 | 278 | 282 | 161 | 161 | 139 | 147 | 389 | 389 | 357 | 369 | 300 | 302 | 194 | 196 | 203 | 217 |
| B13 | | | 239 | 248 | 443 | 443 | 0 | 0 | 161 | 161 | 139 | 139 | 396 | 396 | 367 | 369 | 298 | 302 | 196 | 196 | 215 | 215 |
| B14 | 335 | 350 | 239 | 242 | 455 | 457 | 278 | 282 | 161 | 161 | 139 | 139 | | | 351 | 365 | 300 | 300 | 192 | 196 | 205 | 221 |
| B15 | 335 | 344 | 239 | 239 | 445 | 455 | 278 | 280 | 161 | 161 | 139 | 139 | 385 | 385 | 347 | 375 | 300 | 300 | 194 | 204 | 209 | 217 |
| B16 | 344 | 344 | 239 | 239 | 449 | 455 | 278 | 280 | 161 | 161 | 139 | 139 | | | 369 | 379 | 300 | 302 | 190 | 194 | 209 | 219 |

Greyed out areas indicate missing data

**Appendix 9 Nuclear microsatellite data for *Tetratheca ericifolia* from Bobbin Head Track, Ku-ring-gai National Park**

| | Microsatellite Locus | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Sample** | **Te01BGT** | | **Te02BGT** | | **Te03BGT** | | **Te05BGT** | | **Te09BGT** | | **Te11BGT** | | **Te14BGT** | | **Te15BGT** | | **Te16BGT** | | **Te17BGT** | | **Te18BGT** | |
| BH01 | 344 | 359 | 236 | 239 | 463 | 463 | 278 | 285 | 159 | 161 | 139 | 141 | 377 | 377 | 359 | 379 | 300 | 300 | 172 | 182 | 201 | 233 |
| BH02 | | | 239 | 239 | | | | | 161 | 163 | 131 | 139 | | | 335 | 351 | 300 | 300 | 182 | 192 | 195 | 207 |
| BH04 | | | 239 | 239 | | | | | 165 | 165 | | | | | | | 298 | 300 | | | | |
| BH05 | | | 239 | 239 | 453 | 453 | 278 | 285 | 161 | 161 | 143 | 143 | | | 349 | 349 | 302 | 302 | 182 | 182 | 203 | 227 |
| BH06 | 356 | 362 | 239 | 245 | 447 | 447 | 278 | 285 | 161 | 161 | 139 | 141 | 375 | 375 | 343 | 355 | 300 | 300 | 188 | 190 | 205 | 231 |
| BH07 | 344 | 347 | 239 | 239 | 453 | 453 | 281 | 285 | 161 | 161 | 137 | 139 | 391 | 391 | 335 | 345 | 300 | 300 | 188 | 190 | 199 | 207 |
| BH08 | | | 239 | 239 | 447 | 455 | 285 | 285 | 159 | 161 | 139 | 139 | 395 | 395 | 325 | 375 | 300 | 300 | 172 | 192 | 207 | 211 |
| BH09 | 341 | 347 | 245 | 245 | 445 | 449 | 278 | 285 | 159 | 161 | 141 | 141 | 401 | 401 | 341 | 369 | 300 | 302 | 174 | 192 | 219 | 219 |
| BH10 | 344 | 362 | 242 | 242 | 447 | 449 | 278 | 285 | 159 | 161 | 133 | 141 | 391 | 391 | 343 | 349 | 300 | 302 | 190 | 192 | 207 | 211 |
| BH12 | | | 239 | 239 | | | 278 | 285 | 159 | 161 | 137 | 141 | 375 | 375 | 335 | 359 | 300 | 300 | 190 | 192 | 205 | 215 |
| BH13 | 335 | 356 | 239 | 239 | 449 | 449 | 285 | 285 | 161 | 161 | 123 | 139 | 375 | 397 | 333 | 347 | 300 | 300 | 194 | 194 | 195 | 207 |
| BH14 | | | | | | | 281 | 285 | 159 | 161 | 149 | 149 | | | 337 | 355 | 298 | 300 | | | 213 | 215 |
| BH16 | 353 | 362 | 239 | 239 | 453 | 459 | 280 | 285 | 161 | 161 | 143 | 165 | | | 347 | 347 | 284 | 300 | 192 | 200 | 213 | 221 |
| BH18 | 347 | 365 | 239 | 242 | 447 | 459 | 278 | 285 | 161 | 165 | 137 | 141 | | | 353 | 365 | 302 | 306 | 172 | 192 | 205 | 207 |
| BH19 | 341 | 362 | 239 | 239 | 453 | 457 | 285 | 285 | 159 | 161 | 139 | 147 | | | 335 | 347 | 300 | 300 | 192 | 194 | 205 | 217 |
| BH20 | 341 | 350 | 239 | 245 | 447 | 453 | 278 | 285 | 161 | 161 | 139 | 143 | 391 | 391 | 331 | 353 | 300 | 300 | 172 | 194 | 215 | 219 |

Greyed out areas indicate missing data

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## STATEMENT OF AUTHORS' CONTRIBUTION

(To appear at the end of each thesis chapter submitted as an article/paper)

We, the PhD candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated in the *Statement of Originality*.

| | **Author's Name** (please print clearly) | **% of contribution** |
|---|---|---|
| Candidate | Hannah McPherson | 60 |
| Other Authors | Carolyn Porter | 20 |
| | Paul Rymer | 5 |
| | Darren Crayn | 5 |
| | Maurizio Rossetto | 10 |
| | | |

Name of Candidate: Hannah McPherson

Name/title of Principal Supervisor: Maurizio Rossetto – adjunct Assoc. Prof Ecosystem Management

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Candidate

28 Aug 08
Date

Principal Supervisor

28/08/08
Date

**Journal-Article Format for PhD Theses at the University of New England**

## STATEMENT OF ORIGINALITY

(To appear at the end of each thesis chapter submitted as an article/paper)

We, the PhD candidate and the candidate's Principal Supervisor, certify that the following text, figures and diagrams are the candidate's original work.

| Type of work | Page number/s |
|---|---|
| Text | 867-869 |
| Table 1 | 868 |
| Table 2 | 869 |
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Name/title of Principal Supervisor: Maurizio Rossetto, adjunct Assoc. Prof. Ecosystem Management

Hannah McPherson
Candidate

28 aug 08
Date

Principal Supervisor

28/08/08
Date

**Appendix 11 PCR fragment lengths at 5 loci for 11 populations (158 individuals) of *Tetratheca ciliata***

| | | Microsatellite Locus | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Sample** | **Locality** | **Te11BGT** | | **Te15BGT** | | **Te08BGT** | | **Te18BG** | | **Te17BGT** | |
| 1 | Dukes Highway, SA | 135 | 135 | 346 | 354 | 318 | 318 | 195 | 195 | 169 | 175 |
| 2 | Dukes Highway, SA | 135 | 135 | 365 | 365 | 318 | 318 | 195 | 195 | 169 | 183 |
| 3 | Dukes Highway, SA | 135 | 135 | 361 | 361 | 318 | 318 | 195 | 195 | 167 | 169 |
| 4 | Dukes Highway, SA | 135 | 135 | | | 318 | 318 | 195 | 197 | 169 | 169 |
| 5 | Dukes Highway, SA | 135 | 135 | | | 318 | 326 | 195 | 225 | 169 | 169 |
| 6 | Dukes Highway, SA | 135 | 139 | | | 318 | 318 | | | | |
| 7 | Dukes Highway, SA | 135 | 139 | 356 | 361 | 318 | 318 | 195 | 197 | 169 | 179 |
| 8 | Dukes Highway, SA | 135 | 135 | 356 | 377 | 312 | 318 | 195 | 225 | 169 | 183 |
| 9 | Dukes Highway, SA | | | | | 316 | 318 | 225 | 225 | 169 | 183 |
| 10 | Dukes Highway, SA | 135 | 139 | 344 | 344 | 318 | 318 | 195 | 197 | 169 | 173 |
| 11 | Dukes Highway, SA | 135 | 135 | | | 316 | 316 | 195 | 225 | 167 | 169 |
| 12 | Dukes Highway, SA | 135 | 135 | 363 | 363 | 316 | 316 | 195 | 225 | 169 | 169 |
| 13 | Dukes Highway, SA | 135 | 135 | | | 316 | 318 | 195 | 197 | 169 | 167 |
| 14 | Dukes Highway, SA | 135 | 143 | 356 | 377 | 316 | 318 | 195 | 195 | 169 | 169 |
| 15 | Dukes Highway, SA | 135 | 135 | 354 | 354 | 318 | 322 | 195 | 225 | 169 | 183 |
| 16 | Mt Difficult, Vic | 139 | 139 | 352 | 366 | 320 | 324 | 195 | 227 | 173 | 175 |
| 17 | Mt Difficult, Vic | 135 | 139 | 352 | 366 | | | 195 | 225 | 173 | 183 |
| 18 | Mt Difficult, Vic | 135 | 135 | 399 | 399 | 318 | 320 | 195 | 209 | 173 | 175 |
| 19 | Mt Difficult, Vic | 135 | 141 | 350 | 352 | 320 | 324 | 195 | 197 | 171 | 173 |
| 20 | Mt Difficult, Vic | 139 | 143 | 352 | 369 | 322 | 330 | 195 | 211 | 171 | 175 |
| 21 | Mt Difficult, Vic | 139 | 141 | 350 | 377 | | | 195 | 195 | 167 | 181 |
| 22 | Mt Difficult, Vic | 135 | 148 | 362 | 389 | 318 | 324 | 195 | 229 | 167 | 181 |
| 23 | Mt Difficult, Vic | 135 | 141 | 348 | 364 | 324 | 330 | 195 | 211 | 171 | 175 |
| 24 | Mt Difficult, Vic | 135 | 141 | 350 | 356 | 318 | 328 | 195 | 225 | 171 | 175 |
| 25 | Mt Difficult, Vic | 135 | 135 | 416 | 416 | 318 | 324 | 195 | 225 | 175 | 185 |
| 26 | Mt Difficult, Vic | 135 | 145 | 362 | 362 | 324 | 324 | 195 | 197 | 167 | 185 |
| 27 | Mt Difficult, Vic | 135 | 145 | 356 | 356 | 316 | 320 | 195 | 197 | 171 | 183 |
| 28 | Mt Difficult, Vic | 135 | 135 | 348 | 348 | | | 195 | 201 | 173 | 175 |
| 29 | Mt Difficult, Vic | 135 | 143 | 387 | 387 | 318 | 322 | 195 | 201 | 175 | 183 |

| Sample | Locality | Microsatellite Locus | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Te11BGT | | Te15BGT | | Te08BGT | | Te18BG | | Te17BGT | |
| 30 | Mt Difficult, Vic | 139 | 141 | 350 | 383 | | | | | 167 | 171 |
| 31 | Canadian Ballarat, Vic | 133 | 135 | 334 | 334 | 318 | 324 | 195 | 197 | 175 | 177 |
| 32 | Canadian Ballarat, Vic | 135 | 139 | 344 | 364 | 318 | 324 | 195 | 205 | 175 | 177 |
| 33 | Canadian Ballarat, Vic | 135 | 141 | 338 | 364 | 312 | 318 | 203 | 205 | 175 | 167 |
| 34 | Canadian Ballarat, Vic | 135 | 141 | 364 | 364 | 318 | 324 | 195 | 205 | 175 | 181 |
| 35 | Canadian Ballarat, Vic | 133 | 141 | 350 | 356 | 320 | 324 | 195 | 211 | 175 | 179 |
| 36 | Canadian Ballarat, Vic | 139 | 141 | 364 | 364 | 318 | 334 | 195 | 203 | 175 | 177 |
| 37 | Canadian Ballarat, Vic | 135 | 141 | 338 | 364 | | | 195 | 195 | 175 | 177 |
| 38 | Canadian Ballarat, Vic | 133 | 135 | | | 310 | 324 | 195 | 203 | 171 | 175 |
| 39 | Canadian Ballarat, Vic | 133 | 135 | | | 316 | 324 | 195 | 201 | 175 | 193 |
| 40 | Canadian Ballarat, Vic | 135 | 135 | 332 | 383 | 316 | 322 | 195 | 203 | 167 | 175 |
| 41 | Canadian Ballarat, Vic | 135 | 135 | 344 | 344 | 310 | 320 | 195 | 195 | 175 | 179 |
| 42 | Canadian Ballarat, Vic | 133 | 135 | 350 | 364 | 318 | 334 | 195 | 195 | 175 | 177 |
| 43 | Canadian Ballarat, Vic | 133 | 135 | 364 | 376 | 324 | 328 | 195 | 195 | 173 | 179 |
| 44 | Canadian Ballarat, Vic | 133 | 133 | 364 | 364 | 322 | 324 | 195 | 203 | 175 | 193 |
| 45 | Canadian Ballarat, Vic | 135 | 145 | 338 | 362 | 318 | 324 | 195 | 209 | 175 | 179 |
| 46 | Poverty Gully, Vic | 135 | 135 | 342 | 352 | 318 | 326 | 203 | 225 | 171 | 177 |
| 47 | Poverty Gully, Vic | 133 | 148 | | | 318 | 324 | 195 | 205 | 175 | 183 |
| 48 | Poverty Gully, Vic | 135 | 145 | 342 | 342 | 318 | 322 | 195 | 211 | 175 | 175 |
| 49 | Poverty Gully, Vic | 145 | 158 | | | 318 | 322 | 199 | 223 | 173 | 175 |
| 50 | Poverty Gully, Vic | 133 | 135 | 342 | 342 | 322 | 326 | 195 | 225 | 175 | 183 |
| 51 | Poverty Gully, Vic | 135 | 139 | | | 324 | 324 | 207 | 225 | | |
| 52 | Poverty Gully, Vic | 135 | 135 | 334 | 334 | | | | | 171 | 181 |
| 53 | Poverty Gully, Vic | 135 | 141 | 334 | 365 | 298 | 320 | 225 | 225 | 175 | 185 |
| 54 | Poverty Gully, Vic | 135 | 135 | 365 | 365 | 318 | 324 | 199 | 225 | 175 | 181 |
| 55 | Poverty Gully, Vic | 135 | 135 | 344 | 393 | 318 | 326 | 199 | 203 | 177 | 183 |
| 56 | Poverty Gully, Vic | 135 | 148 | 342 | 383 | 318 | 318 | 223 | 225 | 173 | 183 |
| 57 | Poverty Gully, Vic | 135 | 135 | 365 | 365 | 322 | 322 | 205 | 207 | | |
| 58 | Poverty Gully, Vic | 139 | 148 | 342 | 393 | | | 223 | 225 | 171 | 183 |
| 59 | Poverty Gully, Vic | 139 | 148 | 342 | 393 | | | 223 | 225 | 171 | 183 |
| 60 | Frog Hollow, Vic | 135 | 141 | 350 | 350 | 314 | 320 | 195 | 225 | 175 | 175 |
| 61 | Frog Hollow, Vic | 141 | 141 | | | 314 | 320 | 207 | 225 | 175 | 179 |
| 62 | Frog Hollow, Vic | 135 | 141 | | | 316 | 320 | 195 | 195 | 175 | 183 |

| Sample | Locality | Microsatellite Locus | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Te11BGT | | Te15BGT | | Te08BGT | | Te18BG | | Te17BGT | |
| 63 | Frog Hollow, Vic | 145 | 150 | 334 | 334 | 314 | 322 | 195 | 225 | 175 | 177 |
| 64 | Frog Hollow, Vic | 141 | 150 | | | 312 | 320 | 195 | 195 | 175 | 179 |
| 65 | Frog Hollow, Vic | 135 | 141 | 352 | 352 | 318 | 324 | 195 | 199 | 173 | 177 |
| 66 | Frog Hollow, Vic | 139 | 139 | | | | | 195 | 207 | | |
| 67 | Frog Hollow, Vic | 135 | 141 | | | 316 | 322 | 195 | 205 | 171 | 175 |
| 68 | Frog Hollow, Vic | 133 | 135 | 334 | 350 | 322 | 324 | 195 | 195 | 173 | 181 |
| 69 | Frog Hollow, Vic | 135 | 135 | | | 320 | 324 | 195 | 207 | 175 | 185 |
| 70 | Frog Hollow, Vic | 135 | 150 | 350 | 350 | 320 | 324 | 225 | 225 | 171 | 173 |
| 71 | Frog Hollow, Vic | 135 | 135 | | | 322 | 324 | 195 | 225 | 175 | 183 |
| 72 | Frog Hollow, Vic | 135 | 135 | 342 | 342 | 322 | 322 | 195 | 227 | 171 | 175 |
| 73 | Frog Hollow, Vic | 135 | 145 | 334 | 354 | | | 207 | 209 | 183 | 202 |
| 74 | Frog Hollow, Vic | 135 | 135 | 350 | 350 | 320 | 324 | 195 | 195 | 173 | 179 |
| 75 | Daylesford Road, Vic | | | | | 318 | 322 | 195 | 205 | 175 | 183 |
| 76 | Daylesford Road, Vic | 135 | 135 | 350 | 352 | | | 203 | 227 | 179 | 191 |
| 77 | Daylesford Road, Vic | 139 | 141 | | | 318 | 324 | 195 | 197 | 167 | 181 |
| 78 | Daylesford Road, Vic | 135 | 135 | 346 | 346 | 310 | 320 | 195 | 223 | 171 | 181 |
| 79 | Daylesford Road, Vic | 135 | 141 | 352 | 354 | 318 | 320 | 195 | 201 | 175 | 181 |
| 80 | Daylesford Road, Vic | 135 | 135 | 344 | 344 | 320 | 322 | 195 | 195 | 169 | 183 |
| 81 | Daylesford Road, Vic | 135 | 135 | 344 | 363 | 322 | 322 | 193 | 195 | 169 | 181 |
| 82 | Daylesford Road, Vic | 135 | 141 | 348 | 363 | 324 | 324 | 195 | 203 | 177 | 183 |
| 83 | Daylesford Road, Vic | | | | | 314 | 324 | 195 | 195 | 175 | 181 |
| 84 | Daylesford Road, Vic | 143 | 145 | | | 324 | 326 | 195 | 197 | 175 | 179 |
| 85 | Daylesford Road, Vic | 135 | 135 | 334 | 352 | 314 | 322 | 195 | 203 | 175 | 183 |
| 86 | Daylesford Road, Vic | 135 | 135 | 338 | 354 | 324 | 324 | 195 | 207 | 173 | 181 |
| 87 | Daylesford Road, Vic | 135 | 141 | | | 318 | 322 | 207 | 221 | 175 | 181 |
| 88 | Daylesford Road, Vic | 135 | 141 | 334 | 334 | 324 | 324 | 195 | 195 | 171 | 183 |
| 89 | Daylesford Road, Vic | 135 | 150 | 334 | 350 | 320 | 339 | 195 | 211 | 171 | 183 |
| 90 | Kinglake-Healesville road, Vic | 135 | 145 | 325 | 325 | 328 | 328 | 201 | 201 | 171 | 179 |
| 91 | Kinglake-Healesville road, Vic | 135 | 143 | 329 | 329 | 316 | 332 | 213 | 217 | 181 | 187 |
| 92 | Kinglake-Healesville road, Vic | 135 | 147 | 327 | 327 | | | 201 | 207 | 186 | 191 |
| 93 | Kinglake-Healesville road, Vic | 135 | 147 | 333 | 366 | 320 | 334 | 207 | 217 | 171 | 187 |
| 94 | Kinglake-Healesville road, Vic | 143 | 143 | 327 | 366 | 348 | 357 | 205 | 231 | 177 | 187 |
| 95 | Kinglake-Healesville road, Vic | 141 | 189 | 325 | 325 | 323 | 330 | 205 | 213 | 175 | 204 |

| Sample | Locality | Microsatellite Locus | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Te11BGT | | Te15BGT | | Te08BGT | | Te18BG | | Te17BGT | |
| 96 | Kinglake-Healesville road, Vic | 135 | 143 | 325 | 327 | 320 | 370 | 219 | 219 | 179 | 194 |
| 97 | Kinglake-Healesville road, Vic | 143 | 143 | 326 | 334 | 318 | 322 | 211 | 231 | 173 | 221 |
| 98 | Kinglake-Healesville road, Vic | 135 | 135 | 320 | 325 | 318 | 336 | 201 | 205 | 171 | 214 |
| 99 | Kinglake-Healesville road, Vic | 141 | 143 | 325 | 327 | 332 | 336 | 203 | 221 | 173 | 183 |
| 100 | Kinglake-Healesville road, Vic | 141 | 141 | 325 | 329 | 322 | 336 | 209 | 227 | 171 | 173 |
| 101 | Kinglake-Healesville road, Vic | 143 | 147 | 325 | 327 | 320 | 326 | 201 | 217 | 177 | 177 |
| 102 | Kinglake-Healesville road, Vic | 135 | 152 | 325 | 326 | 322 | 325 | 201 | 201 | 183 | 204 |
| 103 | Kinglake-Healesville road, Vic | 135 | 135 | 327 | 348 | 322 | 322 | 201 | 201 | 185 | 185 |
| 104 | Kinglake-Healesville road, Vic | 141 | 162 | 327 | 336 | 320 | 324 | 201 | 207 | 191 | 204 |
| 105 | Lima South, Vic | 156 | 189 | 344 | 366 | 322 | 322 | 205 | 205 | 189 | 189 |
| 106 | Lima South, Vic | 145 | 156 | 329 | 366 | 312 | 318 | 215 | 215 | 173 | 187 |
| 107 | Lima South, Vic | 143 | 143 | 327 | 335 | 320 | 332 | 207 | 207 | 167 | 183 |
| 108 | Lima South, Vic | 141 | 170 | 335 | 335 | 326 | 326 | 205 | 237 | 183 | 202 |
| 109 | Lima South, Vic | 141 | 145 | 325 | 329 | 324 | 340 | 207 | 207 | 187 | 206 |
| 110 | Lima South, Vic | 143 | 176 | 346 | 374 | 320 | 327 | 209 | 213 | 173 | 189 |
| 111 | Lima South, Vic | 141 | 141 | 329 | 329 | 322 | 322 | 207 | 207 | 187 | 227 |
| 112 | Lima South, Vic | 143 | 145 | 327 | 331 | 320 | 324 | 209 | 217 | 177 | 202 |
| 113 | Lima South, Vic | 141 | 174 | 327 | 337 | 316 | 330 | 201 | 207 | 185 | 202 |
| 114 | Tumbarumba-Tumut road, NSW | 150 | 150 | 327 | 336 | 326 | 330 | 203 | 205 | 187 | 191 |
| 115 | Tumbarumba-Tumut road, NSW | 135 | 150 | 327 | 327 | 316 | 318 | 205 | 207 | 177 | 183 |
| 116 | Tumbarumba-Tumut road, NSW | 135 | 152 | 327 | 327 | 326 | 338 | 203 | 203 | 169 | 204 |
| 117 | Tumbarumba-Tumut road, NSW | 135 | 135 | 327 | 327 | 318 | 326 | 201 | 203 | 169 | 191 |
| 118 | Tumbarumba-Tumut road, NSW | 135 | 150 | 327 | 338 | 320 | 320 | 201 | 205 | 169 | 210 |
| 119 | Tumbarumba-Tumut road, NSW | 135 | 135 | 327 | 338 | 318 | 330 | 205 | 217 | 183 | 183 |
| 120 | Tumbarumba-Tumut road, NSW | 135 | 135 | 338 | 338 | 316 | 326 | 203 | 207 | 185 | 193 |
| 121 | Tumbarumba-Tumut road, NSW | 135 | 135 | 327 | 338 | 316 | 318 | 203 | 203 | 181 | 183 |
| 122 | Tumbarumba-Tumut road, NSW | 135 | 150 | 327 | 337 | 316 | 326 | 203 | 207 | 183 | 185 |
| 123 | Tumbarumba-Tumut road, NSW | 135 | 150 | 325 | 327 | 316 | 320 | 217 | 217 | 169 | 183 |
| 124 | Tumbarumba-Tumut road, NSW | 135 | 135 | 327 | 327 | 328 | 336 | 203 | 203 | 175 | 183 |
| 125 | Tumbarumba-Tumut road, NSW | 139 | 139 | | | | | | | | |
| 126 | Tumbarumba-Tumut road, NSW | 150 | 154 | 327 | 337 | 316 | 322 | 203 | 203 | 183 | 193 |
| 127 | Tumbarumba-Tumut road, NSW | 135 | 150 | 327 | 333 | 324 | 324 | 205 | 205 | 183 | 183 |
| 128 | Tumbarumba-Tumut road, NSW | 135 | 150 | 327 | 327 | 322 | 330 | 203 | 217 | 185 | 191 |

| Sample | Locality | Microsatellite Locus | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Te11BGT | | Te15BGT | | Te08BGT | | Te18BG | | Te17BGT | |
| 129 | Murray Valley Highway, Vic | 135 | 135 | | | 308 | 316 | 205 | 217 | 189 | 197 |
| 130 | Murray Valley Highway, Vic | 141 | 158 | 325 | 325 | 308 | 324 | 201 | 219 | 202 | 210 |
| 131 | Murray Valley Highway, Vic | 135 | 150 | 327 | 327 | 316 | 324 | 201 | 221 | 173 | 183 |
| 132 | Murray Valley Highway, Vic | 141 | 150 | 325 | 327 | 322 | 326 | 205 | 207 | 181 | 181 |
| 133 | Murray Valley Highway, Vic | 152 | 154 | 327 | 327 | 322 | 324 | 201 | 209 | 175 | 193 |
| 134 | Murray Valley Highway, Vic | 141 | 150 | 325 | 327 | | | 201 | 211 | 179 | 181 |
| 135 | Murray Valley Highway, Vic | 150 | 152 | 325 | 327 | 308 | 324 | 205 | 209 | 183 | 204 |
| 136 | Murray Valley Highway, Vic | 135 | 150 | 325 | 327 | | | | | 179 | 212 |
| 137 | Murray Valley Highway, Vic | 150 | 162 | 327 | 327 | 308 | 316 | 211 | 217 | | |
| 138 | Murray Valley Highway, Vic | 135 | 135 | 327 | 327 | 322 | 324 | 209 | 209 | 187 | 189 |
| 139 | Murray Valley Highway, Vic | 135 | 135 | 325 | 327 | 330 | 348 | 201 | 217 | 177 | 195 |
| 140 | Murray Valley Highway, Vic | 141 | 156 | 325 | 327 | 320 | 330 | 201 | 217 | 187 | 204 |
| 141 | Murray Valley Highway, Vic | 150 | 150 | 327 | 327 | 318 | 322 | 205 | 207 | 181 | 187 |
| 142 | Murray Valley Highway, Vic | 135 | 135 | 325 | 327 | 316 | 322 | 205 | 205 | 187 | 214 |
| 143 | Murray Valley Highway, Vic | 135 | 152 | 325 | 325 | 322 | 322 | 205 | 205 | 169 | 181 |
| 144 | Sardine Creek Road, Vic | 141 | 162 | 327 | 327 | 316 | 324 | 207 | 223 | 181 | 199 |
| 145 | Sardine Creek Road, Vic | 150 | 150 | 327 | 327 | 316 | 322 | 203 | 213 | 175 | 175 |
| 146 | Sardine Creek Road, Vic | 150 | 162 | 325 | 327 | 316 | 320 | | | 181 | 183 |
| 147 | Sardine Creek Road, Vic | 143 | 162 | 327 | 327 | 316 | 316 | 207 | 223 | 177 | 177 |
| 148 | Sardine Creek Road, Vic | 150 | 150 | 327 | 327 | 316 | 316 | 223 | 223 | 177 | 181 |
| 149 | Sardine Creek Road, Vic | 150 | 150 | 327 | 327 | 306 | 316 | 203 | 223 | 177 | 181 |
| 150 | Sardine Creek Road, Vic | 150 | 150 | 325 | 325 | 316 | 342 | 203 | 207 | 177 | 181 |
| 151 | Sardine Creek Road, Vic | 150 | 150 | 327 | 329 | | | | | 181 | 181 |
| 152 | Sardine Creek Road, Vic | 135 | 150 | 327 | 327 | 316 | 316 | 207 | 215 | | |
| 153 | Sardine Creek Road, Vic | 150 | 162 | 327 | 327 | 342 | 346 | 211 | 223 | 181 | 181 |
| 154 | Sardine Creek Road, Vic | 150 | 166 | 327 | 327 | 324 | 346 | 207 | 207 | 181 | 199 |
| 155 | Sardine Creek Road, Vic | 135 | 150 | 325 | 327 | 316 | 316 | 203 | 209 | 181 | 181 |
| 156 | Sardine Creek Road, Vic | 135 | 150 | 327 | 329 | 316 | 316 | 207 | 223 | | |
| 157 | Sardine Creek Road, Vic | 150 | 162 | 325 | 327 | 322 | 324 | 203 | 213 | 181 | 183 |
| 158 | Sardine Creek Road, Vic | 150 | 162 | 325 | 327 | 342 | 348 | 203 | 209 | 177 | 183 |

Greyed areas indicate missing data

**Appendix 12 PCR fragment lengths at 5 loci for 4 populations (55 individuals) of *T. stenocarpa***

| Sample | Locality | Microsatellite Locus | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Te11BGT | | Te15BGT | | Te08BGT | | Te18BG | | Te17BGT | |
| 1 | Old Chum Creek, Vic | | | | | 318 | 326 | 197 | 197 | 186 | 198 |
| 2 | Old Chum Creek, Vic | | | | | 318 | 318 | 197 | 197 | 185 | 185 |
| 3 | Old Chum Creek, Vic | 143 | 145 | | | 318 | 318 | 197 | 201 | 186 | 186 |
| 4 | Old Chum Creek, Vic | 139 | 141 | | | 318 | 320 | 197 | 203 | | |
| 5 | Old Chum Creek, Vic | 131 | 143 | 346 | 346 | 318 | 318 | 197 | 203 | 186 | 188 |
| 6 | Old Chum Creek, Vic | 135 | 135 | 338 | 346 | 318 | 320 | 203 | 209 | 188 | 188 |
| 7 | Old Chum Creek, Vic | 131 | 143 | 338 | 444 | 318 | 326 | 197 | 203 | 186 | 194 |
| 8 | Old Chum Creek, Vic | 131 | 131 | 336 | 336 | 318 | 318 | 195 | 197 | | |
| 9 | Old Chum Creek, Vic | 131 | 143 | 348 | 352 | 310 | 320 | 197 | 203 | 186 | 192 |
| 10 | Old Chum Creek, Vic | 131 | 143 | 336 | 338 | 318 | 320 | 197 | 197 | 233 | 233 |
| 11 | Old Chum Creek, Vic | | | 338 | 368 | 310 | 318 | 197 | 201 | 234 | 234 |
| 12 | Old Chum Creek, Vic | 143 | 143 | 346 | 346 | 310 | 318 | 197 | 197 | 194 | 194 |
| 13 | Old Chum Creek, Vic | 131 | 131 | 338 | 346 | 318 | 326 | 197 | 203 | 192 | 192 |
| 14 | Old Chum Creek, Vic | 131 | 143 | 338 | 354 | 318 | 318 | 201 | 203 | 188 | 188 |
| 15 | Old Chum Creek, Vic | 131 | 143 | 338 | 346 | 318 | 320 | 197 | 197 | 194 | 198 |
| 16 | Tentpole Road, Vic | 131 | 131 | 359 | 454 | 310 | 332 | 197 | 197 | 188 | 198 |
| 17 | Tentpole Road, Vic | 145 | 145 | 346 | 368 | 318 | 326 | 201 | 203 | 186 | 186 |
| 18 | Tentpole Road, Vic | 135 | 145 | 342 | 438 | 318 | 320 | 197 | 197 | 194 | 196 |
| 19 | Tentpole Road, Vic | 145 | 145 | 344 | 452 | 310 | 310 | 197 | 201 | 194 | 198 |
| 20 | Tentpole Road, Vic | 131 | 139 | 346 | 456 | 318 | 318 | 197 | 201 | 192 | 194 |
| 21 | Tentpole Road, Vic | 131 | 145 | 362 | 454 | 318 | 318 | 203 | 203 | 192 | 194 |
| 22 | Tentpole Road, Vic | 137 | 137 | | | 318 | 318 | 197 | 215 | | |
| 23 | Tentpole Road, Vic | 131 | 145 | 452 | 452 | 318 | 326 | 197 | 203 | | |
| 24 | Tentpole Road, Vic | 145 | 156 | | | | | | | | |
| 25 | Tentpole Road, Vic | 131 | 131 | 368 | 368 | 318 | 324 | 197 | 197 | 194 | 198 |
| 26 | East Beenak, Vic | | | | | 310 | 318 | 197 | 203 | 186 | 194 |
| 27 | East Beenak, Vic | 131 | 131 | 342 | 433 | 318 | 324 | 197 | 203 | 195 | 195 |
| 28 | East Beenak, Vic | 131 | 131 | 368 | 457 | 318 | 322 | 197 | 201 | 186 | 186 |
| 29 | East Beenak, Vic | 131 | 131 | 368 | 368 | 310 | 310 | 203 | 203 | 198 | 198 |
| 30 | East Beenak, Vic | 135 | 141 | 325 | 327 | 312 | 318 | 209 | 233 | 177 | 181 |

| Sample | Locality | Microsatellite Locus | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Te11BGT | | Te15BGT | | Te08BGT | | Te18BG | | Te17BGT | |
| 31 | East Beenak, Vic | 131 | 145 | 354 | 354 | 322 | 326 | 203 | 203 | 192 | 194 |
| 32 | East Beenak, Vic | 131 | 145 | 348 | 352 | 322 | 322 | 197 | 205 | 186 | 200 |
| 33 | East Beenak, Vic | 131 | 131 | 455 | 461 | 310 | 310 | 197 | 197 | 196 | 200 |
| 34 | East Beenak, Vic | 145 | 145 | 344 | 368 | 310 | 318 | | | | |
| 35 | East Beenak, Vic | 131 | 131 | 325 | 454 | 318 | 318 | 201 | 201 | 185 | 190 |
| 36 | East Beenak, Vic | 131 | 131 | 342 | 452 | 316 | 318 | 201 | 201 | 186 | 200 |
| 37 | East Beenak, Vic | 131 | 131 | 368 | 436 | 318 | 320 | 197 | 197 | 186 | 190 |
| 38 | East Beenak, Vic | 131 | 145 | 368 | 455 | 310 | 322 | 197 | 203 | 204 | 206 |
| 39 | East Beenak, Vic | 131 | 139 | 344 | 344 | 324 | 324 | 197 | 197 | 186 | 195 |
| 40 | East Beenak, Vic | 131 | 145 | 342 | 433 | 318 | 322 | 197 | 201 | 192 | 194 |
| 41 | Forest Road, Vic | 131 | 131 | 364 | 436 | 322 | 326 | 197 | 203 | 196 | 196 |
| 42 | Forest Road, Vic | 131 | 131 | 340 | 348 | 318 | 318 | 201 | 203 | 188 | 198 |
| 43 | Forest Road, Vic | 131 | 131 | 346 | 362 | 318 | 318 | 197 | 199 | 194 | 196 |
| 44 | Forest Road, Vic | 131 | 135 | 354 | 356 | 318 | 318 | 197 | 199 | 188 | 191 |
| 45 | Forest Road, Vic | 131 | 135 | 344 | 358 | 318 | 324 | 195 | 199 | 184 | 184 |
| 46 | Forest Road, Vic | 131 | 139 | 340 | 457 | 318 | 322 | 197 | 197 | | |
| 47 | Forest Road, Vic | 131 | 131 | 346 | 348 | 318 | 328 | 197 | 197 | 192 | 192 |
| 48 | Forest Road, Vic | 131 | 131 | 362 | 452 | 318 | 318 | 197 | 197 | 188 | 191 |
| 49 | Forest Road, Vic | 131 | 131 | | | 308 | 318 | 203 | 203 | 188 | 191 |
| 50 | Forest Road, Vic | 131 | 131 | 342 | 346 | 318 | 318 | 197 | 203 | 192 | 192 |
| 51 | Forest Road, Vic | 131 | 131 | 356 | 459 | 318 | 324 | 197 | 205 | | |
| 52 | Forest Road, Vic | 131 | 135 | 346 | 438 | 318 | 318 | 197 | 199 | 184 | 184 |
| 53 | Forest Road, Vic | 131 | 131 | 340 | 452 | 318 | 320 | 197 | 205 | | |
| 54 | Forest Road, Vic | 131 | 131 | 362 | 438 | 318 | 318 | 205 | 205 | 186 | 192 |
| 55 | Forest Road, Vic | 131 | 131 | 342 | 459 | 318 | 326 | 197 | 197 | 184 | 188 |

Greyed out areas indicate missing data