Identification and the role of hybridisation in New Zealand *Pittosporum*

A thesis

submitted in partial fulfilment

of the requirements for the degree

of

Master of Science

at

The University of Waikato

by

Susan Kathleen Carrodus

Hamilton, New Zealand

2009

ABSTRACT

Godley (1985) proposed that species which have separate juvenile and adult forms may be derived from hybridisation, and proposed that the rare, heteroblastic species *P. turneri* is a putative hybrid. This study aims to identify whether *Pittosporum turneri* is derived from hybridisation between a divaricating shrub (*P. divaricatum*) and a non-divaricating tree (*P. colensoi*), and to improve resolution of relationships among very closely related species within the genus *Pittosporum*. A combined approach was used to test the origin of *P. turneri*. Phylogenetic analysis of the maternally inherited *trn*T-*trn*L region of chloroplast DNA was undertaken to compare with a phylogeny based on the biparentally inherited internal transcribed spacer of nuclear ribosomal DNA (ITS) for all New Zealand *Pittosporum* species. Additionally, inter simple sequence repeats (ISSR's) and allozymes were used in an attempt to identify hybridisation between *P. colensoi* and *P. divaricatum.* A morphological study was undertaken to determine whether *P. turneri* is morphologicaly intermediate to the putative parents. Cross-pollination between the putative parents of *P. turneri* was also undertaken in the wild between female flowers of *P. divaricatum* and male flowers of *P. colensoi* to investigate whether hybridisation between these co-existing species is possible.

The *trn*T-*trn*L region resolved several clades within the New Zealand *Pittosporum* previously unresolved by the ITS region alone. *P. turneri* has the same *tr*T-*trn*L sequence as *P. divaricatum*, implicating *P. divaricatum* as the maternal parent. The profile of ISSR bands in *P. turneri*, exhibit additivity of bands found in *P. colensoi* and *P. divaricatum*, supporting a hybrid origin of *P. turneri.* Morphological analyses also show that *P. turneri* is intermediate to *P. divaricatum* and *P. colensoi*. The cross-pollination experiment was unsuccessful and no seedlings germinated, although four seeds appeared viable. It is proposed that *P. turneri* has a hybrid origin, however this finding needs to be supported by further work.

ACKNOWLEDGEMENTS

Thanks to my supervisors Chrissen Gemmill and Bruce Clarkson for their ideas and their guidance during this project. I acknowledge the University of Waikato for the Masters Research scholarship I received, and Forest and Bird for the Research Grant which helped a great deal with my lab costs.

I would also like to thank the staff and students of the PBRL who were always great people to work with, and who helped me with their technical expertise, and I would also like to thank the other students in our research group for their advice and moral support. Thanks also to the Wehi family for making my life so much easier.

Thankyou to the Department of Conservation, Maniapoto Area Office and the Tongariro-Taupo Area office, and in particular, Nick Singers for his advice on propogation of *Pittosporum turneri*. Thankyou also to Dave Kelly and Andrea Brandon for providing their advice on cross-pollination. Thanks also to Jeff Mcauley at Oratia Native Plant Nursery for providing me with eco-sourced material.

Lastly and most importantly, thanks to Thomas for coming along on my trips to Lochinvar station, and for your proof reading efforts and your support.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

CHAPTER ONE

INTRODUCTION

This study addresses the theory of Godley (1985) that New Zealand plant species with a divaricating juvenile form have descended from a hybrid between a divaricating shrub and a non divaricating tree. This study uses morphology, genetics and cross-pollination experiments to address this theory in respect to a rare Central North Island species, *Pittosporum turneri*. It is proposed that *P. turneri* is a putative hybrid between *P. colensoi,* a common tree found throughout New Zealand, and *P. divaricatum,* an understory shrub closely resembling the juvenile stage of *P. turneri.* Additionally, hybridisation and relationships within the New Zealand species of *Pittosporum* is investigated.

1.1 HYBRIDISATION IN PLANTS

Hybrid speciation occurs when two lineages combine to produce a descendent species that is a combination of both parental lineages (Rieseberg 1997). Hybridisation in plants has been known since the time of Linnaeus who stated " it is impossible to doubt that there are new species produced by hybrid generation" (Linn´e 1760; Rieseberg 1997). However most early experiments found that hybrids tended to be infertile or revert to the parental form, therefore hybridisation was viewed as rare and as having deleterious effects in both plants and animals (Wisseman 2007), a view held by most botanists throughout the eighteenth and nineteenth centuries (Rieseberg 1997).

Hybridisation is now considered to play an important role in evolution and speciation (Barton 2001). Although hybridisation events are thought to be rare, records of hybrid species have been increasing over time and hybrids have been identified within a large number of plant families, comprising around 25% of plant species (Mallet 2005). There have been a number of recent studies in plants demonstrating that hybridisation can lead to rapid radiation and diversification (Hegarty & Hiscock 2005), (Mraz et al. 2005), (Sang et al. 1997) as well as "functional novelty" (Arnold 1995), (Seehausen 2004).

1.1.1 IDENTIFICATION OF HYBRIDS

Hybridisation in plants has been found to be most common in species which have specific life-history characteristics, including perennial habit, outcrossing breeding systems and asexual reproduction (Wisseman 2007). It was proposed by Cockayne (1923) that hybrids can be recognised by intermediacy of characters between the putative parents, that putative parents and hybrids should grow in close proximity, and that the hybrid if fertile should give rise to a polymorphic progeny. However, Cockayne also stated "there are no absolute criteria, so each case of suspected hybridism must be decided on its own merits", usually on the basis of morphological characters. However, more recently Rieseberg (1993) found there was no way to predict the outcome of hybridisation events on gross morphology, and studies have shown that hybrids can show an array of morphological phenotypes relative to parental species (Allendorf et al. 2001).

Hybridisation can occur either by homoploid speciation where there is no change in ploidy level or by allopolyploid speciation where the hybrid has a different ploidy level to either of its parents due to duplication of chromosomes during meiosis (Hegarty & Hiscock 2005). Although hybrids which have arisen through polyploidy can be easily recognised, polyploids do not always arise from hybridisation but can arise through autopolyploidy, where spontaneous doubling of chromosome sets occurs without hybridisation. Additionally, after polyploidy has occurred, species tend to become reduced in their chromosome number and become homoploid over evolutionary time (Wisseman 2007). For these reasons it is a difficult task to document wild hybrids and make generalizations about the frequency and importance of hybridisation in plants worldwide (Wisseman 2007).

1.1.2 HOMOPLOID HYBRID SPECIATION

Hybridisation through polyploidy is usually resistant to introgression because the change in ploidy level establishes genetic isolation (Lai et al. 2006), whereas homoploid speciation requires both ecological and karyotypic divergence (Grant 1981) and is considered rare, with only around 20 homoploid hybrid species rigorously documented (Gross & Rieseberg 2005). Examples are *Stephanomera diegensis* (Gallez & Gottlieb 1982) three species of *Helianthus (Rieseberg et al. 1991; Rieseberg 1991), Encelia virginensis* (Allan et al. 1997), *Iris nelsonii*

(Arnold et al. 1990), and within *Paeonia* (Sang et al. 1995). Theoretically for a homoploid hybridisation event to produce a new species the hybrid must be reproductively isolated from both parents, either genetically or ecologically (Ungerer et al. 1998), otherwise if closely related species living in sympatry or allopatry hybridise then backcross to either parent this will prevent or reduce the rate of divergence (Wu 2001). However, backcrossing and introgression (gene flow between species) could also have important evolutionary effects by introducing new alleles into the population. (Mallet 2005). Homoploid hybrid speciation is theorized to occur through 'recombinational speciation' whereby two parental species differ by two or more chromosomal rearrangements producing a heterozygous F_1 hybrid that will produce a small proportion of gametes which are balanced and viable and with recombinant karyotypes. If self fertilized, a small fraction of F_2 individuals will have a novel homokaryotype and will be fertile and stable as well as partly resistant to introgression. (Grant 1981) (See Figure 1.1.). Such chromosomal rearrangements would theoretically provide a barrier to back-crossing (Gross & Rieseberg 2005).

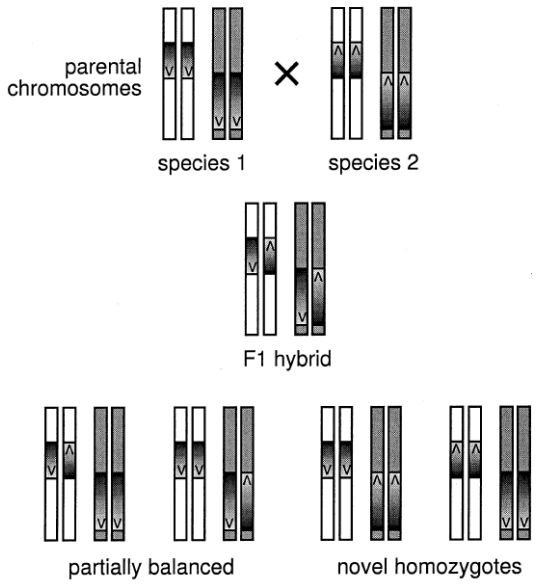


Figure 1.1. Model of recombinational speciation in which two species differ by two chromosomal inversions (marked by v or inverted v). The F1 hybrid is heterozygous for both and has low fertility. Mating among the F1 population or backcrossing will produce two with partially balanced genotypes and novel homozygous genotypes. The novel genotypes will be fertile but partially sterile with the parental species. From Buerkle (2000)

1.1.3 ECOLOGICAL BARRIERS TO INTROGRESSION

External barriers to reproduction may also be an important mechanism of reproductive isolation. Some studies have found that a proportion of hybrids show extreme phenotypes relative to parental phenotypes and can therefore occupy a different ecological niche to either parent, giving the hybrids an ecological advantage over any backcrossed individuals. This phenomenon is called 'transgressive segregation' (Rieseberg et al. 1999) and has been found in both wild and domesticated plant species with many morphological traits found to be transgressive, as well as physiological traits, fecundity, life history traits, composition of organs and tissues, and tolerances to abiotic and biotic factors (Rieseberg et al. 1999). Causes of transgressive segregation may include an increased mutation rate, reduced developmental stability, new combinations of normal alleles, epistasis (whereby genes from different loci interact, masking the effect of one gene on another), unmasking of recessive alleles, or overdominance (where the heterozygote exhibits a phenotype outside the range of either homozygote) (Rieseberg 2000). For example a recent study found that the diploid hybrid *Helianthus paradoxus* has greater salt marsh tolerance due to transgressive segregation of elemental uptake and leaf succulence than either parental species (Lexer et al. 2003). Transgressive segregation may also even lead to the formation of new invasive species (Ellstrand & Schierenbeck 2000). There may be an important role for transgressive segregation in colonizing new habitats in island radiations, where rapid radiation and diversification often happen without postmating reproductive barriers (Givnish & Sytsma 1997).

1.2 EVOLUTION AND HYBRIDISATION IN THE NEW ZEALAND FLORA

Hybridisation has played an important role in the radiation and evolution of plant species in New Zealand (Raven 1973), with a large number of documented hybrids within a number of the plant families (Conner 1985), in genera such as *Phormium* (Houliston et al. 2008), *Hoheria (Heenan et al. 2005), Sophora, (Heenan et al. 2001) Coprosma* (Wichman et al. 2002), and even between genera e.g. *Celmisia* x *Olearia* hybrid (Clarkson 1988). It is recognised that New Zealand plant species tend to show a high level of polymorphy where they are known to hybridise, with great variation within populations, from region to region

and between species of the same genus (Rattenbury 1961). Wardle (1963) proposed that widespread interspecific hybridisation may be due to recent evolution in response to the changing climate throughout the Quarternary where the climate fluctuated between ice ages and interglacial periods. Since the isolation from Gondwana about 80mya, New Zealand experienced frequent geological changes and was reduced from a much greater landmass to a series of archipelagos from the Palaeocene to the Oligocene (65 to 35mya). The land began to rise in the Miocene (35mya) creating its current geography, and a series of glaciations followed in the Pleistocene, with the most recent glaciations occurring 25,000 to 15,000 years ago (Flemming 1979). It is believed that the majority of the New Zealand flora has arrived by dispersal since the mid-Palaeocene, mainly from Australia (Pole 1994).

Rattenbury (1961) suggested that many of the unusual characteristics in the New Zealand flora including the great degree of polymorphy between and among species could be explained by "cyclic hybridization"; where species remain inter-fertile, and are therefore able to adapt to a rapidly changing climate. Theoretically, species which hybridise readily would gain an advantage in rapidly changing climatic conditions, as the increase in number of alleles will produce a wider variety of recombinants, and therefore provide more potential for rapid diversification (Wisseman 2007). Hybrids have also been found to be common in other island ecosystems, with Carr (1995) noting the high frequency of hybrids in the Hawaiian flora, suggesting that oceanic islands maintain the ability to cross among species, using hybridization as a survival mechanism in dynamic habitats. Recent phylogenetic studies have supported this theory, for example a study by Mummenhoff *et al.,* (2004) found that all species of *Lepidium* in New Zealand have low levels of sequence divergence and have radiated through hybridisation, probably coinciding with periods of dramatic climatic change. Another study of *Coprosma* by Wichman *et al*., (2002) found widespread hybridisation in New Zealand within the genus, also suggesting this was likely to be due to climate change.

1.2.1 EVOLUTON OF HETEROBLASTY AND THE DIVARICATE FORM

Heteroblastic development in plants is characterised by great differences between juvenile and adult growth forms, as opposed to homoblastic development where

differences between juvenile and adult stages are slight and gradual (Goebel 1900). Heteroblasty can be defined as habit-heteroblastism: "an abrupt break in the development of the habit of the plant", and leaf-heteroblastism: changes in leaf characters (Philipson 1964). In New Zealand there are around 200 species which show heteroblasty, with differences typically involving changes in the degree of leaf serrations, a change from glabrous to tomentous leaves, or changes from compound to entire leaves, and changes in leaf size either from small to large or vice versa (Godley 1985). Cockayne (1928) noted that about 165 of these species remain purely juvenile for a long period, or in other cases the juvenile form will sometimes reappear in some part of the plant as a reversion shoot. He noted that this occurs in 30 families and 50 genera, of which 10 are endemic, and of which 51 species are trees, 82 are shrubs, 19 are ligneous lianes, 10 are herbs and three are aquatic plants. He also made the observation that most juvenile forms are the more mesophytic (106 species) but some are more xerophytic (17 species), and for others there is no clear difference in ecological tolerance between juvenile and adult (39 cases). Examples of species with strong heteroblastic development in New Zealand include *Pennantia corymobosa* and *Eleocarpus hookerianus* (Heenan 1997). Heteroblasty is also a phenomenon which is very rare on continents but common on islands, with heteroblastic species found in Hawaii (Givnish & Sytsma 1997), Mascarene islands (Hansen et al. 2003) and New Caledonia (Burns & Dawson 2006).

The divaricating growth form is characterised by interlaced, wide-angle branches with small, widely spaced leaves and is relatively common in New Zealand, comprising 10% of New Zealand's flora (Webb & Kelly 1993). There has been much speculation as to why this growth form has evolved in New Zealand. Greenwood and Atkinson (1977) proposed that the divaricating growth form is a response to browsing pressure by moa, as the reduced numbers of leaves on outer branches and tough stems is believed to have deterred moa. Alternatively the divaricating growth form may be a response to climatic conditions, for example an adaptation to xeromorphic conditions which occurred during the "harsh, near tree-less" Pleistocene, as the densely branched structure of divaricating stems may create a wind break to provide a relatively moist interior (Rattenbury 1961). The divaricating form could also provide other advantages such as reduced frost damage due to the networking of stems protecting inner leaves (McGlone & M.S

1981). This growth form has also been considered to be a response of a mainly tropical flora to glaciations that occurred in the Pleistocene, with few species which were adapted to alpine or dessert conditions (Wilson & Galloway 1993). However no studies have yet found the divaricating growth form to be associated with any particular environmental conditions (Burns & Dawson 2006).

Nine New Zealand species are both heteroblastic and have a divaricating juvenile and non-divaricating adult form (Philipson 1964). These include *Pittosporum turneri* (Turner's kohuhu), *Sophora microphylla* (Kowhai), *Streblus heterophylla* (Turepo), *Pennantia corymbosa* (Kaikomako), *Prumnopytus taxifolia* (Matai), *Carpodetus serratus* (Marbleleaf), *Plagianthus regius* subsp. *regius* (Ribbonwood), *Hoheria angustifolia* (Narrow-leaved Hohere), and *Elaeocapus hookerianus* (Pokaka), (Greenwood & Atkinson 1977). It is considered that the change from the divaricating growth form to the non-divaricating adult form is an adaptive feature for New Zealand ecological conditions, which optimises the advantages of different growth forms at different life stages. Day (1998) suggested this could be a response to changing light conditions at different heights in the canopy in broadleaf/conifer forests, where juveniles are exposed to low light intensity and adults are exposed to a greater light intensity. Greenwood and Atkinson (1977) proposed the height where divaricating plants become arborescent is when they reach the height of the "tallest moa", where moa can no longer reach the foliage. More recently Burns and Dawson (2006) supported the latter theory, noting that both New Caledonia and New Zealand have high numbers of heteroblastic species with very similar patterns of ontogeny despite having major climatic differences. They noted that avian herbivores replaced mammalian herbivores in New Zealand, Hawaii and New Caledonia, and also found that the height where the divaricating growth form becomes the adult form is lower in New Caledonia than in New Zealand, where the avian herbivores attained a lesser height than the moa. An experiment by Bond (2004) supported this theory by showing that emus and ostriches could obtain adequate feeding rates from adult foliage but not juvenile foliage of *Plagianthus regius* and *Pennantia corymbosa*. However Burns and Dawson (2006) suggest that we cannot rule out environmental influences as there may be a shared environmental history between the islands where heteroblasty is found which has led to the development of this trait, or the phenomenon may be due to another influence which these

islands have in common such as a similarity in rain regimes. However, it is still unclear what the genetic mechanisms which lead to the dramatic change in growth form are.

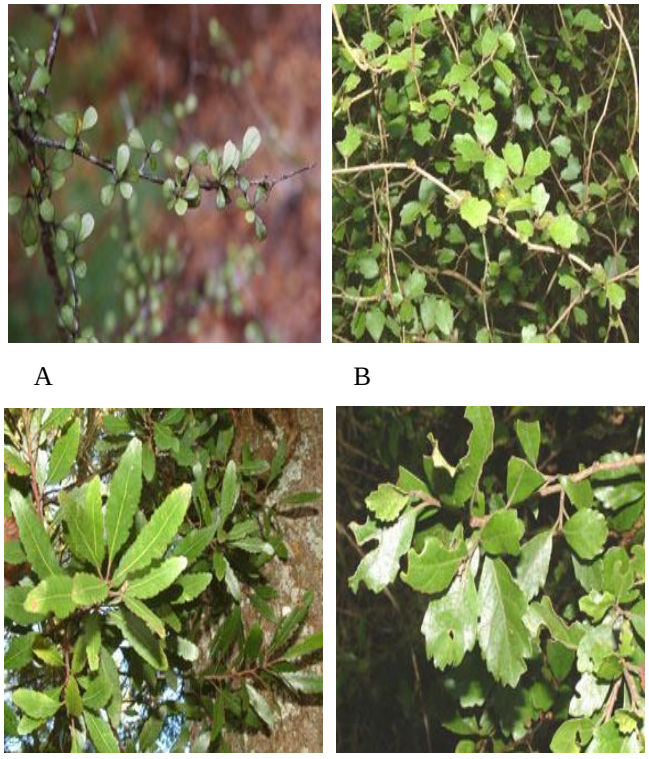


Figure 1.2: Photos of species with a divaricating juvenile and non-divaricating adult A and C: *Elaeocarpus hookerianus*, B and D: *Pennantia corymbosa*

Godley (1979) postulated that dramatic changes in leaf shape and size and growth habit during development is indicative of a hybrid origin. He proposed that all nine species with divaricating juvenile forms and arborescent adult forms have resulted from hybridisation between a divaricating and an arborescent species. Godley suggested that if hybridisation was shown to be the cause for this change during ontogeny it could apply to the origin of all such forms and he proposed a number of genera that have putative hybrids where there is strong leaf heteroblasty including *Pittosporum*, *Sophora*, and *Pseudopanax* (Godley 1985). A previous explanation proposed by Cockayne (1911) was that some ancestral forms in the Tertiary evolved into divaricating shrubs in the Pleistocene, then when warmer conditions returned some species were able to revert to the ancestral stage but retained their juvenile growth form. However Markham (1972) in a study of *Sophora* found that there were too many morphological differences between the juvenile form of *S. microphylla* (a divaricating species) and *S. prostrata* (an

arborescent species) for *S. microphylla* to be a permanent form of *S. prostrata*, having a more 'suckering' habit with stouter stems and branches and much smaller leaves, and containing different phenolic compounds. Godley (1985) suggested that another explanation could be that the divaricating form evolved as the permanent form of a heteroblastic species with a divaricating juvenile form, however if this is true there has been a high number of subsequent extinctions as there are many divaricating species throughout the New Zealand flora with no closely related heteroblastic species which occur in the same or similar environment or region. Greenwood and Atkinson (1977) noted that there are 45 species within 11 genera of divaricating shrubs with no related species with a juvenile divaricating form and non-divaricating adult form, therefore if this is true there have been a minimum of 11 extinctions (the number of genera involved). However Godley identified several species which could be used to test the hybridisation hypothesis, including *P. turneri*, for which he suggested one or both parental species may still exist.

1.3 THE GENUS *PITTOSPORUM* (PITTOSPORACEAE)

The family Pittosporaceae (Apiales) consists of nine genera (Cayzer 1997) and 200-240 species (Carlquist 1981). The Pittosporaceae has recently been identified as a clade within Apiales based on molecular data (Chandler & Plunkett 2004), and morphological data also strongly supports this relationship (Plunkett et al. 2004). Recent advances in the knowledge of relationships of Pittosporaceae have led to the inclusion of the genus *Citriobatus* with *Pittosporum* (Cayzer LW 2000b). There is now a new named genus, *Auranticarpa*, which is probably endemic to Australia, and was previously included within *Pittosporum*, (Cayzer 2000a). Six other genera are entirely endemic to Australia and only *Pittosporum* is widespread, occurring throughout the pacific, eastern Asia, Africa and as far east as Hawaii (Haas 1977).

Pittosporum Banks ex Garten is also by far the largest genus within Apiales with a recent estimate of 100 species (including *Citriobatus*) (Chandler et al. 2007). The genus contains high levels of endemism resulting from species radiations on islands (Gemmill 2002) and species distributions are attributed to long-distance dispersal, as the characteristic resinous seeds are thought to be attractive to birds (Carlquist 1974). Species are typically evergreen shrubs which are sometimes

epiphytic, or small trees reaching around 10m. Leaves are alternate and sometimes whorled and crowded at the branch tips. Flowers are perfect to functionally unisexual, panicled to umbellately arranged, in fascicles, or solitary. Fruit are a globose to ovoid or obovoid capsule, with two to five woody valves. Seeds are often but not always immersed in sticky fluid (Cooper 1956). There are many polymorphic characters which differ between species in *Pittosporum*, however relationships are obscured by the large number of hybrids and by species which intergrade (Allan, 1961). Phenotypic plasticity has also caused difficulty in recognising species which have several growth forms (Chandler et al. 2007). This has proven to be a problem throughout Pittosporaceae, a study by Wilkinson (1992) found that there was a great amount of overlap in morphological characters within each genus during various stages of development and no characters which could be used to define them. Recent molecular work has shown that there is low sequence divergence between the species although there is great morphological variation, creating difficulty in defining relationships between species and suggesting a "decoupling of morphological and molecular evolution" (Chandler et al. 2007). Alternatively this could be due to widespread hybridisation or introgression.

1.4 PITTOSPORUM IN NEW ZEALAND

The New Zealand species of *Pittosporum* includes around 18-26 endemic species (Cooper 1956); (Allan 1961), and all are endemic. However there is more diversity in the North Island, with 11 species endemic to the North Island, and only three species endemic to the South Island (Cooper 1956). *P. tenuifolium subsp. tenuifolium, P. tenuifolium* subsp. *colensoi* and *P. eugenoides* are widespread throughout New Zealand (Cooper 1956), however several species and subspecies have very confined distributions e.g. *P. ellipticum* subsp. *serpintinum* is only found on ultramafic rocks on the Surville Cliffs, North Cape (de Lange 1998). Four species are considered 'nationally endangered', *P. ellicpticum* subsp. *serptintinum, P. obcordatum, P. patulum,* and *P. turneri, P. dallii* is considered 'nationally vulnerable' *P. kirkii* is listed as 'chronically threatened', and *P. ellipticum* is considered 'sparse'. Additionally *P. fairchildii* is listed as range restricted, and *P.* aff. *crassifolium* is in 'serious decline' (de Lange et al. 2004) (see Table 1.1.)

There are also several different growth forms with eight New Zealand species which have distinct juvenile and adult foliage, with juvenile leaves typically lobed or toothed and adult leaves larger and entire. *P. rigidum*, *P. divaricatum*, *P. crassicaule* and *P. anomalum* have a "bewildering series" of polymorphic and heterophyllous forms (Cooper 1956), and all of these are divaricating species along with *P. obcordatum*, which is a trait not known in *Pittosporum* species outside New Zealand (Wilson & Galloway 1993). Hathaway (2001) suggested that the evolution of the divaricating habitat is likely to have occurred three times independently, and therefore there has likely been strong selection pressure for this growth form. Several divaricating species are also known to hybridise with non-divaricating species e.g. *P. obcordatum* x *P. tenuifolium* subsp. *tenuifolium* (Clarkson & Clarkson 1994) and *P. turneri* x *P. divaricatu*m (Druce 1977; Ecroyd 1994`, suggesting weak reproductive barriers between species of different growth forms. Hybrids are common between species found in sympatry and on islands e.g. P. ralphii x P. tenuifolium subsp. tenuifolium and P. crassifolium x P. tenuifolium subsp. tenuifolium {Druce`, 1977 `#164) (Druce 1977) and some of these hybrids are easily cultivated (Metcalf 1987).

Table 1.1. New Zealand endemic *Pittosporum* from (Cooper 1956) and (de Lange et al. 2004).

Taxa	Distribution	Conservation status	Growth form juvenile	Heteroblastic
P. anomalum	N, S		Divaricating shrub	Yes
P. cornifolium	N,S		Epiphytic shrub	No
P. crassicaule	S		Shrub	Yes
P. crassifolium	N		Shrub or Tree	No
*P. aff crassifolium**	Kermadec Is.	Serious decline	Shrub or Tree	No
P. dallii	S	Nationally vulnerable	Small tree	No
P. divaricatum	N,S		Divaricating	Yes

			shrub	
P. ellipticum subsp. *ellipticum*	N	Nationally endangered	Small tree	No
P. ellipticum subsp. *serpintinum*	N		Prostrate shrub	No
P. eugenoides	N,S		Tree	No
P. fairchildii	Three Kings Is.	Range restricted	Shrub	No
P. huttonianum	N		Shrub or small tree	No
P. kirkii	N		Epiphytic shrub	No
P. obcordatum	N,S	Nationally endangered	Shrub	Yes
P. patulum	S	Nationally endangered	Shrub or small tree	No
P. pimeleoides subsp pimeleoides	N		Shrub	No
P. pimeleoides subsp maius	N		Shrub	No
P. ralphii	N		Prostrate shrub	No
P. rigidum	N		Tree	Yes
P. tenuifolium subsp colensoi	N,S, Stewart Island		Tree	No
P. tenuifolium subsp tenuifolium	N,S		Tree	No
P. turneri	N	Nationally endangered	Juv: divaricating shrub, adult: small tree	Juvenile only
P. umbellatum	N		Small tree	No
P. virgatum	N		Small tree	No

* P. aff *crassifolium* is not a described species but is treated as a distinct species in this study. It may be more closely related to *P. bracteolatum* of Norfolk Island

1.4.1 RELATIONSHIPS AND TAXONOMY

Traditionally relationships within *Pittosporum* were based on morphology and groups divided into those species with bivalved and trivalved capsules, with bivalved species showing more diversity in other characteristics (Gowda 1951). Characteristics used for recognising species include capsule number, inflorescence type, absence or presence of hairs on shoots, leaves and inflorescences, along with hair colour, sepal arrangement, presence or absence of extra sepals, petal colour, and valve shape, variation in the length and thickness of the placenta and size and arrangement of the funicles (Cooper 1956). Contemporary studies of relationships within New Zealand *Pittosporum* have used the internal transcribed spacer (ITS)

region of nuclear ribosomal DNA (nrDNA), finding support for an Australian origin of New Zealand species of *Pittosporum*, with all other colonisation events from Australia or from island hopping, with low sequence divergence corresponding to low morphological differentiation, and there has probably been at least two colonisation events into New Zealand as the New Zealand species do not form a separate clade (Chandler et al. 2007).

1.4.2. FLOWER BIOLOGY AND POLLINATION

Pittosporum inflorescences are terminal, lateral or axillary, and flowers can be solitary, few or many flowered, in fascicles, umbels or panicles (Cooper, 1956). Flowers are small, with five petals and five sepals, five stamens, and two-celled anthers. The ovary is formed from 2-5 fused carpels and is sessile or stipulate and superior. In most New Zealand species the corolla are mostly red to deep purple, and a few are yellow, unlike *Pittosporum* elsewhere which typically have white, greenish white, or yellow corolla. Flowers in most species are structurally perfect but functionally unisexual, with either male or female parts reduced. Male flowers are described as having "weakly capitulate to truncate stigmas, long styles, slender ovaries, oblong anthers 2-4mm long borne on slender filaments. Usually the anthers are level with the stigma or exserted." Female flowers are described as having "2-3-4-lobed capitate stigmas, slightly shorter styles, plumper ovaries, apparently abortive, sagittiform anthers 1-2mm long, borne on filaments 1mm or more broad at the base, tapering distally. Usually the anthers are below the stigma." (Cooper, 1956). However flowers with apparently functional anthers, capitate stigmas and plump ovaries occur as well as flowers with 4 long functional anthers and 1 apparently abortive anther 1-2mm long (Cooper, 1956). Clarkson and Clarkson (1994) found that although *Pittosporum obcordatum* has a ratio of 1:1 male to female plants, 6.66% of male plants also produce seed. Flowers of all *Pittosporum* are thought to be entomophilous due to the small flower size and absence of features that are adapted to pollination by birds (Webb et al. 1999). However Anderson (2003) suggests that the importance of birds as pollinators in some species may be underestimated, finding that *P. crassifolium* is likely to be pollinated by endemic honeyeaters such as the tui and bellbird which have declined in abundance on the mainland and may have been important pollinators in the past. Dispersal of *Pittosporum* including all New Zealand species is thought to be mainly by birds due to the resinous seeds, (Burrows 1994).

Figure 1.3. The androecium and gynoecium of a male *Pittosporum turneri* flower (left) and female (centre) and an apparently male flower (right) which was from an individual which also had capsules.

1.4.3 PITTOSPORUM TURNERI

Pittosporum turneri Petrie (Turner's kohuhu) is a rare plant of the Central North Island which has a divaricating juvenile form and an adult larger leaved form more closely resembling one of the larger leaved species of *Pittosporum* (Cooper 1956). *P. turneri* is known from nine main sites and has been sighted in several others ranging from the north-west Ruahine ranges to Pureora and has been reported from several sites which have not since been found, indicating that it may have once been more widespread. *P. turneri* typically grows as a small, emergent tree in shrublands, forest margins, and along stream banks. It grows in acidic soils of about pH 4.7, from 200m in altitude in Taumarunui to 1300m in the Ruahine ranges (Ecroyd 1994). Disturbance may be an important factor for colonisation as many populations consist of even aged stands indicating they may have colonised the site following disturbance and with few seedlings establishing under shade or with competition from other species. Several factors may contribute to the rarity of this species including browse of adult foliage from possums, predation of seedlings by hares, and the impacts of farming and logging (Ecroyd 1994) and land-use changes have undoubtedly affected the distribution of this species.

Figure 1.4. Flowers of *P. turneri* (left), adult foliage (centre) and adult plant with both adult and juvenile foliage (right).

1.4.4 GODLEY'S HYPOTHESIS

Godley (1985) proposed that *Pittosporum turneri* is a putative hybrid, suggesting this species may be a relic of a hybrid swarm, which could help to explain its disjunct distribution. The most morphologically similar divaricating species to the juvenile form of *P. turneri* are *P. divaricatum* and *P. crassicaule*, however *P. crassicaule* only occurs in the South Island (Hathaway 2001). Additionally it was noted by Laing (1935) that in Arthur's Pass there is a "sharp line of demarcation between *P. crassicaule* and *P. divaricatum*", indicating it is possible the two species ranges may not overlap. However it is also possible that either parent could be found in an entirely different range to historical times or could have gone extinct (Godley 1985). Therefore the putative parents should also share a similar habitat and share some morphological similarities. *P. divaricatum* is also found in at least two sites where *P. turneri* is found. *P. colensoi* is widespread in the Central North Island and has the greatest tolerance for colder conditions, being a more montane species than *P. tenuifolium*. Although *P. colensoi* has much larger, broader leaves than *P. turneri* Godley (1985) stated that for all putative hybrids the large leaved non-divaricating putative parent has larger leaves than the putative hybrid.

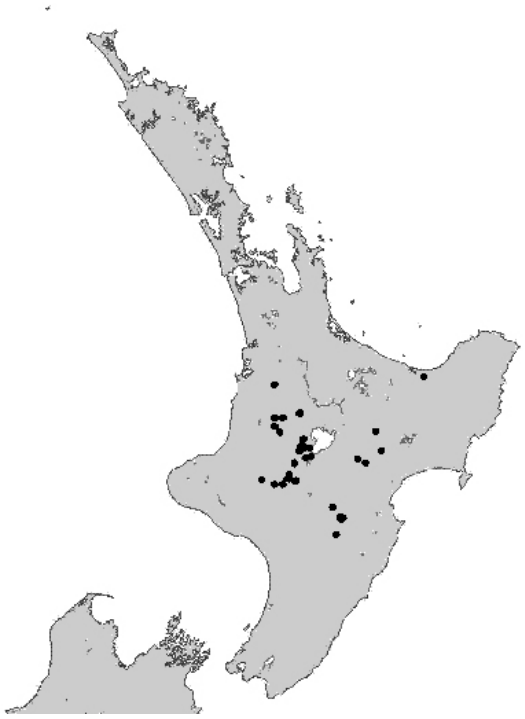


Figure 1.6. Records of sightings of *P. turneri* from the Department of Conservation BIOWEB database.

1.5 IDENTIFICATION OF HYBRIDISATION

1.5.1 ALLOZYMES

Allozymes can be defined as enzymes that have different molecular forms but identical or similar functions (Hunter & Markert 1957). Different molecular forms of isozymes are found both within individual organisms and within different types of tissue (Markert 1975). Differences in the electrophoretic mobility of allozymes reflect differences in the structural genes coding for polypeptides, therefore electroporetic differences are the direct result of genetic differences between enzymes (Crawford 1983). Allozyme studies are a valuable tool for resolving relationships between species because they directly reflect alterations in the gene sequence and exhibit simple mendelian inheritance, expressed as codominant alleles (Crawford 1990), therefore hybrids should have fixed heterozygous patterns reflecting the alleles of both parents (Weeden & Wendel 1990). Allozymes can be used in most cases to support or reject the hypothesis of hybridisation in plants (Crawford 1990), and have been used to show evidence of easily identifiable differentiation between putative parents and hybrid species, for example in European *Tilia* species (Fromm & Hattemer 2003). They have also

been used to study population diversity of *Pittosporum* in the Bonin Islands, Japan (Ito et al. 1997). Allozymes have been used as a tool in a number of studies to support evidence of hybridisation from phylogenetic data using chloroplast DNA (cpDNA) and the internal transcribed spacer of nuclear ribosomal DNA (ITS), and have been used to identify homoploid hybrids e.g. in *Helianthus* (Rieseberg et al. 1990) and *Iris* species (Cruzan & Arnold 1993).

*1.5.2 THE trn*T-*trn*L *REGION OF CHLOROPLAST DNA*

The chloroplast genome (cpDNA) is commonly used for inferring evolutionary processes in plants (Soltis 1999) and typically ranges in size from 135kb to 160kb in angiosperms and contains a ca. 25kb inverted repeat, dividing the rest of the genome into one small and one large single copy region (Olmstead & Palmer 1994). cpDNA is useful for phylogenetic studies due to its large size and large number of protein genes providing a large data base for sequencing and restriction site studies, its low silent substitution rate, and because structural rearrangements are relatively common, with many inversions and intron deletions in angiosperms providing phylogenetically informative data (Olmstead & Palmer 1994). The cpDNA region is maternally inherited in angiosperms and can infer a hybrid origin if a species appears to have inherited cpDNA from more than one maternal source (Clegg et al. 1993) due to chloroplast transfer from one species to another (Soltis 1999). The use of both a biparentally and uniparentally inherited marker is a commonly used method for resolving relationships between plant species and detecting reticulation events (Arnold 1997). This is because when using only biparentally inherited markers it is not possible to determine whether a species which contains common alleles or shows morphological intermediacy to its putative parents is ancestral to the putative parents or is derived by hybridisation (Rieseberg et al. 1990). Additionally phylogenetic reconstruction is a much more powerful method when combining multiple data sets using defined genetic markers (Avise 1994). The cpDNA genome is commonly used in studies which also employ a marker such as the ITS region.

The region between the *trn*T and *trn*F genes (Taberlet et al. 1991) is a non-coding region which has been shown to have a large number of phylogenetically informative characters The *trn*T – *trn*L region is the most variable region within this larger region, averaging around 752 base pairs and containing several large

indels (Shaw et al. 2005). The *trnT-trnL* region has been used in a number of studies to resolve angiosperm phylogenies (Borsche et al. 2003), (Fukuda et al. 2003).

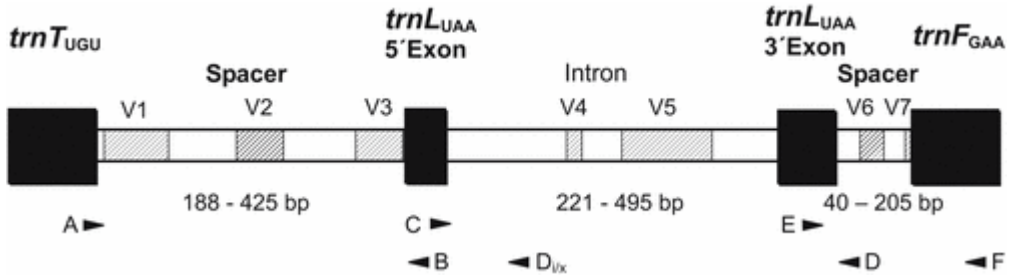


Figure 1.6. The *trn*T-*trn*F region of chloroplast DNA of bryophytes with forward and reverse primer sites applicable to all plants from (Quandt and Stech, 2004)

1.5.3 THE INTERNAL TRANSCRIBED SPACER OF NUCLEAR RIBOSOMAL DNA (ITS) REGION

Internal transcribed spacers (ITS) are located in the 18S – 26S nuclear ribosomal DNA (rDNA) region and consist of an external transcribed spacer (ETS), the 18S gene, an internal transcribed spacer named ITS1, the 5.8S gene, another internal transcribed spacer named ITS2 and the 26S gene. The ITS region is often used to obtain phylogenetic information due to the level of variation both within and among genera and can contain evidence of hybridisation when a species appears to have inherited repeat types from two parental species (Baldwin et al. 1995). Incongruence of relationships resolved by cpDNA and ITS markers is often indicative of hybridization. (Soltis & Kuzoff 1995).

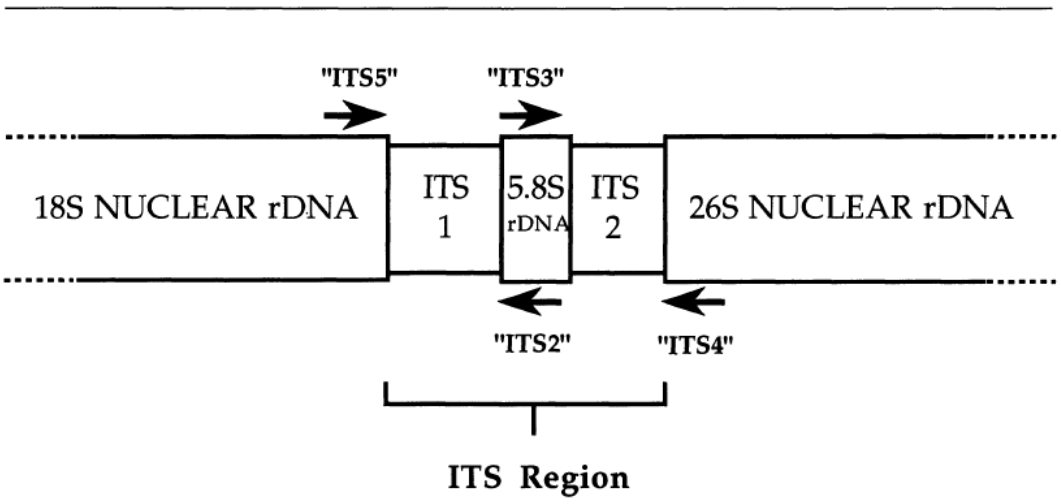


Figure 1.7. The ITS region and primer sites most commonly used to amplify this region. From (Baldwin et al. 1995)

1.5.4 INTER-SIMPLE SEQUENCE REPEATS (ISSR'S)

ISSR's are PCR-based markers used to amplify multiple DNA fragments between microsatellites (tandem repeats or SSR's) (McCauley & Ballard 2002). Microsatellites are short tandem repeat DNA sequences of around 2-6 base pairs of up to 10 bp long. These are distributed throughout the entire genome and are flanked by highly conserved sequences. (Rakoczy-Trojanowska & Bolibok 2004). ISSR's are regions between the closely spaced inverse, adjacent microsatellites and are typically 100 – 300bp. These are amplified using a single oligonucleotide primer (16-18bp), anchored on either the 5′- end or 3′- end of the microsatellite. The resulting PCR product reveals multiple genomic loci on each lane of a gel, providing a great deal of information (Zietkiewicz et al. 1994). The number of bands produced reflects the frequency of the SSR motif within the genome, which varies for each ISSR marker (Blair et al. 1999). ISSR's have been shown to be 'hypervariable', exhibiting more bands and more polymorphisms than other markers. For example they have been shown to be more variable than SSR's in ramie (Zhou et al. 2005), and AFLP's in rice (Blair et al. 1999). ISSR's have been recently used to identify hybridisation between closely related species e.g. among oak species (Conte et al. 2007) and *Coffea* species (Ruas et al. 2003). They are inherited in a dominant or codominant mendelian fashion so that if a species has bands of both the putative parents, this is indicative it may be a hybrid. Absence of a band is interpreted as primer divergence or loss of a locus through deletion of the SSR site or chromosomal rearrangement (Wolfe & Liston 1998). A study by Wolfe (1998) found that ISSR's were an effective way to identify hybrids, providing support for previously identified hybrids using other molecular techniques such as allozymes and restriction fragment analyses of chloroplast DNA.

1.5.5 MORPHOLOGY

Morphological studies are a valuable tool for use in combination with molecular techniques in identifying a hybrid origin and are often used in studies of hybridisation (Allendorf et al. 2001). Traditionally, measures of morphological intermediacy to the putative parents were used to resolve the parentage of a putative hybrid. Hybrids are usually intermediate to the parental species for a

large number of characters and character states, and inference is based on the number of characters which are intermediate. This is based on the assumption that alleles will combine additively, but can sometimes be untrue if there is dominance or epistasis. The genetic basis for inheritance of individual traits is unknown in a study of morphology, so care must be taken not to assume hybridisation rather than divergence, when similar patterns can occur for each (Wilson 1992), and not all variation in morphological characters has a genetic basis (Yüzbaşioğlu E 2008).

There are many considerations which need to be addressed in a morphological study, including the number of characters to study and which character states to measure or score. Those that are highly correlated may provide an unrealistic account of intermediacy if there are pleitropic or functional correlations involved (Wilson 1992). However, a morphological study is important to show that putative parents and hybrids can be distinguished on a morphological basis.

1.6 OBJECTIVES

It is hypothesized that *Pittosporum turneri* has a homoploid hybrid origin due to (i) its change in ontogeny resembling 'morphological intermediacy' between a divaricating shrub and arborescent tree (ii) its occurrence in a zone where there is overlap between other *Pittosporum* species which can be considered putative parents based on morphology (iii) its disjunct distribution and its occurrence in an extreme environment relative to other species of *Pittosporum*. This study addresses this theory in *Pittosporum turneri* by considering putative parents and also attempts to identify reticulation and resolve relationships among New Zealand *Pittosporum*.

This hypothesis is addressed using cpDNA and ITS sequencing as well as allozymes and ISSR's. A cross-pollination experiment was also conducted to determine whether hybridisation is possible in the wild between *P. colensoi* and *P. divaricatum* in the same area and to characterise the morphological attributes of any seedlings produced.

Specifically the questions addressed are:

1. Is there molecular evidence that hybridisation has occurred between *Pittosporum colensoi* and *P. divaricatum*?
2. Is there morphological evidence that hybridisation has occurred between these species?
3. How does a seedling of *P. divaricatum* x *P. colensoi* compare to a *P. turneri* seedling?
4. Where are reticulation events likely to have occurred in *Pittosporum*?

CHAPTER TWO

PHYLOGENETICS OF THE NEW ZEALAND *PITTOSPORUM*

2.1 INTRODUCTION

The sequencing of chloroplast and nuclear ribosomal genes is the most commonly used method of inferring plant phylogenies (Shaw et al. 2005). By combining these independent data sets with different phylogenetic histories it is possible to improve the resolution of relationships among species. Combining a chloroplast and nuclear data marker can be used to identify hybridisation events where topological incongruence occurs between trees (Howarth & Baum 2005), as chloroplasts are maternally inherited in angiosperms, whereas nuclear genes are biparentally inherited (Kim & Donoghue 2008). This study uses phylogenetic analysis of the *trn*T-*trn*L region of chloroplast DNA and the nuclear ribosomal internal transcribed spacer (ITS) region to investigate whether *Pittosporum turneri* has a hybrid origin, identify any other putative hybrids, and improve our knowledge of relationships among closely related New Zealand *Pittosporum.*

2.2 MATERIALS AND METHODS

2.2.1 THE trnT-trnL REGION OF CHLOROPLAST DNA

2.2.1.1 *Plant collection and DNA isolation*

Twenty-five taxa from different localities throughout New Zealand were sampled, including one taxa from Norfolk Island (*P. bracteolatum*), a taxon nested within the New Zealand clade according to ITS sequences (Hathaway, 2001). This includes all New Zealand *Pittosporum* except for *P. ellipticum* subsp. *serpintinum* and *P. crassicaule*, which may be a subspecies of *P. rigidum* (Wilson & Galloway 1993), and a number of other varieties which are not considered species or have doubtful status (Cooper 1956). A large proportion of the DNA samples used were already available as they had been used for a previous study of the ITS region by Hathaway (2001), and had been collected from wild populations and botanic gardents throughout New Zealand (Table 2.1). DNA samples used for sequencing

were either from the same individual or population as those used in the earlier study where possible, however some DNA samples of Hathaway's (2001) were degraded and new DNA extractions were necessary. Genomic DNA was extracted, using either the PureLink Plant total DNA purification kit (Invitrogen Inc) or a modified version of the CTAB method (Appendix 1). Samples were collected from the same population or area as the earlier study if possible, or from the Auckland Botanical Gardens or Oratia Native Plant Nursery in Auckland, where provenance information was obtained where possible. Additionally a putative hybrid produced in cultivation between *P. crassifolium* and *P. obcordatum* was sequenced along with one of its progeny to investigate patterns of inheritance and whether the parental species of the putative hybrid could be identified by phylogenetic incongruence (Figure 2.2). All specimens used in the study and their provenance information are listed in Table 2.1.

Table 2.1 Taxa used in this study for sequencing of the *trn*T-*trn*L and ITS regions. Individuals marked with ♦ = only used in the ITS study (Hathaway, 2001) ♠ = only used in the *trnT-trnL* study.* = additional samples used for ITS and *trnT-trnL* sequences in this study. N.I: North Island, S.I: South Island.

Species	Source	Locality	Collection number
New Zealand			
P. anomalum♦	wild	Castle Basin, S.I	DG7505
P. anomalum♠	Oratia Native Plant Nursery	Cultivation, N.I.	SKC004
P. cornifolium	Otari Native Botanic Garden	Waikanae, N.I	OBG9200494
P. crassifolium♦	wild	Kauaeranga, N.I.	CEC316
P. crassifolium♠	wild	Mokau, N.I.	SKC005
P. crassifolium x P. obcordatum F_1	cultivated	Hamilton, N.I.	SKC007
P. crassifolium x P. obcordatum F_2	cultivated	Hamilton, N.I.	SKC008
P. aff crassifolium	Auckland Regional Botanical Garden	Raoul Island, Kermedec Islands	ABG942199
P. dallii	Landcare Research, Lincoln	Cobb dam, S.I	LC16439
P. divaricatum 1	wild	Ripia Valley, N.I.	SKC006
*P. divaricatum 2**	wild	Arthurs Pass, S.I	CECG229
P. ellipticum subsp. *ellipticum*	Auckland Regional Botanical Garden	Waitakere ranges, N.I.	ABG941495
P. eugenoides	Cultivated	Christchurch, S.I.	CECG301
P. fairchildii	Oratia Native Plant Nursery	Three Kings Islands	SKC003
P. huttonianum	wild	West coast, N.I.	Bcsn5
P. kirkii♦	Otari Native Botanical Garden	Great Barrier Island	OBG9100137
P. kirkii♠	Auckland Regional Botanical Garden	Cultivation, N.I.	ABG20030274
P. obcordatum	wild	Waikura Valley, N.I.	CECG293
P. patulum	Landcare Research, Lincoln	Lee Creek, Wairau river, S.I.	LC16564
P. pimeleoides subsp. pimeleoides	Landcare Research, Lincoln	Timaru Botanic gardens	LC15/90A
P. pimeleoides subsp. majus	Landcare Research, Lincoln	North Cape, N.I.	LC11564
P. ralphii	wild	Rangataiki, N.I.	CECG291
P. rigidum	wild	West Coast, N.I.	BCsn4

P. tenuifolium subsp colensoi	Wild00	Pureora Forest Park, N.I.	SKC001
P. tenuifolium subsp tenuifolium	wild	Waipunga, N.I.	CECG290
P. turneri 1	wild	Ripia Valley, N.I	CECG287
*P. turneri 2**	wild	Bog Pine Reserve, Pureora N.I	SKC008
P. umbellatum♦	Auckland Regional Botanic Garden	Great Barrier Island	ABG940216
P. umbellatum♠	Oratia Native Plant Nursery	North Cape, N.I	SKC007
P. virgatum	Otari Native Botanical Garden	Peketi, N.I.	OBG8800096
'P. Stephens Island"♠	Oratia Native Plant Nursery	Stephens Island, S.I.	SKC002
Norfolk Island			
'P. bracteolatum	Auckland Regional Botanical Garden	Norfolk Island National Park	ABG980883

2.2.1.2 PCR amplification and sequencing

The *trn*T-*trn*L Taberlet region was amplified using the forward primer Tab 'a' and Tab 'b' (Taberlet et al. 1991). PCR was performed in 25 µL volumes, with 1X PCR buffer, 200 µmol/L each dNTP, 3.0 mmol/L $MgCl_2$, 0.1 µmol/L each primer, 1.25 units of *Taq* and 2 µL of unquantified DNA, diluted to 1:10. 1:100, or 1:1000 dilution to prevent co-precipitation of secondary compounds with the DNA, which may inhibit amplification. Reactions included a positive control (sample which had previously amplified) and a negative control (containing an equivalent measure of distilled water instead of DNA) to check for contamination of the mastermix. The PCR was performed using an Eppendorf Mastercycler gradient thermal cycler with the following parameters: An initial denaturation of one cycle at 96 ˚C for 5 minutes; followed by 35 cycles of denaturation at 96 ˚C for 1 minute, annealing at 53 °C for 2 minutes, and extension at 72 °C for 2 minutes; followed by a final extension at 72˚C for 5 minutes to complete polymerisation. The lid of the thermal cycler was heated to 105 °C to prevent evaporation of the reactions.

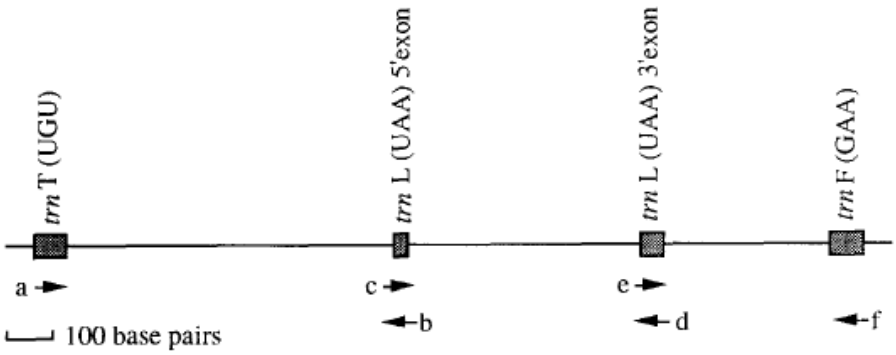


Figure 2.1. The *trn*T-*trn*F region and primer sites with primers 'a' to 'f'. From Taberlet (1991)

After the PCR reaction was completed, PCR products were electrophoresed using a 1% agarose gel with 1XTBE buffer stained with ethidium bromide (0.1 mg/L). Samples were loaded into the gel with loading buffer (0.0083% bromophenol blue, 2.5% ficol (MW 400 000) and 5mM EDTA (disodium salt). Gels were run for approximately 30 minutes at 55 volts. Bands were then visualized and photographed under UV light using an Alpha-imager (Alpha Innotech Corporation). A 100 base pair ladder (Invitrogen Inc) was used to determine the size of the PCR product. Products

that produced a clear band of around 700 base pairs were purified using EXO-SAP (Invitrogen Inc), and electrophoresed again and visualised using the Alpha-imager to ensure quality of the purified product before sending to sequencing. Samples that did not amplify were tried again with a different dilution of DNA. Sequencing was performed at the University of Waikato sequencing unit with Big Dye chemistry on an ABI automated sequencer.

2.2.2 ITS REGION

Sequencing of the ITS1 and ITS2 region for four DNA samples was undertaken using the higher plant primer ITS5HP (5'-GGAAGGAGAAGTCGTAACAAGG-3') (Laboratory of molecular systematic, Smithsonian Institution (LMS), and the universal eukaryote primer ITS4 (White et al. 1990). These sequences were aligned with unpublished sequences of New Zealand *Pittosporum* by Hathaway (2001) and Chrissen Gemmill of the University of Waikato. This included the F_1 and F_2 putative hybrid between *P. obcordatum* and *P. crassifolium* (see figure 2.2) and a second *P. divaricatum* and *P. turneri* specimen from different regions to the original sequenced specimens to determine whether there is sequence variation within these species. PCR was performed in 25 µL volumes with 1XPCR buffer, 5 mmol/L $MgCl_2$, 200 µmol/L each dNTP, 0.2 µL 1% BSA, 0.2 µmol/L each primer, 1 unit of *Taq* and 2 µL of DNA (diluted 1:10 or 1:100). PCR was performed using an Eppendorf Mastercycler with the following parameters: An initial denaturation of 94 °C for 5 minutes, 35 cycles of denaturation period of 94°c for 1 minute, a primer annealing period of 50°c for 1 minute, an extension of 72°c for 1 minute) followed by a final extension of 72°c for 1 minute. PCR products were then checked for quality and purified as for the *trnT-trnL* region. Sequencing was performed using both primers ITSHP5 and ITS4 as well as the internal primers ITS2 and ITS3 (White et al. 1990) when necessary to improve the quality of the consensus sequences, as ITS4 generally provided poor sequencing results.

Figure 2.2. Hybrids of putative hybrids included with both cpDNA and ITS sequencing F_1 *P. crassifolium* x *P. obcordatum* F_1 (left) and F_2 (right).

2.2.3 SEQUENCE ALIGNMENT

Sequences were edited and aligned manually using Sequencher 4.8 software (Gene Codes, Inc). All individuals that differed in nucleotide sequence were included in phylogenetic analyses. Sequences were truncated so that all started and ended in the same position (Appendix 2 and 3).

2.2.4 SEQUENCE CHARACTERISTICS

The uncorrected pairwise distance between all taxa was calculated using PAUP* version 4.0b10 (Swofford 2003). The g_1 statistic was calculated using 10000 random trees to determine the level of phylogenetic signal in the data (Hillis & Huelsenback 1992).

2.2.5 PHYLOGENETIC ANALYSIS

Phylogenetic trees were constructed using maximum parsimony and maximum likelihood for both the ITS data and cpDNA data using PAUP*. For maximum parsimony, searches were performed using a heuristic search method with characters treated as unordered and of equal weight, with gaps treated as missing, with accelerated transformation (ACCTRAN) character state transformation, tree-bisection-reconnection (TBR) branch swapping, and simple, random addition. Bootstrap tests (Felsenstein 1985) were carried out to evaluate support for each node using 100 replicates with the same heuristic search settings. A model search was conducted using PAUP* to determine the best model of evolution for maximum likelihood using MODELTEST 3.7 (Posida & Crandall 1998), testing 56 models of evolution before implementing the best model in PAUP*. Heuristic search methods

were used for maximum likelihood with TBR branch swapping, (MULPARS and ACCTRAN optimization). Bootstrap tests were performed using 100 replicates with sub-tree pruning, regrafting (SPR) branch swapping. For the combined ITS and *trnT-trnL* regions MODELTEST was also used to search for the best model of evolution, which selected a different model from that of either data sets (Table 2.6.). All trees were rooted using the outgroup *P. cornifolium*, *P. pimeleoides* subsp. *pimeleoides* and *P. pimeleoides* subsp. *majus*, a separate lineage from all other New Zealand *Pittosporum* which form a monophyletic group most closely related to the New Caledonian species of *Pittosporum* (Chandler et al. 2007). Additionally, relationships based on ITS sequences were depicted using neighbour-net, implemented in Splits-tree 4.0 (Huson 1998). This method was used to visualize all conflicting signal in the data, using all characters to connect nodes and create a network rather than a tree, to illustrate relationships which are strongly conflicting, and to identify where hybridization events may have occurred.

2.2.6 INCONGRUENCE TESTS

Incongruence was assessed using two approaches. (1) An incongruence length difference (ILD) test (Farris et al. 1994) was conducted using the partition homogeneity test in PAUP* to determine whether the two data sets were comparable, using simple taxon addition, TBR branch swapping, and a heuristic search of 1000 repartitions. (2) Paired-site tests were implemented in PAUP* to assess the level of conflict between each tree produced by maximum parsimony and maximum likelihood analysis of the two data sets. The Templeton (Wilcox signed-rank) test (Templeton 1983) was used to determine whether the topologies of the strict consensus tree from the most parsimonious trees produced from each data set were incongruent. The Kishino and Hasegawa (Kishino & Hasegawa 1989) test was used to test whether the topologies of the two maximum likelihood trees were significantly incongruent. The null distribution for this test was generated by nonparametric bootstrapping using the RELL method (Kishino & Hasegawa 1989). This was done for both maximum likelihood trees of the cpDNA and ITS data, and four constrained "test" trees that had nodes constrained that were found in the *trn*T-*trn*L trees and not in the ITS tree.

2.3 RESULTS

2.3.1 SEQUENCE CHARACTERISTICS OF THE trnT-trnL REGION

Sequences were obtained for all taxa, however only a poor sequence was obtained for the subspecies *P. aff crassifolium* from the Kermedec Islands. *P. aff crassifolium* produced a poor sequence and was therefore not included in the analysis. The entire *trn*T-*trn*L region was 796 characters long and was truncated to an alignment of 708 characters in length, including gaps to account for insertions and deletions (indels). Most taxa were uniform in sequence length with 689 bases. *P. kirkii* has the longest sequence with 703 bases, *P. bracteolatum* has the shortest sequence (688 bp) and *P. rigidum* and *P. pimeleoides* subsp. *majus* have an extra base pair (690bp) (see Table 2.2). All other sequences differed in length by a maximum of one base pair. Sequences were AT rich, with a GC content of 25.8%, ranging from 25.02% to 26.02%. A chi-squared test of homogeneity of base frequencies across taxa using PAUP* showed that there is no significant heterogeneity of base frequencies between taxa (p= 1).

2.3.2 SEQUENCE VARIATION OF THE trnT-trnL REGION

A pairwise identity of 97% was found for all taxa, with 688 identical sites. Of the 20 variable characters, 16 were parsimony informative (synapomorphies found in two or more taxa). Sequence divergence ranged from 0.00 to 1.7%, with an average of 0.6%. For those taxa which were sampled from two locations (*P. turneri* and *P. divaricatum*), sequences were identical between the two populations, therefore only one sequence was used in further analyses. Some taxa had identical sequences: *P. turneri* has the same sequences as *P. divaricatum*, both F_1 and F_2 *P. obcordatum* x *P. crassifolium,* have identical sequences to *P. obcordatum, P. colensoi, P. tenuifolium* and *P. "Stephens Island*" have identical sequences, and .*P. bracteolatum* and *P. umbellatum* shared identical sequences.

Table 2.2. Percentage of nucleotides which were a G or C in the *trn*T-*trn*L region and total number of bases for each taxon.

Taxon	GC content	Total no. bases
1. *P. anomalum*	25.98%	689
2. *P. cornifolium*	25.98%	689
3. *P. crassifolium*	25.98%	689
4. *P. dallii*	25.83%	689
5. *P. divaricatum*	25.97%	689
6. *P. ellipticum*	25.83%	689
7. *P. eugenoides*	25.68%	689
8. *P. fairchildii*	25.83%	689
9. *P. huttonianum*	25.98%	689
10. *P. kirkii*	25.20%	703
11. *P. obcordatum*	25.83%	689
12. *P. obcordatum x P.*	25.83%	689
13. *P. obcordatum x P. crassifolium* F_2	25.83%	689
14. *P. patulum*	25.98%	689
15. *P. ralphii*	25.83%	689
16. *P. rigidum*	25.94%	690
17. *P. pimeleoides* subsp. *majus*	25.79%	690
18. *P. pimeleoides* subsp *pimeleoides*	24.83%	689
19. *P. "Stephens Island"*	25.83%	689
20. *P. colensoi*	25.83%	689
21. *P. tenuifolium*	25.83%	689
22. *P. turneri 1*	25.97%	689
23. *P. umbellatum*	25.83%	689
24. *P. virgatum*	26.02%	689
25. ***P. bracteolatum***	25.66%	688
Mean	**25.84%**	**689**

2.3.3. INDELS

A total of 17 gaps were required to align the data matrix. There were seven indels throughout the whole *trn*T-*trn*L region, including two insertions found in only *P. kirkii*, one four bp long and another 9bp long. The rest of the insertions were of a single base pair, and none of the informative indels were used in phylogenetic analyses as they were found only in the designated outgroup (*P. cornifolium*, *P. pimeleoides* subsp. *pimeleoides* and *P. pimeleoides* subsp. *majus*) or found in the outgroup and *P. rigidum* only.

Table 2.3 Indels needed to align the *tnr*T-*trn*L matrix. Informative indels are highlighted in grey.

Size	Position	Characteristics
4	110	Insertion in *P. kirkii* ATAC
9	116	Insertion n *P. kirkii* TATTATTTT
1	126	Insertion in *P. rigidum*, *P. cornifolium*, both *P. pimeleoides* subspecies T
1	129	Insertion in *P. rigidum* C
1	137	Insertion in *P. cornifolium*, both *P. pimeleoides* subspecies C
1	494	Insertion in *P. pimeleoides* subsp. *majus* A
1	736	Gap in *P. bracteolatum* A

2.3.4 SEQUENCE CHARACTERISTICS AND VARIATION OF THE ITS REGION

The ITS data contains 569 characters and a pairwise identity of 95.8%. 440 characters are constant (77.3%), and 62 variable and parsimony-informative. The GC content is 60%, differing slightly from the results of Hathaway (2001) due to the addition of extra sequences. Hathaway (2001) discusses the length and GC content of the ITS region for the New Zealand *Pittosporum* in length. Pairwise sequence divergence averages 4.2%, 7 times greater than the *trn*T-*trn*L region. 26 indels were needed to align the ITS region, a much greater number of indels than the *trn*L-*trn*T region. Most of these are 1 base in length and seven are phylogenetically informative. The two *P. divaricatum* DNA samples from different regions have the same sequence, however the two *P. turneri* samples differed by one base pair. The *P. crassifolium* x *P. obcordatum* F_1 and F_2 individuals have a different sequence, with 18 differences in nucleotide bases, and the F_2 hybrid contains a number of gaps (Table 2.4).

Table 2.4. Indels needed to align the ITS data matrix. Informative indels are highlighted in grey.

Size	Position	Characteristics
5	1	Gap in *P. crassifolium* x *P. obcordatum* F_2
1	32	Insertion in *P. bracteolatum* T
1	36	Gap in *P. bracteolatum* C
1	66	Gap in *P. ralphii*
4	67	Gap in *P. fairchildii*
1	74	Gap in *P. fairchildii*
6	77	Gap in *P. fairchildii*
1	85	Gap in *P. fairchildii*
1	115	Insertion in *P. colensoi, P. crassifolium, P. crassifolium x P. obcordatum* F_1, *P. huttonianmum, P. ralphii* C
1	115	Insertion in *P. crassifolium* x *P. obcordatum* F_2 G
1	120	Gap in *P. crassifolium* x *P. obcordatum* F_2
1	125	Gap in *P. crassifolium* x *P. obcordatum* F_2
1	181	Gap in *P. crassifolium* x *P. obcordatum* F_2
1	185	Insertion in *P. crassifolium* x *P. obcordatum* F_2 T
1	188	Insertion in *P. colensoi, P. crassifolium, P. crassifolium* x *P. obcordatum* F_1, *P. crassifolium* x *P. obcordatum* F_2, *P. ellipticum, P. ralphii, P. tenuifolium* and *P. huttonianum*. T
1	369	Insertion in *P. eugenoides* and *P. umbellatum* G
1	369	Insertion in *P. umbellatum* A
1	373	Gap in *P. umbellatum*
1	375	Gap in *P. bracteolatum*
1	381	Insertion in *P. dallii, P. umbellatum, P. fairchildii, P. virgatum* and *P. eugenoides* C
1	382	Insertion in *P. dallii* and *P. eugenoides* C
1	388	Gap in *P. umbellatum*
1	389	Gap in *P. ellipticum*
1	417	Insertion in *P. bracteolatum* and *P. eugenoides, P. cornifolium,* both *P. pmeleoides* subspecies A
1	422	Gap in *P. dallii* and *P. kirkii, P. cornifolium* ,both *P.pimeleoides* subspecies
1	424	Insertion in *P. eugenoides* G

2.3.5 G_1 STATISTIC

A G1 statistic was calculated using 10000 random trees, with a statistic of -1.6589 for the *trn*L-*trn*F region. This compares to a G_1 statistic of -2.59 for the ITS region and a combined statistic of -2.837 for the two regions combined. These results indicate that there is sufficient signal in both data sets to make phylogenetic inferences as they deviate significantly from zero ($p<0.01$) (Hillis & Huelsenback 1992).

2.3.6 ILD TEST

An ILD test was performed using both data sets with all taxa including the *P. crassifolium* x *P. obcordatum* F_1 and F_2 hybrids. The test produced an insignificant result of $p=0.29$, indicating that there is no major conflict between data sets. Therefore sequences of the *trn*T-*trn*L region and the ITS region were combined for phylogenetic analyses.

Table 2.5. Uncorrected pairwise distances for the *trn*T-*trn*L region of cpDNA

		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1.	*P. anomalum*												
2.	*P. cornifolium*	.0131											
3.	*P. crassifolium*	.0029	.0131										
4.	*P. dallii*	.0043	.0116	.0014									
5.	*P. divaricatum*	.0058	.0160	.0058	.0072								
6.	*P. ellipticum*	.0014	.0116	.0014	.0029	.0043							
7.	*P. eugenoides*	.0101	.0175	.0101	.0087	.0072	.0087						
8.	*P. fairchildii*	.0014	.0116	.0014	.0029	.0043	.0000	.0087					
9.	*P. huttonianum*	.0029	.0131	.0000	.0014	.0058	.0014	.0101	.0014				
10.	*P. kirkii*	.0043	.0145	.0072	.0058	.0101	.0058	.0116	.0058	.0072			
11.	*P. obcordatum*	.0014	.0116	.0014	.0029	.0043	.0000	.0087	.0000	.0014	.0058		
12.	*P. ob x P. crass* F_1	.0014	.0011	.0014	.0029	.0043	.0000	.0087	.0000	.0014	.0058	.0000	
13.	*P. ob x P. crass* F_2	.0014	.0116	.0014	.0029	.0043	.0000	.0087	.0000	.0014	.0058	.0000	.0000
14.	*P. patulum*	.0000	.0131	.0029	.0043	.0058	.0014	.0101	.0014	.0029	.0043	.0014	.0014
15.	*P. ralphii*	.0029	.0131	.0029	.0043	.0058	.0014	.0101	.0014	.0029	.0072	.0014	.0014
16.	*P. rigidum*	.0058	.0145	.0058	.0072	.0000	.0043	.0073	.0043	.0058	.0102	.0043	.0043
17.	*P. pimeleoides* subsp. *majus*	.0116	.0145	.0116	.0102	.0145	.0102	.0160	.0102	.0116	.0131	.0102	.0102
18.	*P. pimeleoides* subsp. *pimeleoides*	.0116	.0014	.0116	.0102	.0145	.0102	.0160	.0102	.0116	.0131	.0102	.0102
19.	*P. "Stephens Island"*	.0058	.0160	.0058	.0072	.0087	.0043	.0043	.0043	.0058	.0101	.0043	.0043
20.	*P. tenuifolium* subsp. *colensoi*	.0058	.0160	.0058	.0072	.0087	.0043	.0043	.0043	.0058	.0101	.0043	.0043
21.	*P. tenuifolium*	.0058	.0160	.0058	.0072	.0087	.0043	.0043	.0043	.0058	.0101	.0043	.0043
22.	*P. turneri 1*	.0058	.0160	.0058	.0072	.0000	.0043	.0072	.0043	.0058	.0101	.0043	.0043
23.	*P. umbellatum*	.0043	.0117	.0043	.0029	.0072	.0029	.0087	.0029	.0043	.0058	.0029	.0029
24.	*P. virgatum*	.0000	.0131	.0029	.0043	.0014	.0014	.0072	.0014	.0029	.0043	.0014	.0014
25.	*P. bracteolatum*	.0043	.0117	.0043	.0029	.0072	.0029	.0087	.0029	.0043	.0058	.0029	.0029

	13	14	15	16	17	18	19	20	21	22	23	24
14. *P. patulum*	.0014											
15. *P. ralphii*	.0014	.0029										
16. *P. rigidum*	.0043	.0058	.0058									
17. *P. pimeleoides* subsp. *majus*	.0102	.0116	.0116	.0131								
18. *P. pimeleoides* subsp. *pimeleoides*	.0102	.0116	.0116	.0131	.0000							
19. *P. "Stephens Island"*	.0043	.0058	.0058	.0087	.0145	.0146						
20. *P. colensoi*	.0043	.0058	.0058	.0087	.0145	.0146	.0000					
21. *P. tenuifolium*	.0043	.0058	.0058	.0087	.0145	.0146	.0000	.0000				
22. *P. turneri*	.0043	.0058	.0058	.0000	.0145	.0145	.0087	.0087	.0087			
23. *P. umbellatum*	.0029	.0043	.0043	.0072	.0102	.0102	.0072	.0072	.0072	.0072		
24. *P. virgatum*	.0014	.0000	.0029	.0014	.0116	.0116	.0058	.0058	.0058	.0014	.0043	
25. *P. bracteolatum*	.0029	.0043	.0043	.0073	.0102	.0102	.0072	.0072	.0072	.0072	.0000	.0043

Table 2.6. Uncorrected pairwise distances for the ITS region

	1	2	3	4	5	6	7	8	9	10	11	12
1. *P. anomalum*												
2. *P. cornifolium*	.0804											
3. *P. crassifolium*	.0089	.082										
4. *P. dallii*	.0143	.084	.023									
5. *P. divaricatum*	.0053	.0769	.014	0.017								
6. *P. ellipticum*	.0072	.0788	.008	0.021	0.012							
7. *P. eugenoides*	.0355	.091	.037	0.030	0.033	0.039						
8. *P. fairchildii*	.0073	.084	.016	0.016	0.012	0.014	0.036					
9. *P. huttonianum*	.0089	.082	.007	0.023	0.014	0.008	0.037	0.016				
10. *P. kirkii*	.0268	.085	.030	0.019	0.030	0.030	0.040	0.025	0.030			
11. *P. obcordatum*	.0125	.082	.014	0.016	0.017	0.019	0.040	0.016	0.014	0.023		
12. *P. ob x P. crass* F_1	.0161	.089	.007	0.030	0.021	0.012	0.044	0.023	0.007	0.037	0.037	
13. *P. ob x P. crass* F_2	.0291	.092	.030	0.036	0.034	0.034	0.056	0.031	0.030	0.041	0.034	0.034
14. *P. patulum*	0.007	0.084	0.016	0.021	0.012	0.014	0.043	0.014	0.016	0.033	0.033	0.023
15. *P. ralphii*	0.012	0.086	0.003	0.023	0.017	0.012	0.037	0.016	0.003	0.030	0.030	0.010
16. *P. rigidum*	0.000	0.080	0.008	0.014	0.005	0.007	0.035	0.007	0.008	0.026	0.026	0.016
17. *P. pimeleoides* subsp. *majus*	0.080	0.000	0.082	0.084	0.076	0.078	0.091	0.084	0.082	0.085	0.085	0.089

18. P. pimeleoides subsp. *pimeleoides*	0.080	0.000	0.082	0.084	0.076	0.078	0.091	0.084	0.082	0.085	0.085	0.089
19. P. colensoi	0.008	0.082	0.000	0.023	0.014	0.008	0.037	0.016	0.000	0.030	0.030	0.007
20. P. tenuifolium	0.008	0.082	0.000	0.023	0.014	0.008	0.037	0.016	0.000	0.030	0.030	0.007
21. P. turneri 1	0.001	0.080	0.008	0.016	0.005	0.009	0.035	0.009	0.010	0.028	0.028	0.017
22. P. turneri 2	0.016	0.082	0.010	0.014	0.007	0.007	0.037	0.007	0.010	0.026	0.026	0.016
23. P. umbellatum	0.003	0.084	0.012	0.017	0.008	0.010	0.039	0.011	0.012	0.030	0.030	0.019
24. P. virgatum	0.005	0.084	0.014	0.019	0.010	0.012	0.037	0.012	0.014	0.032	0.032	0.021
25. P. bracteolatum	0.043	0.096	0.052	0.038	0.044	0.050	0.035	0.044	0.052	0.050	0.050	0.059

	13	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**
14. P. patulum	0.036											
15. P. ralphii	0.034	0.019										
16. P. rigidum	0.029	0.007	0.012									
17. P. pimeleoides subsp. *majus*	0.092	0.084	0.086	0.080								
18. P. pimeleoides subsp. *pimeleoides*	0.092	0.084	0.086	0.080	0.000							
19. P. colensoi	0.030	0.016	0.003	0.008	0.082	0.082						
20. P. tenuifolium	0.030	0.016	0.003	0.008	0.082	0.082	0.000					
21. P. turneri 1	0.030	0.008	0.014	0.001	0.082	0.082	0.010	0.010				
22. P. turneri 2	0.029	0.007	0.012	0.000	0.080	0.080	0.008	0.008	0.001			
23. P. umbellatum	0.032	0.010	0.016	0.003	0.084	0.084	0.012	0.012	0.005	0.003		
24. P. virgatum	0.034	0.012	0.017	0.005	0.084	0.084	0.014	0.014	0.007	0.005	0.008	
25. P. bracteolatum	0.063	0.050	0.055	0.043	0.096	0.096	0.052	0.052	0.045	0.043	0.047	0.045

2.3.7 MAXIMUM PARSIMONY ANALYSIS

Analysis of the *trn*T-*trn*L region failed to find a single most parsimonious tree, retaining 130 trees of 31 steps. Three clades were recovered with moderate to strong support in the strict consensus tree (Figure 2.3) (1) *P. turneri* forms a clade with *P. divaricatum* and *P. rigidum* with 76% bootstrap support. (2) *P. bracteolatum* and *P. umbellatum* form a clade with 66% bootstrap support, and (3) *P. colensoi*, *P. tenuifolium, P. "Stephens Island*" and *P. eugenoides* form a clade with 66% bootstrap support. Additionally a clade of *P. crassifolium, P. huttonianum* and *P. dallii* and a clade of *P. anomalum, P. patulum, P. virgatum* and *P. kirkii* is found in the 50% majority-rule consensus tree only, *however* both clades have below 50% bootstrap support.

Maximum parsimony analysis of the ITS data produced 20,000 most parsimonious trees. In both 50% majority-rule (Fig. 2.4) and strict consensus (Fig. 2.6) trees none of the clades resolved by the *trn*T-*trn*L region are recovered in the ITS region. Three clades were recovered with moderate to strong support in the strict consensus trees. (1) A clade of *P. eugenoides* and *P. bracteolatum* with 93% bootstrap support, (2) a clade of *P. tenuifolium*, *P. ralphii, P. colensoi*, *P. huttonianum*, *P. crassifolium* and the F_1 hybrid between *P. crassifolium* and *P. obcordatum* as a sister taxon with 71% bootstrap support, and (3) *P. ellipticum* as a sister taxon to this group with 59% bootstrap support. A clade of *P. obcordatum* and *P. crassifolium* x *P. obcordatum* F_2 hybrid was also recovered in the strict consensus tree despite having low bootstrap support.

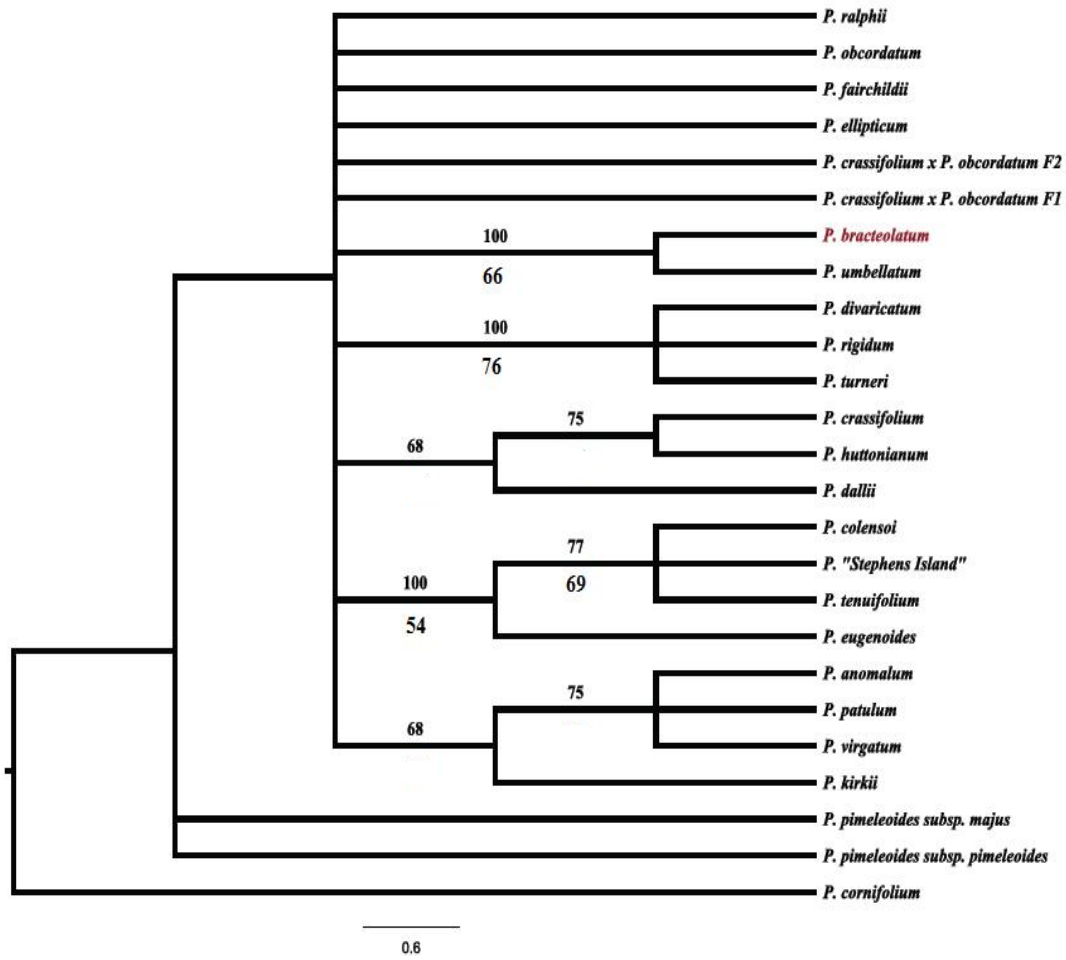


Figure 2.3. 50% majority- rule tree of 130 most parsimonious trees of *trn*T-*trn*L sequences. CI= 0.800, HI= 0.200, RI = 0.881, RC = 0.7048. Numbers above the branches indicate the percentage each clade is represented in the most parsimonious trees. Numbers below the branches indicate bootstrap values over 50%.

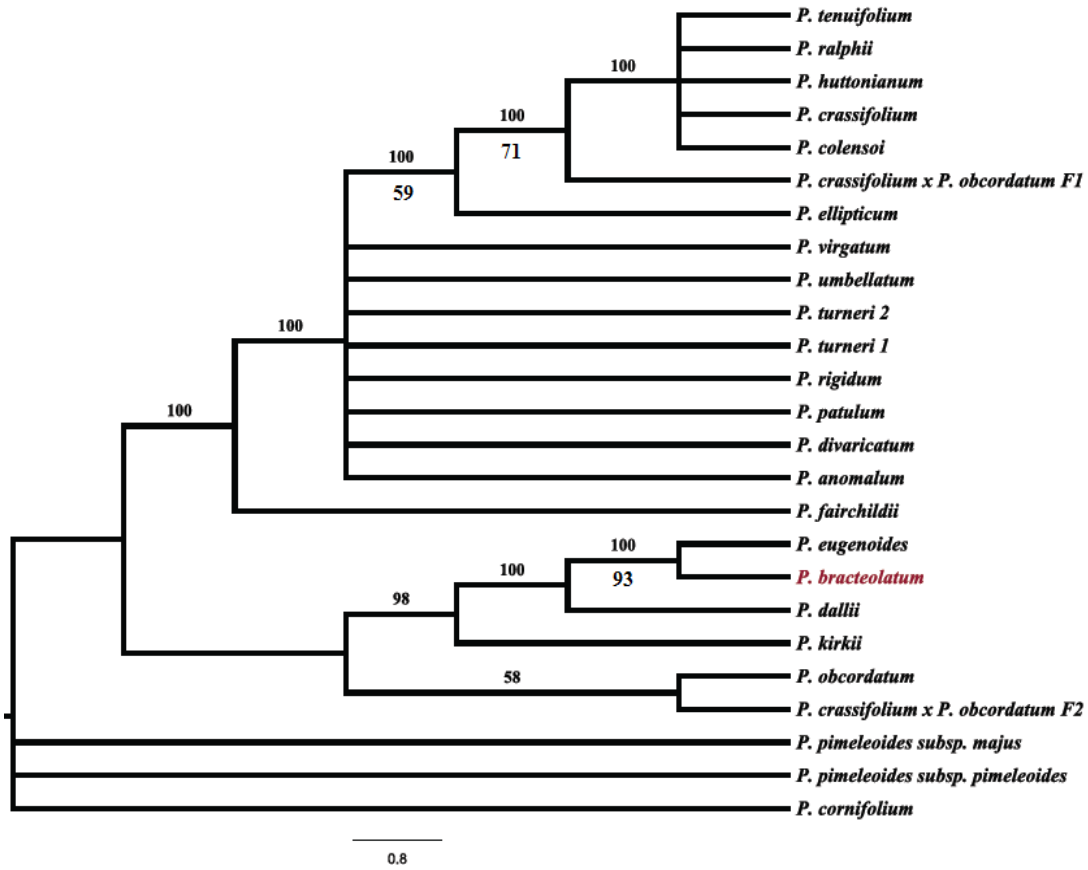


Figure 2.4. 50% majority rule of 20000 most parsimonious trees of ITS sequences. CI = 0.7941, HI = 0.2059, RI = 0.8409, RC = 0.8409. Values above branches represent percentage of times clades found in the most parsimonious trees, values below the branches represent bootstrap values over 50%.

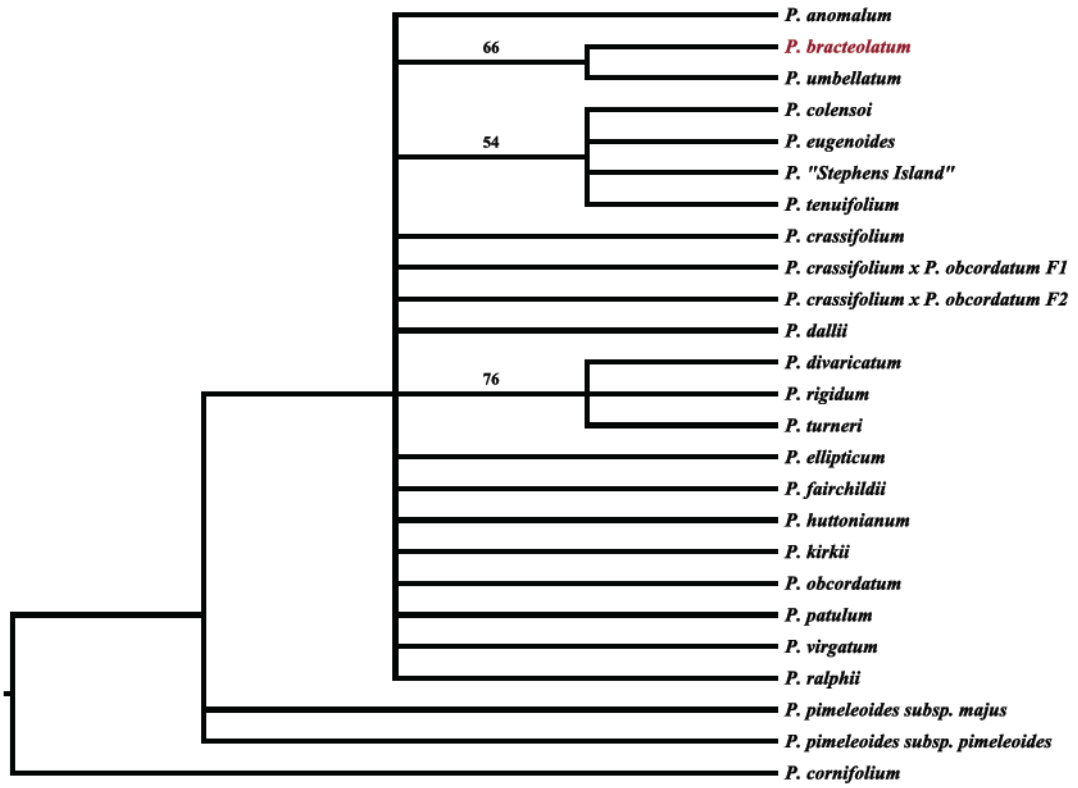


Figure 2.5. Strict consensus of the *trn*T-*trn*L region. Values above branches indicate bootstrap values over 50%. CI= 0.800, HI= 0.200, RI = 0.881, RC = 0.7048.

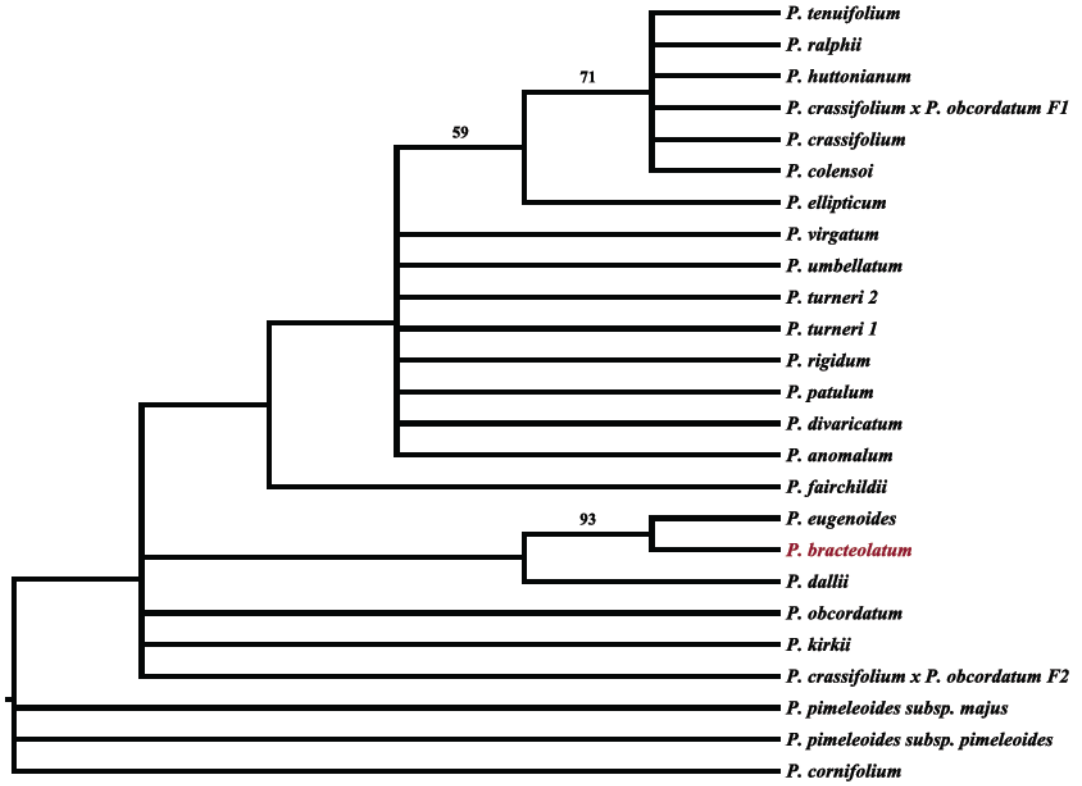


Figure 2.6. Strict consensus of the ITS region. Values above branches represent bootstrap values over 50%. CI = 0.7941, HI = 0.2059, RI = 0.8409, RC = 0.8409.

2.3.8 TEMPLETON TEST OF INCONGRUENCE

The results of the Templeton signed-rank test showed that the topologies of the cpDNA and ITS strict consensus tree did not differ significantly (p= 0.07). Therefore the two data sets were combined to provide a more resolved tree. Maximum parsimony analysis produced 396 most parsimonious trees of 168 steps. This increased the bootstrap values of two clades, *P. obcordatum* and *P. crassifolium* x *P. obcordatum* $F_{2,}$ and *P. tenuifolium* and *P. colensoi,* but reduced the bootstrap support found in other clades, showing that there is still some degree of conflict between data sets. Overall, combining the two data sets produced more resolved trees with more clades (Fig. 2.7 and Fig 2.8).

Table 2.7. Templeton signed-rank (1983) test of strict consensus of most parsimonious trees. N: sum of steps gained in the ITS tree compared to the *trn*T-*trn*L tree. P: *P* value with 95% confidence.

Data set	Tree length	N	*P*
ITS	46		
trnT-trnL	31	8	0.0703

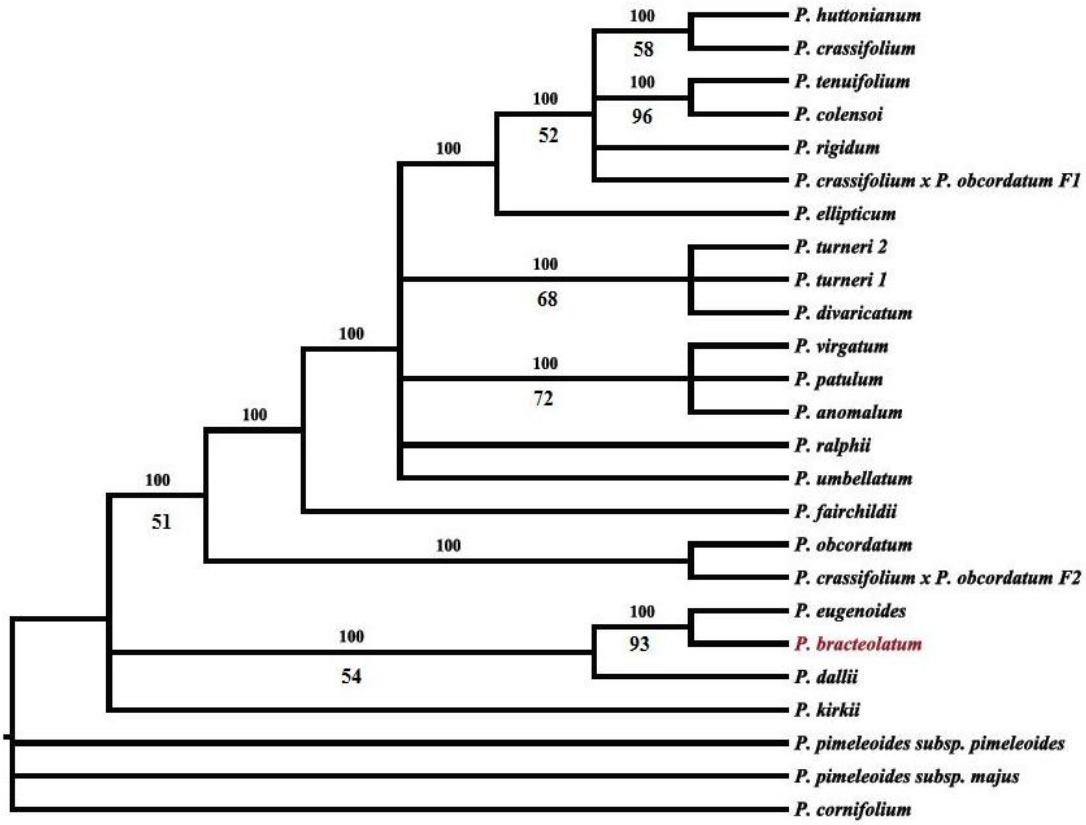


Figure 2.7 Maximum parsimony 50% majority rule of the 396 most parsimonious trees of combined *trn*T-*trn*L and ITS region CI = 0.7671, HI = 0.2329, RI = 0.8381, RC = 0.6429. Values above branches indicate percentage of times these clades are recovered in the 396 most parsimonious trees. Values below branches indicate bootstrap support values over 50%.

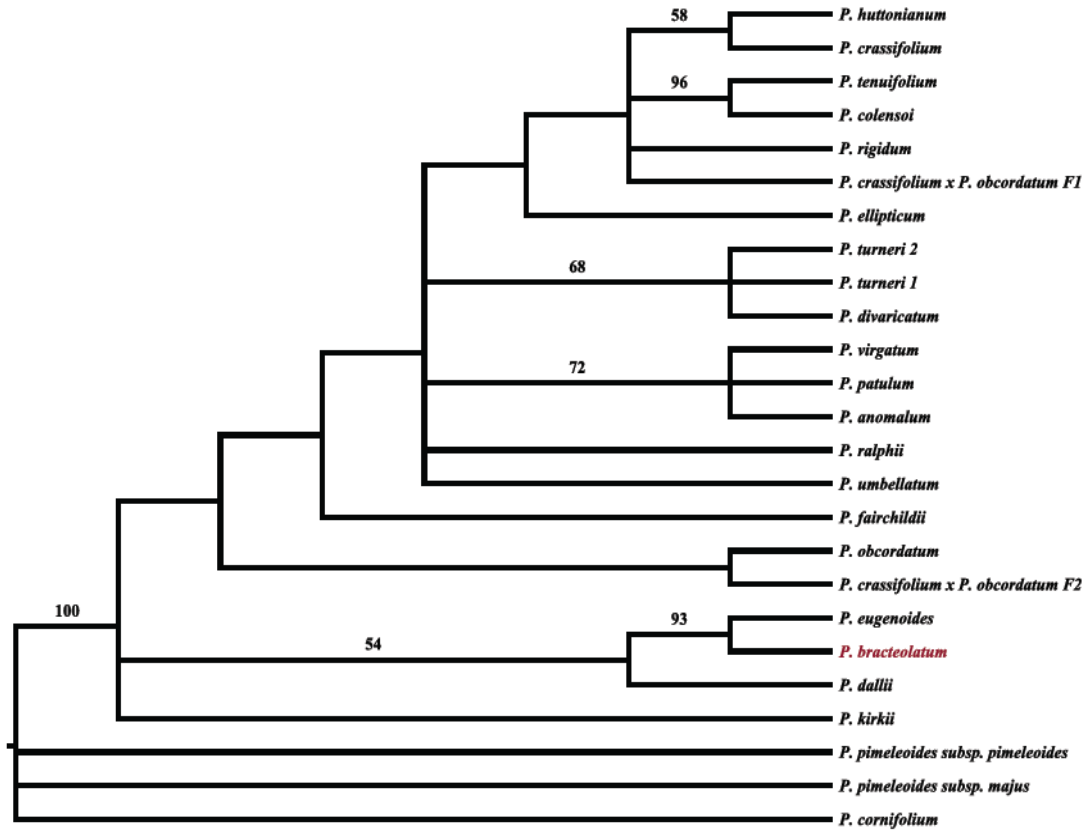


Figure 2.8. Strict consensus of parsimony analysis of combined *trn*T-*trn*L and ITS regions. Values above branches indicate bootstrap support values over 50%. CI = 0.7671 HI= 0.2329, RI= 0.8381, RC= 0.6429

2.3.9 MAXIMUM LIKELIHOOD ANALYSIS

MODELTEST found that the K81uf+I+G model is the most suitable for the *trn*T-*trn*L region, with base frequencies of: A= 0.38722, C=0.1216, G=0.1325, T= 0.3588, and a substitution rate matrix of (A-C: 1.000000, A-G: 0.0392, A-T: 0.2407, C-G: 0.2407, C-T: 0.0392, G-T:1.0000), with the proportion of variable sites = 0.9046, and a gamma distribution of 0.8159. The best model for the ITS region is the TrN+G model, with base frequencies of A=0.2150, C=0.2979, G=0.2858, T=0.2013 and a substitution rate matrix of (A-C: 1.000000, A-G: 2.651200, A-T: 1.000000, C-G: 1.0000, C-T: 8.0417, G-T: 1.0000), proportion of invariable sites = 0 and a gamma distribution of 0.2367. The best model of evolution combining both data sets is the TrN+I+G model, with base frequencies of A=0.3075, C=0.2025, G=0.2062, T=0.2838 and a substitution rate matrix of (A-C: 1.000000, A-G: 1.4401100, A-T: 1.000000, C-G: 1.0000, C-T: 4.3996, G-T: 1.0000), with the proportion of invariable sites = 0.6541, and gamma distribution of 0.7514.

Table 2.8. Model scores of the evolutionary models used for maximum likelihood analyses of the *trn*T-*trn*L region, ITS region and combined regions

Region	Model	Model score (-lnL)
trnT-trnL	K81+uf+I+G	1068.4465
ITS	TrN+G	1637.7467
trnT-trnL and ITS	TrN+I+G	2797.7783

Maximum likelihood analysis of then *trn*T-*trn*L region produced similar results to that of maximum parsimony. Five clades were recovered: 1) *P. bracteolatum* and *P. umbellatum* with 68% bootstrap support, 2) *P. divaricatum*, *P. rigidum* and *P. turneri*, with 58% bootstrap support, 3) *P. crassifolium*, *P. huttonianum* and *P. dallii* form a clade but with low bootstrap support, 4) *P. tenuifolium*, *P. colensoi*, *P. "Stephens Island*" and *P. eugenoides* form a clade with 58% bootstrap support. 5) *P. anomalum*, *P. patulum*, *P. virgatum* and *P. kirkii* form a clade with 51% bootstrap support.

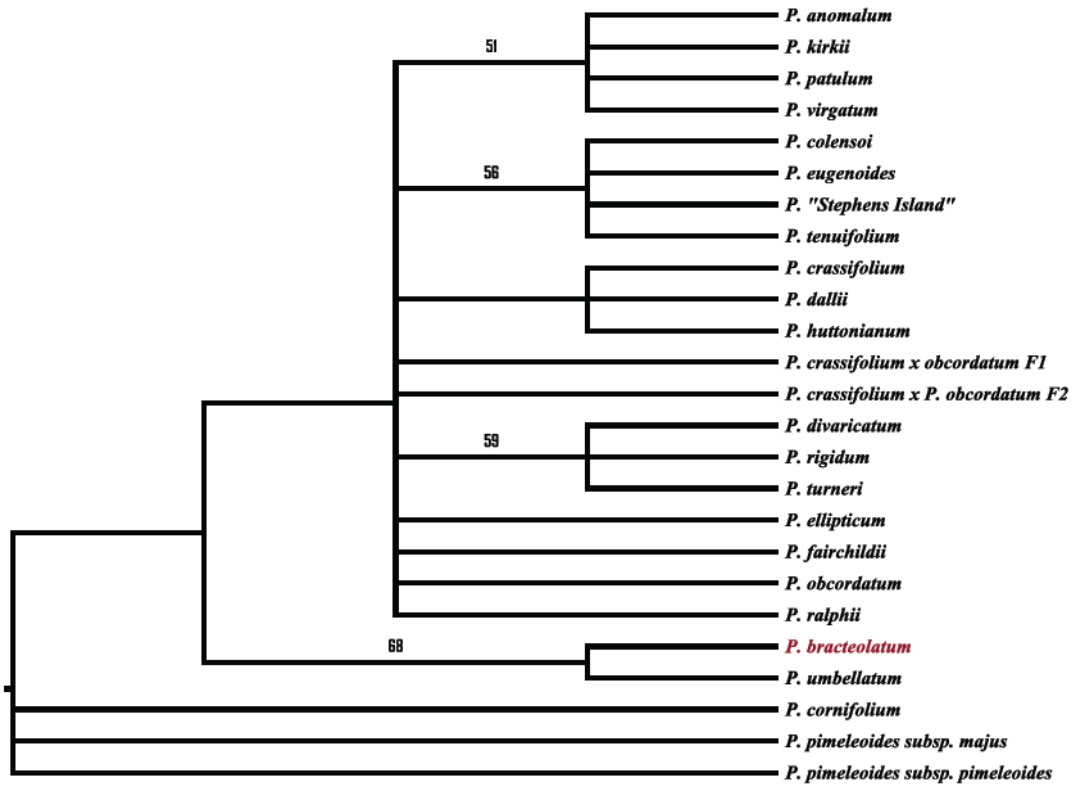


Figure 2.9. Maximum likelihood analysis of the *trn*T-*trn*L data. Numbers indicate bootstrap values from 100 bootstrap replicates. Log likelihood = 1062.50251.

Maximum likelihood analysis of the ITS region revealed three clades: 1) *P. bracteolatum* and *P. eugenoides* with 95% bootstrap support, with *P. dallii* as a sister taxon (63% bootstrap support), and *P. kirkii* as a sister taxon to these three taxa (58% bootstrap support), 2) *P. obcordatum* and *P. crassifolium* x *P. obcordatum* F_2 hybrid form a clade with 52% bootstrap support 3) *P. tenuifolium*, *P. ralphii*, *P. huttonianum*, *P. crassifolium*, *P. crassifolium* x *P. obcordatum* F_1 hybrid and *P. colensoi* form a clade with 58% bootstrap support, with *P. ellipticum* as a sister taxon to this group but with low bootstrap support.

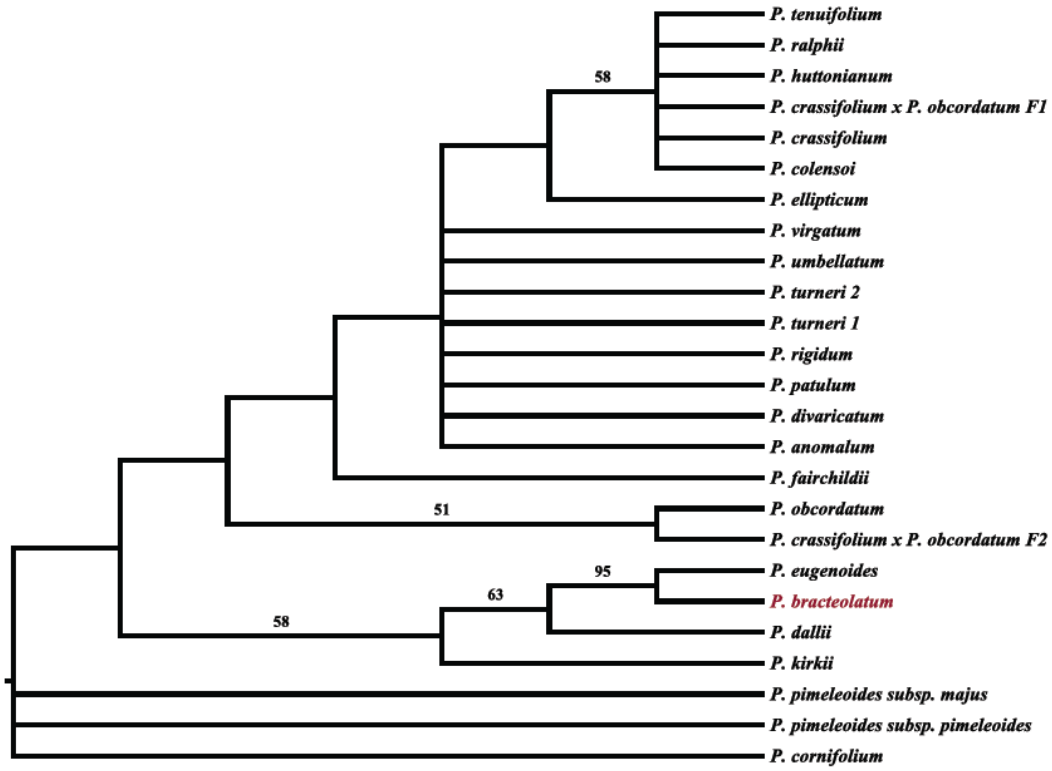


Figure 2.10. Maximum likelihood analysis of the ITS data. Numbers indicate bootstrap values for 100 bootstrap replicates. Log likelihood = 1142.30568

2.3.10 KISHINO-HASEGAWA TEST OF INCONGRUENCE

Maximum likelihood analysis showed a significant result ($p<0.05$) for the Kishino-Hasegawa test, indicating that there is significant topological disagreement between the data sets. Four ITS test trees were produced with constrained nodes where clades were moderately to well supported by the more resolved *trn*T-*trn*L data set, but not supported by the ITS data set. These constrained test trees contained clades of 1) *P. anomalum*, *P. patulum*, *P. kirkii* and *P. virgatum*, 2) *P. colensoi*, *P. eugenoides*,

P. tenuifolium, 3) *P. crassifolium*, *P. dallii* and *P. huttonianum*, 4) *P. bracteolatum* and P. *umbellatum*. Only the first test tree produced an insignificant result, therefore the taxa contributing to the incongruence are considered to be *P. eugenoides, P. dallii* and *P. umbellatum* as these taxa are those incongruent between trees produced by the two data sets.

Table 2.9. Kishino-hasegawa (1989) test scores

Tree	**-lnL**	**Difference in -lnL**	***P***
trnT-trnL	1062.50251		
ITS	1142.30568	79.80317	0.001*
Test tree			
1	1545.47618	18.12999	0.092
2	1557.18789	29.84170	0.015*
3	1560.25138	32.90520	0.000*
4	1554.78667	27.44049	0.005*

The two data sets were also combined to produce a single tree based on maximum likelihood and to determine whether this increased the support for some clades and reduced support for others. This tree produced two resolved clades with more support than either region alone: 1) *P. colensoi* and *P. tenuifolium*, a clade with 90% bootstrap support, 2) *P. anomalum*, *P. patulum* and *P. virgatum* form a clade with 63% bootstrap support. There is also a greater level of support for a split between the main group of closely related New Zealand taxa and *P. fairchildii* (53% bootstrap support). Other relationships are clearly conflicting between data sets as bootstrap support diminishes for most clades. However, overall, the tree reflects the general support for two main clades found in the ITS phylogeny, indicating that the signal from the ITS set overwhelms that of the *trn*T-*trn*L data set, and the *trn*T-*trn*L region provides more resolution between the very closely related taxa, creating overall a more resolved tree. (1) *P. bracteolatum* forms a clade with *P. eugenoides* with 86% bootstrap support, with (2) *P. dallii* as a sister taxa with 51% bootstrap support and (3) *P. kirkii* as a sister taxa to this group with 52% bootstrap support. (4) *P. crassifolium* x *P. obcordatum* F_2 is placed with *P. obcordatum* in a clade with 53% bootstrap support. (5) *P. turneri* and *P. divaricatum* form a clade but with low bootstrap support. (6) *P. anomalum*, *P. patulum* and *P. virgatum* form a clade with 63% bootstrap support. (7) *P. colensoi* and *P. tenuifolium* form a clade, with 90% bootstrap support. (8) *P.*

crassifolium and *P. huttonianum* form a clade with 65% bootstrap support. (9) *P. anomalum, P. patulum, P. virgatum, P. colensoi, P. tenuifolium, P, crassifolium, P. huttonianum, P. crassifolium x P. obcordatum F1, P. rigidum, P. ellipticum, P. divaricatum, P. turneri, P. umbellatum* and *P. ralphii* form a clade with 53% bootstrap support.

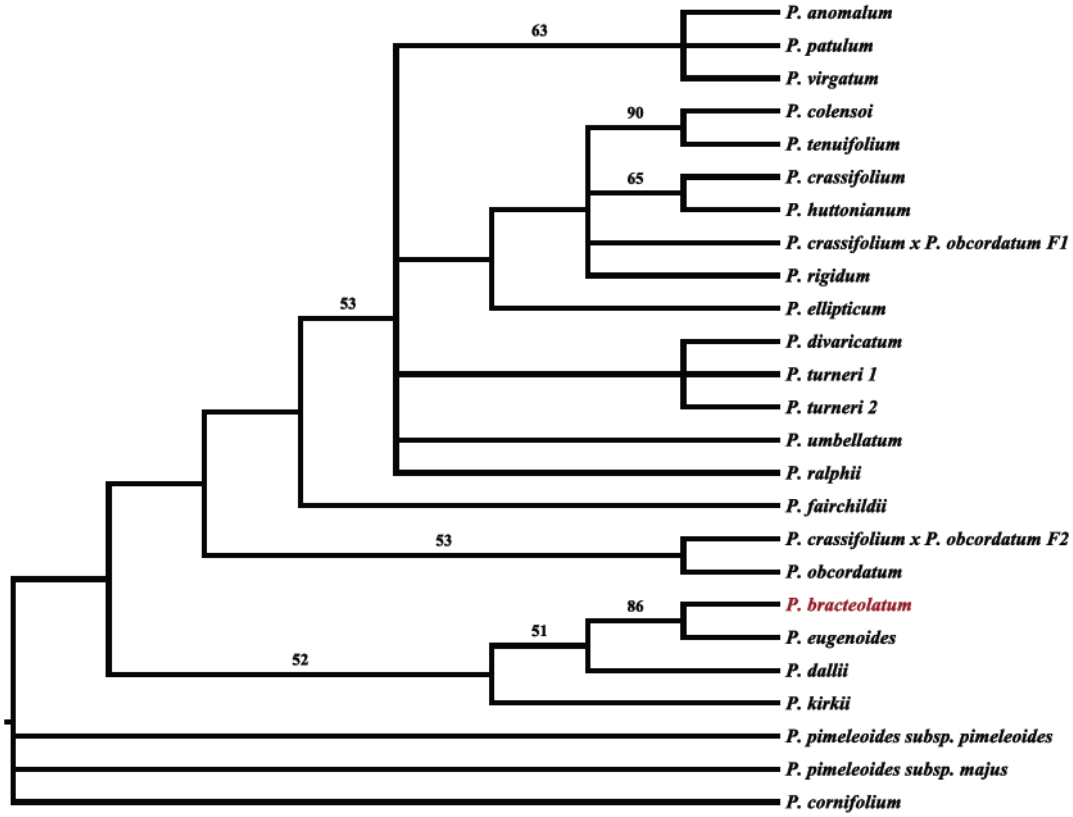


Figure 2.11. Maximum Likelihood tree of combined ITS and *trn*T-*trn*L regions. Log likelihood = 2811.20745. Numbers represent bootstrap values for 100 bootstrap replicates.

2.3.11 CONFLICT IN THE PLACEMENT OF TAXA

The *trn*T-*trn*L region suggests different placements of *P. eugenoides, P. dallii, P.* and *P. umbellatum* to the ITS analyses for both maximum parsimony and maximum likelihood. The putative hybrid *P. turneri*, however, shows no conflict between data sets, and forms a clade with *P. divaricatum* in the *trn*T-*trn*L region but is unresolved in the strict consensus maximum parsimony tree and maximum likelihood of the ITS data. The two *P. crassifolium* x *P. obcordatum* F_1 and F_2 hybrids have different ITS sequences and are placed in different positions in the ITS trees but have identical chloroplast sequences to that of *P. obcordatum*, however their placement is only

resolved in the maximum likelihood analysis of the ITS region. *P. rigidum* forms a well supported clade with *P. turneri* and *P. divaricatum* in maximum parsimony and maximum likelihood trees of the *trn*T-*trn*L data but appears more closely related to *P. colensoi* and *P. tenuifolium* in the combined analysis of ITS and *trn*T-*trn*L data, but this relationship has low bootstrap support. *P. kirkii* is also placed in different clades between data sets, however ITS trees constraining the node containing this taxon in the *trn*T-*trn*L tree does not cause significant incongruence between trees.

2.3.12 NEIGHBOUR-NET ANALYSIS

Analysis using neighbour-net of the ITS data set indicates that there is low conflict for the highly resolved taxa, but graphically represents the conflict which occurs within the data set. In particular, the taxa that appear to have the most conflict are *P. turneri* and *P. rigidum* as all other taxa have split from the network to some degree, with *P. bracteolatum* and *P. eugenoides* showing the longest splits. The F_1 and F_2 *P. crassifolium* x *P. obcordatum* hybrids also show a long split from the rest of the network. Other taxa involved in conflict of the data set are *P. divaricatum*, *P. virgatum*, *P. umbellatum*, *P. fairchildii*, and *P. patulum*.

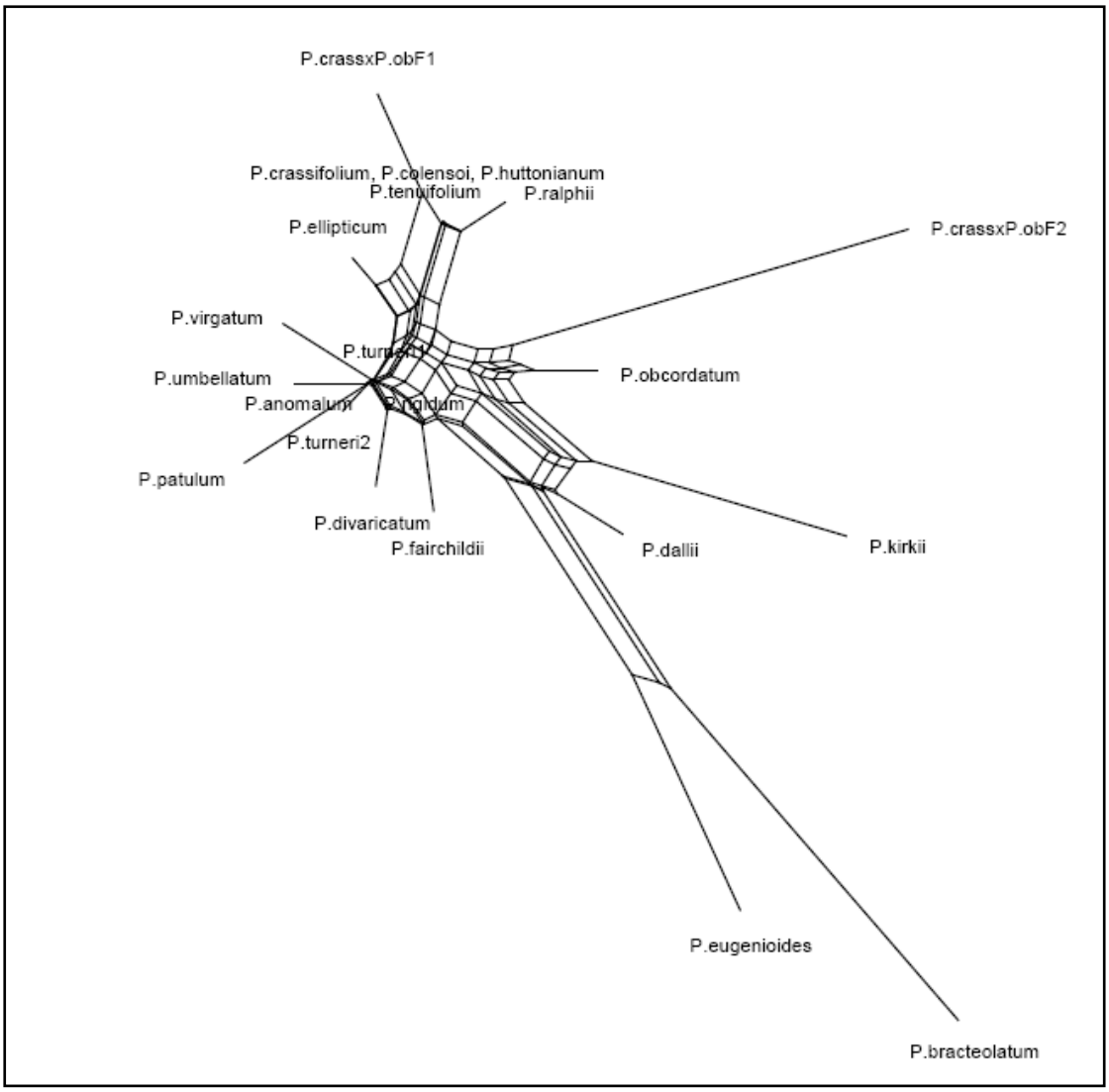


Figure 2.12 Neighbour-network analysis of uncorrected pairwise distances of ITS sequences, excluding the outgroup (*P. pimeleoides* taxa and *P. cornifolium*).

2.4 DISCUSSION

2.4.1 TAXA REPRESENTATION

Only one individual was included in this analysis for most taxa. This may be adequate for elucidating relationships using the *trn*T-*trn*L region due to the slow rate of evolution of this marker, as *P. turneri* or *P. divaricatum* both had identical sequences between the two sampled populations, however, *P. turneri* had different ITS sequences for the two samples from different locations. Nuclear genes have an effective population size four times that of organellar genes for dioecious species, therefore relationships inferred by nuclear markers may partially reflect stochastic sorting processes, creating "noise" in the data and causing erroneous reconstructions

(Linder & Rieseberg 2004). In future studies more than one population should be sampled for other closely related taxa, especially from those taxa which exhibit a wide range of morphological variation. This may also increase the likelihood of detecting hybridisation and introgression, which may have occurred in some populations and not others.

*2.4.2 SEQUENCE VARIATION AND UTILITY OF THE trn*T-*trn*L *AND ITS REGION*

The *trn*T-*trn*L region amplified in total was 794 bp long. This is close to the average size range of this region of 752bp as described by (Shaw et al. 2005). The variation observed between sequences was mostly due to point mutations with few indels, and alignment was achieved easily, but sequence variation was very low. The *trn*T-*trn*L region exhibited a high AT content characteristic of the chloroplast genome (Shaw et al. 2005). The GC content did not differ significantly between taxa indicating that there is unlikely to be any compositional bias introduced, as taxa which share a greater GC content can be linked erroneously (Wendel & Doyle 1998). The G_1 statistic was significantly different from 0 ($p<0.01$) (Hillis & Huelsenback 1992), with a value of -0.659 indicating that the data is highly skewed and contains enough phylogenetic signal to provide meaningful interpretation of relationships. However there are few phylogenetically informative sites compared to the ITS region, indicating that the ITS region is likely to depict relationships more accurately than the *trn*T-*trn*L region alone. This is also because the chloroplast genome can be exchanged easily between taxa, which can lead to erroneous phylogenetic reconstructions when it is used as the only source of phylogenetic information (Comes & Abbott 1999).

2.4.3 MAXIMUM PARSIMONY AND MAXIMUM LIKELIHOOD

Maximum parsimony produces a tree which requires the fewest number of character state changes, assuming an equal rate of substitution changes between nucleotides and across sites (Yang 1996), whereas maximum likelihood uses an explicit evolutionary model, and produces the tree most likely to be the 'true' tree given the chosen model of evolution (Lewis 1998). According to recent studies, maximum likelihood is more likely to represent the species tree when the appropriate model is selected (Hall 2005), as maximum parsimony is essentially a subset of maximum likelihood where random

variables are estimated, leading to a greater potential for statistical error (Goldman 1990). This study found very similar reconstructions between the maximum parsimony 50% majority rule tree and the maximum likelihood tree for both the *trn*T-*trn*L region and the ITS region, therefore these relationships are supported by both the model of evolution used for maximum likelihood and by maximum parsimony. However, some relationships differ between maximum parsimony and maximum likelihood. In the strict consensus tree of the most parsimonious trees for the *trn*T-*trn*L region, *P. bracteolatum* and *P. umbellatum* do not form a clade separate to the main New Zealand clade as they do in maximum likelihood. Also, in the strict consensus tree of the most parsimonious trees of the ITS region, *P. kirkii* does not form a clade with *P. eugenoides*, *P. bracteolatum* and *P. dallii*. However, when combining the two data sets there is very little difference between the strict consensus of the most parsimonious trees and the maximum likelihood tree.

2.4.4 *TESTS OF INCONGRUENCE*

Incongruence between trees based on different markers does not always reflect differences in the evolutionary history of the taxa in question. Other causes may include statistical error, such as when clades are weakly supported and there is not enough 'signal' in the data or when the gene used has a high rate of evolution relative to the level of taxon divergence. Incongruence can also be caused by technical difficulties such as sequencing error (Wendel & Doyle 1998). Therefore, the level of statistical support for each clade is very important in determining whether phylogenetic incongruence reflects evolutionary processes such as hybridisation. The ILD test assesses the significance of any incongruence between data sets against the null hypothesis of homogeneity in the distribution of phylogenetic information (Johnson & D.E 1998), assessing whether the conflict between datasets is significantly greater than the conflict within each data set (Planet & Sarkar 2005). This is done by comparing the difference in tree lengths between the most parsimonious trees of each data set. The difference is the sum of the total incongruence (the number of homoplasius steps required to explain the shortest trees) in each data set when the data sets are combined minus the number of homoplasius steps required to explain the shortest tree within each data set. This result can range from 0-1. When the result is zero, the shortest trees recovered are identical. When it equals 1, there is no homoplasy and the two topologies are distinct (Johnson & Soltis

1998). This test showed that the ITS and *trn*T-*trn*L data sets are compatible because the level of homoplasy within each data set indicates that the same tree topology could be reached with either data set. Therefore the best representation of relationships is likely to be when both data sets are combined to reduce the level of homoplasy. However, this is only considered a starting point, as congruence is only accepted if there are a large number of sites, specific tree topology conditions such as asymmetry, and a high mutation rate and trees are not incongruent if they have different branch lengths (Darlu & Lecointre 2002).

The Templeton (Wilcox signed-rank) two-tailed test (significantly less parsimonious test of Templeton) compares the number of steps required for each character for the separate topologies using maximum parsimony (Cunningham 1997), testing the null hypothesis that characters which differ in numbers of steps are equally likely to favour both topologies (Lee & Hugall 2003). The result is insignificant when the increase in steps required by some characters on the alternate topology (the most parsimonious topology based on the other data set) is not significantly different to the decrease in number of steps required by other characters (Johnson & D.E 1998). The Kishino-Hasegawa test is used to compare topological incongruence between trees based on maximum likelihood. This test used the ln-likelihood score of each tree, which is made up of the ln-likelihood for all sites, and computes the variance of the sum of all ln-likelihoods for each tree. It uses the variance to determine whether the difference in ln-likelihood between the two trees is statistically significant (Lewis 1998). Either of these tests can be used to compare topologies using constraint trees (trees that have been altered to include nodes with conflicting relationships between trees).

Although the strict consensus trees were not statistically significantly different between ITS and cpDNA data sets based on the Templeton-signed rank test this may be because, for the ITS data there were 20,000 most parsimonious trees, indicating many combinations were equally parsimonious. However, using maximum likelihood the scores of the trees were significantly different. This is likely to be because the model selected corrects for problems that lead to erroneous reconstructions, e.g. correcting for multiple hits that can otherwise lead to long-branch attraction (where long branches are erroneously linked because they have more sites in common)

(Bergston 2005). Therefore, the trees produced are superior to any other reconstruction under the particular model of evolution.

2.4.5 TOPOLOGICAL INCONGRUENCE

The significant result of the Kishino-hasegawa test ($P<0.05$), indicates that there are some clades which cause conflict in topology between trees using maximum likelihood. The taxa that were placed in different clades between data sets and were significantly incongruent were *P. eugenoides, P. dallii, and P. umbellatum*. The placement of *P. eugenoides* and *P. dallii* within the main group in the *trn*T-*trn*L tree, relative to the position in the other main New Zealand clade the ITS tree, could indicate that these taxa have been involved in introgression at some stage in their evolutionary history, as morphologically distinct taxa can contain the same chloroplast (chloroplast capture), (Rieseberg 1991). However, the *P. eugenoides* sample was from a cultivated specimen in Christchurch, indicating that this introgression event may have occurred in cultivation. The *P. dallii* individual was sampled from Landcare Research in Christchurch and was a wild specimen. However *P. dallii* is a rare species that only occurs in the South Island and the two taxa it is placed with based on the *trnT-trnL* region, *P. crassifolium* and *P. huttonianum*, are restricted to the North Island. This indicates that the introgression event may have been ancient, when these species had different geographical ranges to the present. In the combined analysis *P. dallii* is placed with *P. eugenoides* and *P. bracteolatum*. This seems to be a more likely relationship as these species all share the same white/yellow flower colour and inflorescence type (Cooper 1956). *P. umbellatum* may, on the other hand, be derived from hybridisation between one of the more basal species in the New Zealand phylogeny, or a now extinct taxon and one of the species within the main New Zealand clade. However, this needs to be investigated further as this species is unresolved in its placement in the ITS tree. *P. kirkii* is also incongruent between trees, however test trees showed that the grouping of this taxa in a clade with *P. virgatum*, *P. patulum* and *P. anomalum* was not incongruent with the ITS data set.

2.4.6 COMBINED trnT-trnL AND ITS REGIONS

Some clades that were well resolved in the *trnT-trnL* region were not resolved in the ITS region and vice versa, but did not cause any conflict, indicating that overall relationships are most likely to be best represented by combining the data sets. Some relationships were better supported with higher bootstrap values when combining data sets. This can be due to homoplasy masking the phylogenetic signal in one of the data sets but providing "secondary signal" when both data sets are combined (Nixon & Carpenter 1996). Relationships found in the ITS tree, which are recovered when including the *trnT-trnL* region, include the clade of *P. bracteolatum* with *P. eugenoides, P. kirkii* and *P. dallii*, which is incongruent between the data sets. However, there is less bootstrap support for this clade. There is also increased support for relationships found based on both maximum parsimony and maximum likelihood using the *trnT-trnL* region, which were more supported when combining data sets. This includes a clade of *P. tenuifolium* and *P. colensoi,* and a separate clade of *P. huttonianum* and *P. crassifolium,* a clade of *P. crassifolium* x *P. obcordatum* F_2 and *P. obcordatum,* and a clade of *P. anomalum, P. patulum* and *P. virgatum* in both the strict consensus of the most parsimonious trees and the maximum likelihood tree of the combined data sets. Additionally, some relationships are apparent when combining data sets, which were not found in either data set alone. For example, *P. rigidum* changes position from being in a clade with *P. divaricatum* and *P. turneri* in the cpDNA data set and being unresolved within the main New Zealand clade in the ITS data set to being included within a clade that contains *P. colensoi, P. tenuifolium, P. huttonianum* and *P. crassifolium* in both maximum parsimony and maximum likelihood analysis of the combined data sets. Overall, the analysis of combined data sets using maximum parsimony and maximum likelihood exhibit the relationships strongly supported by each data set and appears to be a more accurate representation of relationships within the genus.

2.4.7 NEIGHBOUR-NET ANALYSIS

Phylogenetic analysis allows conflicting signals and ignores alternate phylogenetic histories. However, methods that allow the visualization of these conflicting signals such as neighbour-net can help identify ambiguous relationships and identify where further investigation is needed. The use of networks also better represents relationships when there is a history of hybridisation, because reticulated graphs rather than a bifurcating tree (Bryant & Moulton 2004 224) better represent these

relationships. However, ambiguous relationships can be caused by sampling error, or other processes than hybridisation, and this method does not address these hypotheses specifically. There are conflicting signals within closely related taxa (*P. turneri*, *P. divaricatum*, *P. rigidum* and *P. patulum*), as well as *P. umbellatum* and *P. fairchildii*. *P. turneri* and *P. rigidum* exhibit the strongest conflict. This conflict indicates that these taxa are more likely to have a history of hybridisation but this needs to be investigated further.

2.4.8 THE ORIGIN OF PITTOSPORUM TURNERI

Clearly *P. turneri* has a close relationship to the divaricating species *P. divaricatum* and *P. rigidum*, with *P. divaricatum* being the closest relative in combined analyses. However, the findings from this study do not provide enough resolution to determine whether *P. turneri* has evolved from hybridisation between *P. divaricatum* and a large-leaved species because both *P. turneri* and *P. divaricatum* are unresolved in all ITS phylogenies within the internal node containing most of the taxa with ambiguous relationships. When the number of polymorphisms between sequences are low, hybrids can cluster towards one of the parental taxa, however this may change when more sequence information is added (Soltis et al. 2008), as found in *Collomia (Plemnoniaceae)* (Johnson & RL 2006). This is likely to be the case in *P. turneri*, where the addition of more regions may change the relationships inferred. However, there is a tendency for one of the parental alleles of nuclear genes to become fixed in the hybrid, which can occur in many nuclear genes when the hybrid is ancient, due to genetic drift, population bottlenecks and inbreeding (Pan et al. 2007). This has important implications in *P. turneri* as most populations are severely fragmented with few individuals and therefore introgression with other closely related taxa (such as *P. divaricatum*) is also highly plausible, "erasing" evidence of hybridisation within these slowly evolving markers. There is no evidence to suggest any of the larger-leaved species are likely to be the parent of *P. turneri,* however *P. umbellatum* has the least uncorrected pairwise distance of the larger-leaved tree species from *P. turneri* 2 (collected from Pureora), where it is not found in sympatry with *P. divaricatum*. *P. umbellatum* also shares some character traits with *P. turneri,* such as having flowers in umbels and leaves in whorls. It is also possible that *P. turneri* is the result of hybridisation between more than two taxa, or an extinct taxa. However, the unresolved status of *P. turneri* may also reflect stochastic population processes, which

may be uncovered by sequencing of a larger number of samples from different populations and the inclusion of more nuclear regions as markers.

2.4.9 HETEROBLASTY AND THE DIVARICATE FORM IN PITTOSPORUM

This study supports the findings of Hathaway (2001) that the divaricating form has evolved at least three times independently in *Pittosporum*. Analyses support a close relationship between the divaricating species *P. turneri*, *P. divaricatum*, and *P. rigidum*, with the other heteroblastic taxa *P. obcordatum* and *P. anomalum* having likely acquired this growth form independently. There is also no evidence for any of the heteroblastic species of *Pittosporum* having evolved from hybridisation as all heteroblastic species are unresolved in the ITS trees within the main New Zealand clade. It is possible that hybridisation could have increased the amount of homoplasy within the ITS region, as homoplasy is often caused by the effects of recombination in nuclear genes (Small et al. 2004) and rates of homoplasy are relatively high in the ITS region (Alvarez & Wendel 2003). This is because allelic recombination can also result in alleles which are chimeric (combining sequences of the parental taxa), violating the assumptions of phylogenetic analysis (Small et al. 2004). Therefore, sequencing of a larger number of independent nuclear markers may help elucidate and change relationships within the heteroblastic taxa and address the question of the origin of heteroblasty more thoroughly.

2.4.10 TAXONOMY AND RELATIONSHIPS WITHIN NEW ZEALAND PITTOSPORUM

The strict consensus of maximum parsimony and maximum likelihood of the combined data sets are identical. Two main New Zealand clades are apparent, one *P. eugenoides, P.dallii* and *P. kirkii*, which form a clade with *P. bracteolatum* from Norfolk Island, and another containing the rest of the New Zealand *Pittosporum*.

Hathaway (2001) proposed that *P. bracteolatum* shares a common ancestor with the New Zealand clade, and may be descended from a dispersal event from New Zealand to Norfolk Island (Gemmill, Botany 2008 conference). *P. obcordatum* and *P. fairchildii* are the next most distinct taxa from the main New Zealand clade as they are both placed in a distinct clade from the rest of the main New Zealand clade. These

are also two of the rarest taxa, with *P. fairchildii* occurring only on the Three Kings Islands, and *P. obcordatum* occurring in only twelve small isolated populations throughout New Zealand (Clarkson & Clarkson 1994). *P. fairchildii* appears to be more closely related to *P. crassifolium* based on morphology and these species hybridise readily (Cooper, 1956). However, *P. fairchildii* has some morphologically distinct traits such as yellow/green glabrous capsules (Allan 1961). *P. obcordatum* is more morphologically distinct from other New Zealand *Pittosporum.* so its placement is less unexpected. It has a divaricating form but is dissimilar in leaf shape to all other divaricating forms within the genus, with distinctive heart-shaped leaves. A clade of *P. turneri* and *P. divaricatum* is apparent, along with a clade including *P. patulum*, *P. anomalum* and *P. virgatum* within the main New Zealand clade, separating the remaining divaricating taxa into two groups.

2.4.11 THE EVOLUTION OF MORPHOLOGICAL CHARACTERS IN NEW ZEALAND PITTOSPORUM

When combining the two data sets there is still conflict with previous hypotheses regarding relationships based on morphology. No correlation has been found between relationships based on ITS sequences and morphology, implying that many of these characters have evolved more than once in different clades, or that the markers used do not evolve at a fast enough rate comparable to the rate of morphological evolution involved. The separate clade of taxa from the main group according to combined analyses are those with several to many flowered fascicles or umbels (*P. eugenoides* and *P. dallii*), consistant with the hypothesis previously proposed by (Cooper 1956) that reduced numbers of flowers are the derived form in New Zealand. These taxa are also bivalved, while the main New Zealand clade has both bivalve and trivalved taxa. However, valve number may be an unstable characteristic, unsuitable for inferring relationships (Haas 1977). These taxa also have white or yellow flowers, suggesting

that the red flower colour may have evolved within New Zealand. The apparent polyphyly of morphological characters could also be due to convergence, with repeated evolution of the same characters (Wendel & Doyle 1998), or could be a result of hybridisation. The one taxon identified as a putative hybrid in this study (*P. umbellatum*) shares the characteristic red flowers of the closely related New Zealand taxa with the many flowered inflorescences seen in the distinct clade including *P.*

bracteolatum for with which it clusters according to ITS sequences. This taxon is therefore considered a putative hybrid but this needs to be investigated further. Gemmill (2002) suggest that morphological evolution and molecular evolution are "decoupled" in this genus.

2.4.12 FUTURE DIRECTIONS

The use of multiple unlinked nuclear markers may be needed to resolve relationships within closely related *Pittosporum*, as more phylogenetic information would help overwhelm the conflicting signals of homoplasy in the ITS region (Sang & Zhong 2000). Linder and Rieseberg (2004) suggest that the use of a large set of biparentally inherited markers are needed to reconstruct each hybrid speciation event, due to the problem of population genetic and stochastic issues. They also suggest that the most powerful way to detect hybridisation is through combining studies of phylogenetic incongruence with searching for linkage disequilibrium (genetically linked markers), as closely linked markers are significantly more likely to come from the putative parent than from convergence or common descent (Rieseberg 2000). Another way to detect hybridisation is to combine multiple independent loci into one analysis and look for two or more evolutionary histories by performing analyses such as splits decomposition (similar to neighbour-net), which examines the level of conflict in the data (Bandelt & Dress 1992). Future studies could include sequencing of other nuclear regions, including the External transcribed spacer of nuclear ribosomal DNA (ETS) region, which has been shown to have great utility in phylogenetic studies, and to improve resolution of trees based on ITS data (Baldwin 1998). For example, Howarth and Baum (2005) used four introns of nuclear genes, the ITS region, LEAFY, NITRATE REDUCTASE, and GLYCERGLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE to identify hybridisation in *Scaevola,* finding all markers were needed to resolve relationships between taxa adequately. More nuclear and chloroplast markers may also be available in the future (Linder & Rieseberg 2004).

2.5 CONCLUSION

Phylogenies produced from the ITS region and *trn*T-*trn*L region show distinct clades which appear to be incongruent in their placement using both maximum parsimony and maximum likelihood analyses. There is no evidence that *P. turneri* is a hybrid

based on these two markers. However, one taxon, *P. umbellatum* may have a history of hybridisation and two other taxa, *P. eugenoides* and *P. dallii* appear to have experience introgresson. Combining the two data sets however increases the support for some clades and provides greater resolution of relationships among New Zealand *Pittosporum*. Further investigation using more nuclear and chloroplast markers may improve the resolution of relationships depicted by these markers.

CHAPTER THREE

ALLOZYMES AND INTER-SIMPLE SEQUENCE REPEATS (ISSR's)

3.1 INTRODUCTION

A variety of different molecular methods have been used for the identification of plant hybrids, however it can be necessary to employ several methods to attain an adequate understanding of relationships between closely related species and putative hybrids (Wolfe 1998). Allozymes are widely used to identify hybrids as they are a codominant marker with a known genetic basis, and they show easily identifiable evidence for hybridisation. First generation hybrids are identified by having patterns of fixed heterozygosity, combining the alleles of both parents, which then segregate in the next generation (Weeden & Wendel 1990), however later generation hybrids should contain alleles found in either parent for different enzyme systems. Studies of ancient, putative hybrids have confirmed a hybrid origin based on the presence of alleles of both parental taxa, for example (Rieseberg et al. 1990) found the species *Helianthus paradoxus* combined the alleles of its putative parents *H. annus* and *H. petiolaris* for several different enzymes. An alternative approach for identifying hybrids is the use of inter-simple sequence repeats (ISSR's): random hypervariable nuclear markers generated by PCR amplification. Hybrids can be identified by having a unique profile, with an additive banding pattern of bands of both parental species as they are inherited in a dominant fashion (Wolfe & Liston 1998). ISSR's have been used to successfully differentiate between very closely related species when other markers do not show any variation (Wolfe 1998). For example (James & Abbott 2005) found 11 bands which were exclusive to the putative hybrid *Senecio squalidus* and one putative parent, and 13 bands found exclusively in *S. squalidus* and the other putative parent, providing strong evidence for a hybrid origin of this species. This preliminary study investigates whether allozymes and ISSR's show the level of variation useful for investigating the putative hybrid origin of *P. turneri*, and aims to determine whether there is any evidence that *P. turneri* is a diploid hybrid derivative of *P. divaricatum* and *P. colensoi.*

3.2 METHODS

3.2.1 ALLOZYMES

Thirty-eight samples were collected in total, ten leaf samples were collected from *Pittosporum turneri*, *P. divaricatum* and *P. colensoi* at Lochinvar station in the Ripia Valley, Bay of Plenty (GPS reference: E2800102, N6240083) and eight individual *P. turneri* samples were collected at the Bog Pine reserve near Pureora Village, Waikato (GPS reference: E2730662, N6296368). Samples were ground with a mortar and pestle to homogenize the leaf tissue using a Tris-HCL grinding buffer with 12.5 ml 0.1 M Tris-HCL, ph 7.5, 0.75 g PVP-40, 0.005g EDTA, 0.009 g Potassium Chloride, 0.025 g Magnesium chloride, 3 drops ß-mercaptoethanol and 1.25 ml DMSO modified from (Soltis et al. 1983). Homogenates were placed in two separate 0.5 ml eppendorf tubes then stored at -70˚c prior to use. Electrophoresis using gels made of 12% hydrolyzed potato starch were used to resolved three enzyme systems out of 16 enzyme systems trialled (Appendix 4). Staining recipes were those used by (Ranker et al. 1989). Resolved enzyme systems included PGI (phosphoglucoisomerase), ALD (aldolase) and PGM (Phosphoglucomutase). The buffer systems used to resolve these enzyme systems were System 8 and System HC, modified from (Soltis et al. 1983), using the same gel and electrode buffer concentrations. System 8 resolved PGI and ALD and system HC resolved PGM. System 8 had an electrode buffer of 0.04M lithium hydroxide and 0.26 M boric acid adjusted to pH 8.0 and a gel buffer of 0.033 M Tris, 0.005 M citric acid, 0.004 M lithium hydroxide and 0.03 M boric acid, adjusted to pH 7.6. System HC was a continuous system using 0.26 M histadine-free base and 0.03 M citric acid titrated to pH 6.5. Homogenates were loaded on to the gels using paper wicks, and gels were then run in a fridge to prevent loss of enzyme activity, at 35-45 mAmps (system 8) and 200 volts (system HC) for three to four hours (see figure 3.1). Gels were sliced horizontally three times to apply three different stains. They were stained immediately and left in the dark to develop until bands were clearly visible. Bands were scored as present or absent, where the presence of two bands was interpreted as two alleles of the same locus.

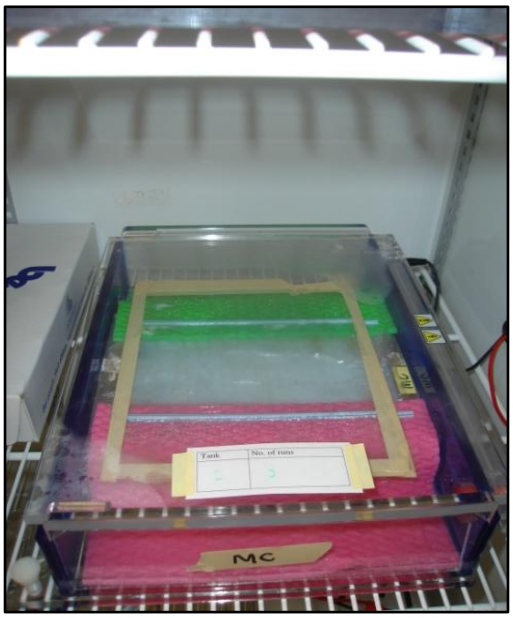


Figure 3.1. Allozyme set up using starch gel electrophoresis, with electrode buffer in chambers on either end to conduct current through the gel and sponges used to transfer buffer onto the gel. Plastic wrap is placed on the exposed part of the gel to prevent drying of the gel surface during electrophoresis. A glass plate is placed on the gel, with straws on top of the sponges to maintain contact of the sponges on the gel surface.

3.2.2 INTER-SIMPLE SEQUENCE REPEATS (ISSR'S)

DNA was extracted from leaf tissue of three of the samples from each taxon collected for the allozyme study, including a putative hybrid between *P. divaricatum* x *P. turneri (Ecroyd 1994).* A sample of a more distantly related taxon, *P. cornifolium* was also included in the study to examine the number of bands common to all New Zealand *Pittosporum.* Samples were extracted using an Invitrogen kit (Invitrogen Corporation) (see appendix 2 for DNA isolation protocol). Eight ISSR primers were trialled, these were developed by (Wolfe, 1992) which were obtained from Invitrogen Corporation. A temperature gradient was performed for each primer between 39°c and 45°c. PCR amplification was performed using 1XPCR buffer, 4 mmol/L $MgCl_2$, 200 µmol/L each DNTP, 0.2 µl 1% 1% BSA, 0.2 µmol/L each primer, 2.5 units of *Taq* and 1µl of unquantified DNA. Cycle parameters were as follows: An initial denaturing period of 94°c for 2 minutes, followed by 40 cycles of (denaturing at 94° for 40 seconds, an annealing adjusted for each primer of 45 seconds, followed by an extension at 72°c for 1 minute) and a final extension of 72°c for 20 minutes. All PCR's included a positive control, which had been previously amplified to check for reproducibility, and a negative control to ensure there was no contamination of

DNA in the mastermix. PCR products were electrophoresed following PCR's on a 1.8% agarose gel, which was stained with ethidium bromide (0.1 mg/L). A 100 base pair ladder (Invitrogen Inc) was used as a reference on the first and last lanes of the gel for sizing of bands. The gel was run for around four hours at 44 volts (or until the bottom blue dye marker had ran about 10cm). Gels were then visualised under UV light and photographed using an Alphaimager (Alphainnotech). Five primers produced clear bands (table 3.1). Bands which lined up on the gel as the same size were assumed to be homologous (of the same origin) and all clearly visible bands were scored by eye as present or absent and included in a data matrix. A replicate to ensure reproducibility of bands was performed using primer '1' and any anomalies between results reported.

Table 3.1. ISSR primers and sequences and the annealing temperature used for each primer.

Primer	*Sequence*	*Annealing temp*
ISSR 'A'	$(CA)_8$ TC	40°c
ISSR ' B'	$(CAC)_4$ GC	40.8°c
ISSR 'C'	$(CT)_7$ AC	42.2°c
ISSR 'F'	$(CA)_6$ GC	43.3°c
ISSR '1'	$(CA)_6$AT	42.2°c

3.2.3 *DATA ANALYSIS*

For allozyme data, mean number of alleles per locus, mean number of alleles per polymorphic locus, percent polymorphic loci, number of loci per enzyme system was calculated and expected heterozygosity was calucated using TFPGA (Miller 1997). For ISSR data, percent polymorphism and number of shared 'marker bands' (polymorphic bands shared among taxa and present in all accessions of a taxon) were calculated using Statistica 8.0 (Statsoft Inc, 2008). An Analysis of Molecular Variance (AMOVA) was performed using Arlequin version 3.0 (Excoffier et al. 2005) to examine the level of within and between population variation. An UPGMA (unweighted pair group method algorithm) using Nei's (1978) unbiased distance was performed on all data also using TFPGA. This

measure was used because it doesn't consider band absences as matches, an assumption which can overestimate the level of similarity between populations and overwhelm the significance of band matches, as absence of a band may be due to a number of reasons e.g. a mutation in either priming site, structural rearrangement of the chromosome during meiosis, or an insertion or deletion large enough to interpret the band as a separate locus due to differences in band size (Black 1997). Bootstrapping (Felsenstein 1985) using 100 replicates was performed to indicate the strength of each support node for the UPGMA and thus provide an indication of the confidence of relationships inferred. Additionally a Principal Components Analysis was performed using Statistica 8.0 (Statsoft Inc, 2008) to investigate how genetic variation appears in multivariate space.

3.3 RESULTS

3.3.1 ALLOZYMES

The three enzyme systems assayed were interpreted as being encoded by three putative loci: PGI-1, PGM-1, and ALD-1. For all three systems only seven individuals per taxa were used as all 30 sampled did not fit on the starch gel, and later repeats of the procedure were unsuccessful using the rest of the samples. There was no variation in ALD-1 or PGM-1, with all loci monomorphic (homozygous for the same allele). One *P. colensoi* individual was a heterozygote for PGI-1 while all other individuals were homozygotes but for different alleles. *P. turneri* individuals were also monomorphic for this enzyme system, showing no variability for any of the three enzyme systems, while *P. divaricatum* and *P. colensoi* contained two alleles. The samples which were collected from a separate population of *P. turneri* at Bog Pine Reserve, Pureora were low in activity and could not be resolved for any enzyme systems to show whether allele patterns differed between the two *P. turneri* populations. Enzyme activity appeared to diminish after time spent in the ultracold freezer at -70°, and this may have been due to degradation of the homogenates during storage.

Table 3.2 Allelic frequencies for 3 putative loci.

Population Loci		*P. divaricatum*	*P. turneri*	*P. colensoi*
PGI-1	a	0.500	1.000	0.857
	b	0.500	-	0.428
ME-1	a	1.000	1.000	1.000
	b	-	-	-
ALD-1	a	1.000	1.000	1.000
	b	-	-	-

Table 3.3 Percent loci polymorphic (*Ps*), number of alleles per polymorphic locus (*As*), mean number of alleles per locus (*Acs*) and expected heterozygosity over all loci.

Population	*Ps*	*As*	*Acs*	*He*
P. divaricatum	33.3	1.00	1.00	0.16
P. turneri	0.0	1.00	1.00	0.00
P. colensoi	33.3	2.00	1.05	0.11

3.3.2 ISSR's

All primers produced bands which were polymorphic between taxa, and the average number of bands scored per primer ranged from four to ten (average of 6.2 bands per primer). Overall 42 bands were scored with an average of 23 bands per taxon. The number of polymorphic bands was 31 (73.8%). Bands scored ranged from 600 bp to approximately 2072 bp, as bands smaller than 600bp tended to be faint and those below 500 bp tended to run off the gel. A replicate using primer '1' showed no discrepancies above the size range of 600bp, bands smaller than this were faint in both replicates, and it was therefore assumed there were no problems with reproducibility at the larger size range. There were also several bands above 600bp which were difficult to score as they were also faint and where bands were questionable they were left as missing data.

Table 3.4. Summary of ISSR bands scored per taxon for each primer.

Primer	Primer sequence	Total no. bands	Size range of bands	*P. divaricatum*	*P. turneri*	*P. colensoi*
ISSR 'A'	$(CA)_8$ TC	7	600-2072	5.3	6	3
ISSR ' B'	$(CAC)_4$ GC	10	600-2072	7	9	7
ISSR 'C'	$(CT)_7$ AC	7	800-1600	2	4	3
ISSR 'F'	$(CA)_6$ GC	4	700-2072	5	6	5
ISSR '1'	$(CA)_6$AT	9	600-2072	3	7	3
Total				22.3	34	21

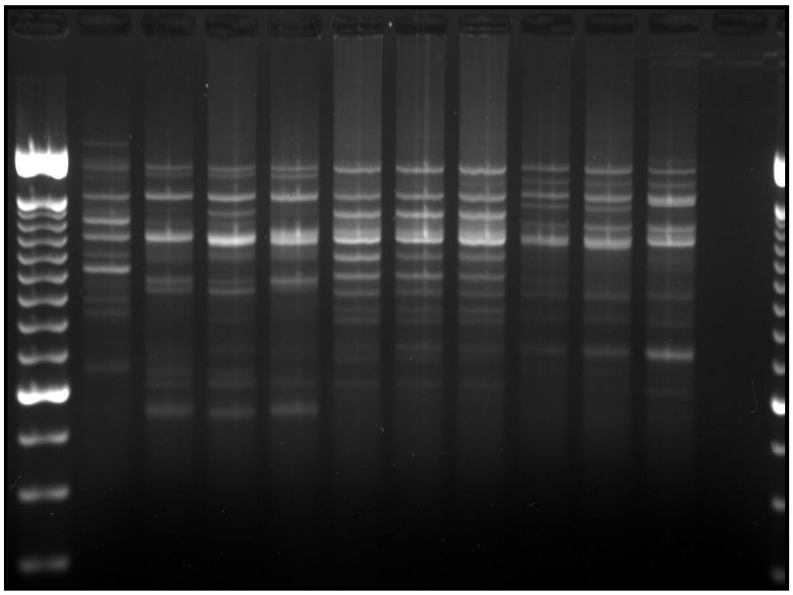


Figure 3.2. Agarose gel showing appearance of ISSR bands using 'ISSR A'. Lane 1: *P. cornifolium*, lane 2-4: *P. divaricatum*, lane 5-7: *P. turneri*, lane 8-10: *P. colensoi*. *P. cornifolium* exhibits a different profile with some bands which are shared by all taxa and some unique bands. *P. turneri* has bands which are found in some *P. divaricatum* accessions and some *P. colensoi* accessions and contains several unique bands.

40.5% of bands scored were polymorphic between *P. divaricatum*, *P. colensoi* and *P. turneri.* The putative hybrid between *P. turneri* and *P. divaricatum* did not exhibit any differences to *P. turneri* at any loci and was therefore included in all analyses as *P. turneri.* *P. divaricatum* had one unique, taxon specific band found in all accessions and *P. turneri* had five unique, taxon specific bands, however *P. colensoi* had no unique taxon specific bands. All taxa contained one to three bands

which were unique to an individual and found in no other accessions of the other taxa.

Marker bands used to identify hybridisation were considered as those bands shared between all accessions within a taxon, but are polymorphic between taxa. *P. turneri* shares four marker bands with *P. colensoi* and no other taxa and two marker bands with *P. divaricatum*, and no other taxa, combining the bands of both taxa and exhibiting an additive profile consistent with the hypothesis of hybridisation. No bands are specific to *P. colensoi* and only one band is specific to *P. divaricatum*, however both *P. cornifolium* and *P. turneri* have a high number of taxon specific bands (six and five respectively). *P. colensoi* shares the greatest number of bands with *P. cornifolium* of the other taxa, with two bands in common which are not observed in *P. divaricatum* or *P. turneri.*

Table 3.5 Total number of ISSR bands shared between taxa. Number of shared marker bands between taxa in parenthesis.

	P. cornifolium	*P. divaricatum*	*P. turneri*	*P. colensoi*
P. cornifolium	**			
P. divaricatum	11	**		
P. turneri	11	20 (2)	**	
P. colensoi	13 (2)	18 (0)	22 (4)	**

3.3.2.1 Cluster analysis using UPGMA

P. cornifolium was the most distant taxa with 100% bootstrap support and a consistency index of 100% (see figure 3.3), indicating strong support for this relationship. *P. divaricatum* and *P. colensoi* cluster together, however the support for this node is relatively weaker (61% bootstrap support, and a consistency index of 16.67%). There is strong support for the relationship between *P. turneri* and the taxa (*P. colensoi* and *P. divaricatum*) (96% bootstrap support, 64.29% consistency index). This shows that *P. colensoi* individuals have more bands in common with *P. divaricatum*, and reflects the number of unique bands of *P. turneri*. The genetic distance between *P. colensoi* and *P. divaricatum* is very low (0.19) and also low between both of these taxa and *P. turneri* (0.2449). The genetic distance between taxa is highest between *P. cornifolium* and both *P. divaricatum* and *P. colensoi*

(0.7302), and lowest between *P. divaricatum* and *P. colensoi* (0.1902), and P. turneri has equal distance to *P. colensoi* and *P. divaricatum* (0.2449).

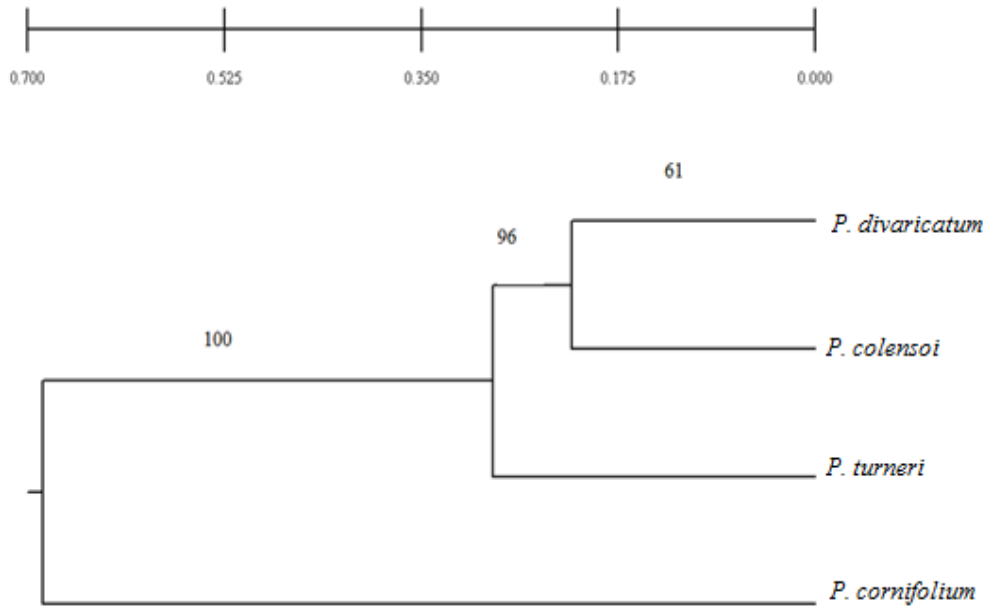


Figure 3.3. UPGMA using Nei's (1978) unbiased genetic distance. Values of 100 bootstrap replicates are shown. Consistency indices (CI's) at each node are 1 (between 1 and (2, 3, 4) = 100%, 2 (between 2, 3, 4) = 64.29% and 3 (between 2 and 4) = 16.67%.

Table 3.6. Nei's (1978) unbiased genetic distance values between taxa.

	P. cornifolium	*P. divaricatum*	*P. turneri*	*P. colensoi*
P. cornifolium	****			
P. divaricatum	0.7302	****		
P. turneri	0.4853	0.2449	****	
P. colensoi	0.7302	0.1902	0.2449	****

3.3.2.2 *Analysis of Molecular Variance (AMOVA)*

29 polymorphic loci were used for the AMOVA, excluding loci with missing data. The majority of variation is between groups when comparing genetic differences between *P. cornifolium* and (*P. divaricatum* and *P. colensoi*) (48.1%), with less variation between populations (46.1%). 5.71% of variation was found within populations. These results are significant for both within populations and between populations ($p<0.05$), however not between groups ($p=0.25$), (due to only one individual DNA sample of *P. cornifolium* being used).

Table 3.5. AMOVA of variation between taxa, within taxa and between the groups *P. cornifolium* vs. (*P. divaricatum*, *P. turneri* and *P. colensoi*).

Source of variation	d.f.	Sum of squares	Variance components	Variation (%)
Within taxa	1	11.489	3.74	5.71
Between taxa	2	22.44	3.59	46.19
Between groups	6	2.667	0.44	48.10

3.3.2.3 *Principal Component Analysis*

A principle component analysis was performed using 29 loci without missing data. Figure 3.4 shows the first two factors of the PCA with a demarcation of *P. cornifolium* from the other taxa and clustering of individuals of the three other taxa, with no overlapping of taxa. Table 3.7 shows that only the first seven loci out of all 29 loci were needed to explain the total variation between taxa.

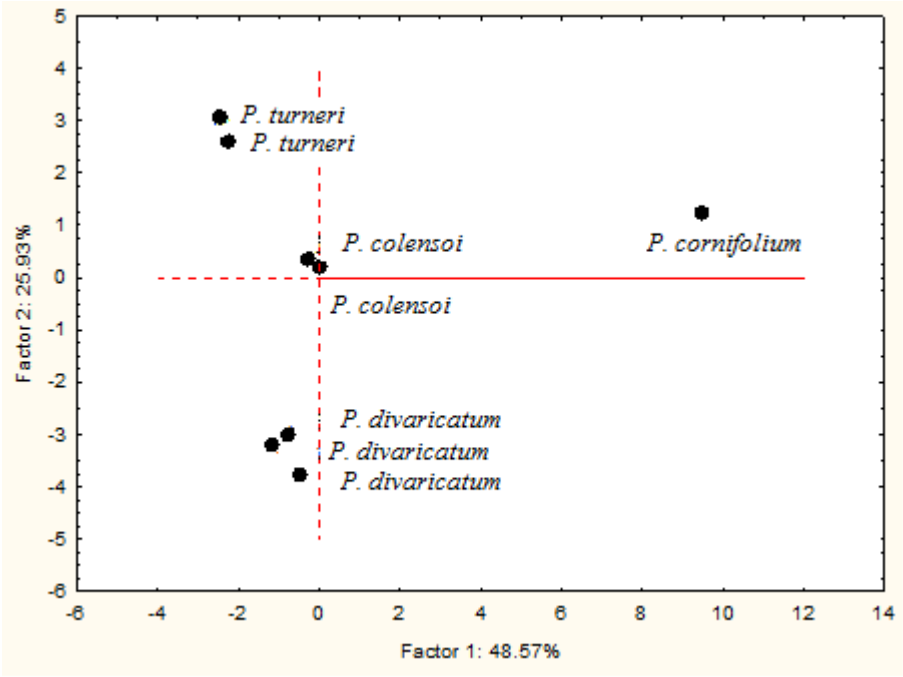


Figure 3.4. First two factors in a Principle Components Analysis of ISSR loci between all four species.

Table 3.7 Eigenvalues for the first seven loci in the Principle Component Analysis.

Eigenvalue	Cumulative %
13.6	48.57
7.26	74.5
4.16	89.37
1.55	94.93
0.71	97.49
0.55	99.48
0.15	100

3.4 DISCUSSION

3.4.1 ALLOZYMES

Allozymes have traditionally been used as evidence for hybridisation, however several studies have been unsuccessful (Wolfe 1998). For example, a study of a putative diploid hybrid *Arisaema* found that this species contained alleles which were in either one or the other putative parent for different enzyme systems but not both (Maki & Murata 2001), suggesting that the putative hybrid could be either derived from hybridisation or divergence. Although F_1 hybrids are expected to show fixed heterozygosity (Weeden & Wendel 1990) points out that after the first generation, the alleles segregate and can reflect the pattern of either of the parents. Therefore the allele patterns found in *P. turneri* with PGM-1 may be due to either divergence or hybridisation, as the one allele found in all accessions of *P. turneri* was also found to be a common allele in *P. divaricatum* and *P. colensoi.* Additionally, because only one loci was polymorphic there is little basis to make inferences in the origin of *P. turneri.*

In this study, taxa were sampled from only one location where they occur in sympatry (as the second population sampled did not show any activity).In this population *P. turneri* shows no variation, while the other two taxa have individuals with either allele. The low diversity in *P. turneri* may be due to a population bottleneck in the evolutionary history of *P. turneri,* as found to be the case in the diploid hybrid *Helianthus deserticola* (Gross et al. 2003), or this could only be occurring at the one site at Ripia Valley where samples were taken. Other studies have also found putative hybrids to be monomorphic for loci while the

parental taxa are not e.g. (Rieseberg et al. 1990) found the hybrid *Helianthus paradoxus* to be monomorphic for ADH, 6PGD and PGI while the parental taxa contained heterozygotes. This species however has been shown to be a hybrid through sequencing of chloroplast DNA and nuclear ribosomal DNA (Rieseberg et al. 1990).

3.4.2 ISSR's

3.4.2.1 *Sample representation*

Because the sampling within each population was very low in this study, bands which are considered to be unique to each taxon cannot be ruled out as bands potentially found in other taxa if the sampling scheme was larger. Further sampling could show that some of the bands which are considered 'marker bands' found only in *P. turneri* and one other taxon also occur in the other putative parent, which would reduce the number of bands shown to be 'additive' in *P. turneri*. This also could be the cause of some of the non-parental bands found in *P. turneri*. Sampling error and incompletely explored parental genomes can be the underlying cause of additive band patterns in putative hybrids (Neuffer & Jahncke 1997).

3.4.2.2 Shared 'marker bands'

The number of bands shared between *P. turneri* and *P. divaricatum*, and *P. turneri* and *P. colensoi* implies an additive profile of banding patterns, as *P. turneri* shares four marker bands with *P. colensoi* (bands found in all accessions of *P. colensoi* and not found in *P. divaricatum* or *P. cornifolium*) and two marker bands with *P. divaricatum* (bands found in all accessions of *P. divaricatum* and not found in *P. colensoi* or *P. divaricatum*). This could indicate that these two taxa have hybridized to form *P. turneri*.

3.4.2.3 UPGMA

The UPGMA based on Nei's (1978) genetic distance illustrates clearly that *P. cornifolium* is the most distant taxon from the other taxa. However, the node separating *P. turneri* from *P. colensoi* and *P. divaricatum* was an unexpected

result anomalous with ITS and cpDNA sequencing results, which suggest that *P. turneri* is more closely related to *P. divaricatum* than *P. colensoi* (see chapter two). This pattern reflects the fact that while a high number of bands are shared between *P. divaricatum, P. colensoi* and *P. turneri, P. turneri* has five unique taxon specific bands. The UPGMA also illustrates that the distance between *P. cornifolium* and the remaining taxa is great, with many unique bands found in P. cornifolium. This suggests that ISSR's could be useful in clarifying relationships within the New Zealand *Pittosporum*.

3.4.2.4 AMOVA

Table 3.6 shows that there is a high percentage of variation between taxa and between *P. cornifolium* and the other taxa, with low variation within taxa. A greater genetic distance between *P. cornifolium* and the other three species is expected as it is a distinct lineage (Chandler et al. 2007). There is also a greater difference between *P. turneri* and *P. cornifolium* than all other pairwise differences, this could suggest that *P. turneri* is more distantly related to *P. cornifolium,* however this needs to be further investigated. This more distant relationship to *P. cornifolium* probably also reflects a history of hybridisation because recombination has led to the distinct banding pattern found in *P. turneri*, with many taxon specific bands. The low variation found within taxa is a positive result, as this indicates that there is a low likelihood of finding more bands within taxa if the sampling was broader.

3.4.2.5 Principle Components Analysis

The PCA showed differentiation between species, with no overlap between taxa or outlying data to indicate any anomalous results. There was enough differentiation between taxa to distinguish them with seven loci. This is consistent with the findings of Wolfe (1998), who found that only one to three primers were needed to fingerprint each accession. This suggests that a low number of primers are needed to distinguish between taxa and to gain adequate resolution among closely related *Pittosporum* species. However a larger number of loci may be necessary to confirm with confidence whether there is an additive profile among hybridizing populations. Based on the number of unique bands scored in *P. turneri* we would expect *P. turneri* to appear outside the ranges of values for *P.*

divaricatum and *P. colensoi.* This illustrates the unusually high number of taxon specific bands in *P. turneri* relative to the number of additive bands found.

3.4.3 EVIDENCE FOR HYBRIDISATION?

The criteria used to support hybridization using ISSR's is an additive profile of parental bands (Wolfe 1998). *P. turneri* shares two marker bands with *P. divaricatum* and four marker bands with *P. colensoi.* This is consistent with a hybrid origin of *P. turneri.* The extra 'non-parental' bands found in *P. turneri* could also be indicative of a hybrid origin, as they may be due to crossing-over events during meiosis, and mutation in the binding sites (Neuffer & Jahncke 1997). Many studies have found a similar result, with many non-parental bands found in diploid hybrids e.g. (Godwin et al. 1997). This result could also be caused by problems with PCR amplification such as heteroduplex bands (non-independent bands caused by degenerate repeats or multiple priming sites within a locus) (Wolfe & Liston 1998), sampling error, or incompletely sampled parental genomes (Neuffer & Jahncke 1997). However the occurance of bands due to any artifacts caused by the PCR procedure should be no more common in interspecific hybrids than in any intraspecific progeny (Rieseberg 1998). As very few novel bands were found in *P. divaricatum* and *P. colensoi*, it is highly likely the extra bands found in *P. turneri* have a genetic basis.

3.4.4 POTENTIAL FOR USE OF ISSR'S IN PITTOSPORUM

This study provided adequate resolution between closely related taxa with a low number of loci and exhibited low variation within each population. This is important as any marker used to identify hybridisation depends on being able to distinguish parental taxa from one another (Wolfe & WJ 1995). Although the main concern with arbitrary markers such as ISSR's is the assumption of homology (the assumption that bands shared indicate a common origin), this is less of a problem in closely related taxa (Wolfe & Liston 1998). This study shows that ISSR's may be a valuable tool to study relationships at the species level in *Pittosporum* and resolve relationships between problematic taxa, as well as potentially an excellent tool to study hypotheses of hybridisation.

3.4.5 FUTURE STUDIES

Amplified fragment length polymorphisms (AFLP's) are a more accurate method than ISSR's, and have been shown to a have greater level of reproducibility and information content than RAPD's and ISSR's (Mcgregor et al. 2000). However AFLP's are also prone to the problem of the assumption of homology and they are a more expensive method, requiring two rounds of PCR amplification and the use of radio-active primers and acrylamide gels (Wolfe & Liston 1998). ISSR's could be used to study the origin of *P. turneri*, employing a larger number of samples and larger number of primers, however it is recommended that other taxa than *P. divaricatum* and *P. colensoi* be included in the study as *P. turneri* may be derived from more than one hybridization event.

3.5 CONCLUSIONS

The results of this study support a hybrid origin of *P. turneri* as this species combines ISSR bands which are found exclusively in this species and *P. divaricatum* and other bands in this species and *P. colensoi*. *P. turneri* also has a number of non-parental bands consistent with a history of recombinational speciation. Allozymes did not provide any evidence for hybridisation as *P. turneri* did not exhibit additivity of bands, therefore the pattern of inheritance was consistent with either hybridisation or divergence.

CHAPTER FOUR

MORPHOLOGY STUDY

4.1 INTRODUCTION

Morphological studies are a useful tool where molecular methods do not resolve relationships between taxa because hybrids often show greater morphological variation than molecular variation (Gottlieb 1984). Hybridisation is inferred from the degree of morphological intermediacy between the putative parents over a large number and range of characters (Wilson, 1992), and this has been found to be true in many cases of suspected hybridisation which have combined morphological data with molecular data (Milne 1999). *Pittosporum turneri* appears morphologically intermediate between several of the closely related divaricating and large-leaved species of *Pittosporum* (Cooper, 1956), however the extent to which it is morphologically intermediate has never been investigated. This study uses the methods of Wilson (1992) to address the hypothesis that *P. turneri* is a hybrid derived from hybridisation between *P. divaricatum* and *P. colensoi*.

4.2 METHODS

A study of herbarium specimens throughout the geographical ranges of *P. turneri*, *P. colensoi* and *P. divaricatum* was conducted, as well as a morphological study of the three species at one site at Lochinvar station in the Ripia Valley, Central North Island where they occur in sympatry. This was done to measure variation over a large number of morphological characters between species and to identify whether *P. turneri* is intermediate in adult vegetative, floral and growth form characters. 29 character traits were observed for the herbarium study and 33 for the site study. A number of categorical characters were included in both studies if they could be shown to differ between species and were consistent between individuals of the same species. These characters were taken from Allan (1961) and Cooper (1956) for the herbarium study and confirmed with herbarium specimens (see appendix 7).

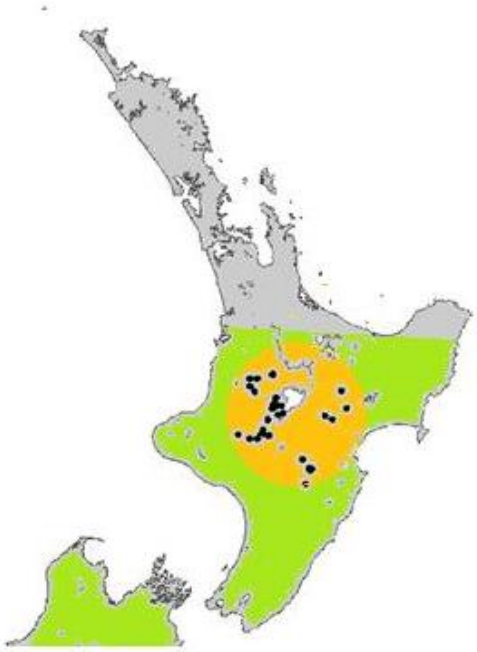


Figure 4.1. Ranges of *P. divaricatum*, *P. colensoi* and *P. turneri* in the North Island. Records of *P. turneri* (black dots) from the BIOWEB data base, Department of Conservation, Generalised distribution of *P. divaricatum* (yellow) and *P. colensoi* (green) in the North Island. *P. colensoi* occurs from 38° South while *P. divaricatum* is found " throughout" the central North Island (Allan, 1961).

4.2.1 HERBARIUM STUDY

Herbarium specimens used were from the University of Waikato Herbarium (WAIK), the Allan Herbarium at Landcare Research Lincoln (NZCHAR), and the National Forestry Herbarium at Scion Research (NZFRI). These specimens were selected to include the diversity of ecotypic variation from throughout their natural ranges (see appendix 7 for list of specimens). 22 adult *P. divaricatum* specimens were observed along with 27 adult *P. colensoi* and 15 adult *P. turneri* herbarium specimens. 27 characters were observed, including 11 categorical character traits which were scored and judged as to whether or not they were intermediate (see table 4.1). All characters taken from Allan (1961) and Cooper (1956) (Appendix 8) were verified using herbarium specimens except one character, seed number, as there was no overlap in the range stated by these authors. Information was taken from herbarium labels where possible (e.g. height and growth form) and measurements were made using a ruler and a stereomicroscope. Specimens which had good examples of floral and fruit characters were photographed.

Table 4.1. Characters observed in the herbarium and Ripia Valley studies.

1. Leaf length (mm)	18. Capsules (0) smooth (1) weakly rugose (2) rugose
2. Leaf width (mm)	19. Stigma (0) glabrous (1) Sparsely hairy (2) densely hairy
3. Petiole length (mm)	20. Capsules (0) glabrous (1) sparsely hairy (2) densely hairy
4. No. lateral veins	21. Stems (0) glabrate (1) ciliate (2) tomentose
5. Petal length (mm)	22. Leaf texture (0) coriacious (1) submembranous (2) membranous
6. Petal width (mm)	23. Leaves (0) glabrous (1) ciliate (2) tomentose
7. Sepal length (mm)	24. Leaf apex (0) obtuse (1) sub-acute (2) acute
8. Sepal width (mm)	25. Leaf arrangement (0) altermate (1) both alternate and fascicled (2) mainly fascicled
9. Length of pedicle (mm)	26. Venation (0) not netted (1) netted over some of the leaf surface (2) netted over the whole leaf surface
10. Length of anthers (male flowers) (mm)	27. Sepal apex (0) obtuse (1) sub-acute (2) acute
11. Length of filament (male) (mm)	28. Sepals (0) glabrous (1) sparsely hairy (2) densely hairy
12. Length of carpel (female) (mm)	29. Pedicels (0) glabrous (1) Sparsely hairy (2) densely hairy
13. No. Flowers in inflorescences	Ripia valley study only:
14. Length of stigma (mm)	30. Basal diameter (cm)
15. No. valves on capsules	31. Height (m)
16. Capsule diameter (mm)	32. No. seeds per capsule
17. Capsule shape (0) ovoid (1) subglobose (2) globose	33. Degree of leaf indentation

4.2.2 RIPIA VALLEY SITE STUDY

Two visits were made to the Ripia Valley site in November and December 2008 (GPS reference: E2800102, N6240083). A wider variety of measurements were taken, including basal diameter and height, and some floral characters which were difficult to determine on pressed herbarium specimens (e.g. anther length, filament length, and carpel length). A branch about 50cm long was selected which had flowers and/or capsules present. This was done for tall trees by using a long handled (approximately four metre, extendable) pair of secateurs. Ten adult *P.*

divaricatum adult plants were found at this location, and 13 *P. turneri* and 13 *P. colensoi* flowering adults were included in the study.

Figure 4.2. Study site at Ripia Valley, with *Pittosporum turneri* adult in the foreground.

4.2.3 DATA ANALYSIS

Box-plots were produced to show the range, upper and lower quartiles and extremes for each variable. This was done to compare the ranges of each character between species and identify outliers which may dramatically affect results. Graphical representation of the continuous data was produced using scattergrams to illustrate diagrammatically any patterns of intermediacy for characters which had a significant difference between two or more species. For multivariate analyses, the character count procedure developed by (Wilson 1992) was used to determine the number of continuous characters for which *P. turneri* is intermediate to the other taxa and whether this number is significantly greater than would be expected for a divergent species. A Tukey's HSD test was used for pairwise comparisons of means to determine whether characters successfully distinguished between the putative parents and whether the measurements for *P. turneri* were statistically significantly different from *P. divaricatum* or *P. colensoi.* This is a conservative test which reduces the chance of type 1 Error (the chance of finding differences when there are none). A traditional Andersonian hybrid index was calculated for *P. turneri* individuals for the Ripia Valley data

using the method of (Anderson 1949), with a total of eight characters of which there was no missing data. A Principle Component analyses (PCA) was conducted using Statistica 8.0 (Statsoft Inc, 2008), to examine patterns within the data. PCA is used to compare data in multivariate space. This is done by converting data into "eigenvalues", a method which allows the comparison of any number of variables on a common scale (Shaw 2003), which helps to identify any outlying or anomalous results, and can also be used to identify how many characters are needed to explain the total variation found. This was performed using a correlation matrix of Pearson's product moment correlation coefficients.

4.3 RESULTS

4.3.1 HERBARIUM STUDY

4.3.1.1 Sample representation

Many data values were missing for a number of variables (see Table 4.1) as individuals usually had either flowers or fruit, and this problem is compounded by the overlap in ranges for some characters. Several characters could not be confirmed using herbarium specimens or showed no identifiable difference between species such as those characters associated with the degree of hairiness present on floral parts and leaf shape, and the results for these characters were then disregarded and not used for further analysis. Sepal width was not easily measured and was replaced with sepal shape (from Allan, (1961 and Cooper, (1956)). The range of characters was therefore reduced from 27 to 20 (including both continuous and categorical characters). Table 4.2 shows the number of individuals contributing to the means shown in table 4.3.

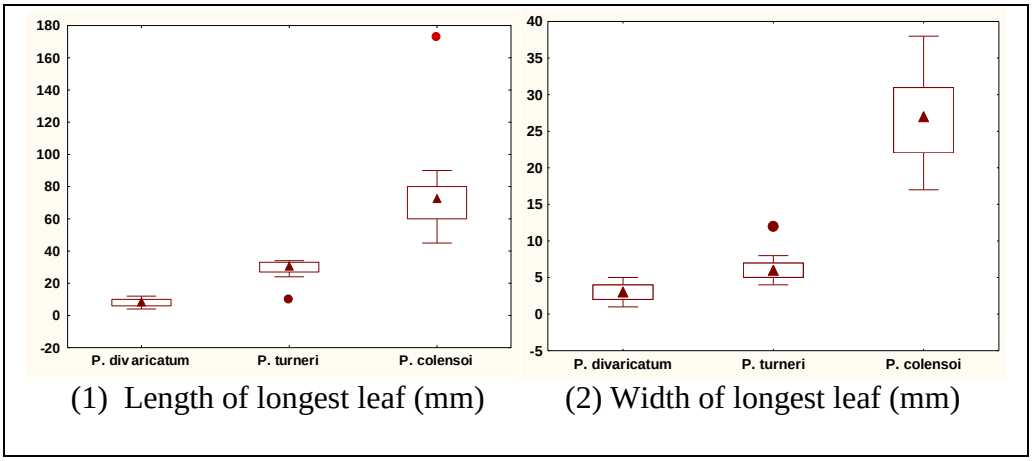
Table 4.2. Number of individuals observed for each character in the herbarium study.

	P. colensoi	*P. turneri*	*P. divaricatum*
Length of longest leaf (mm)	23	14	23

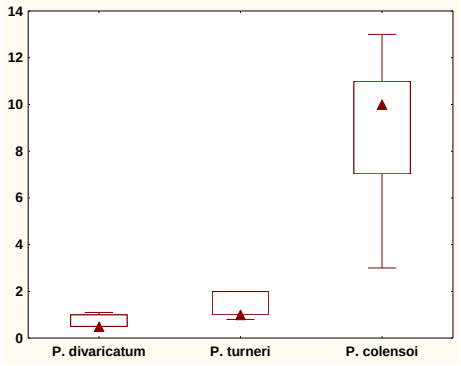
Width of longest leaf (mm)	23	14	18
Petiole length of longest leaf (mm)	10	10	10
Petal length (mm)	7	7	6
No. flowers per inflorescence	9	8	7
Sepal length (mm)	3	6	5
Pedicle length of capsules (mm)	8	5	3
Seed length (mm)	16	4	4
Capsule thickness (mm)	10	5	4

4.3.1.2 Data summary

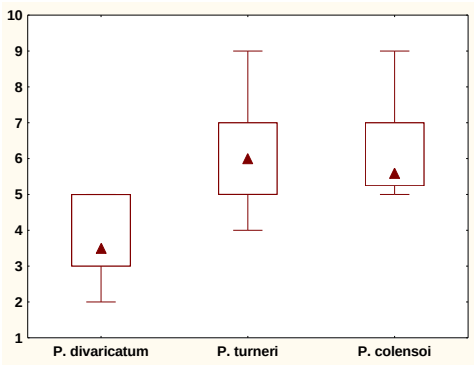
Figure 4.3 shows that there are a number of outliers in the data and that the range varies dramatically between species for many characters. Maximum and minimum values for some characters lie close to the ranges of other species. This may reflect the nature of *Pittosporum* species with many overlapping character states (see appendix 7). *P. colensoi* contains values which are outliers for leaf length, seed length, and capsule pedicle length. *P. turneri* contains outliers for leaf length, leaf width, sepal length and pedicle length, and *P. divaricatum* has no outliers.



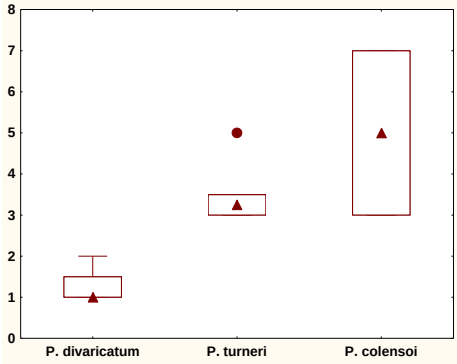
(1) Length of longest leaf (mm) (2) Width of longest leaf (mm)



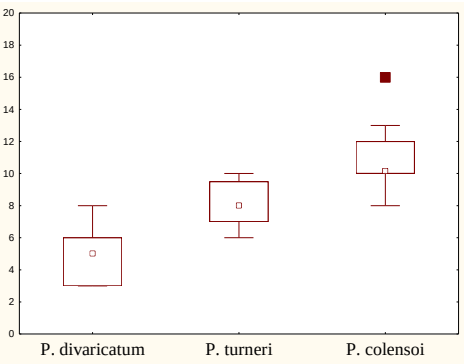
(3) Petiole length



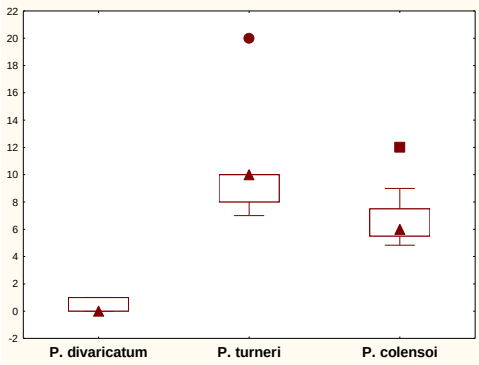
(4) Petal length (mm)



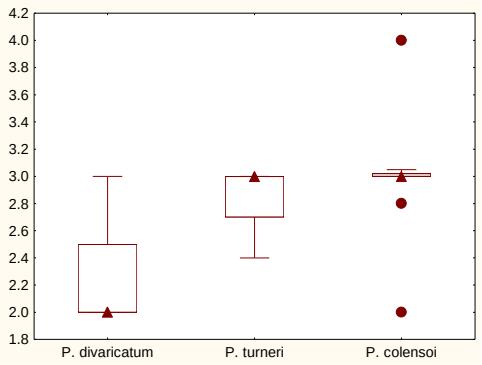
(5) Sepal length (mm)



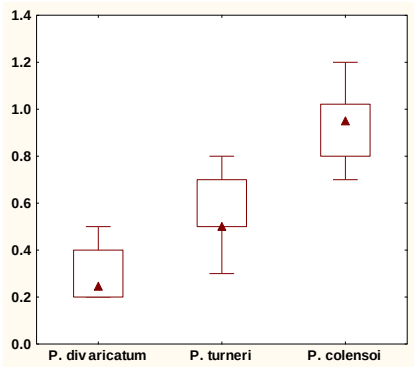
(6) Capsule length (mm)



(7) Length of capsule pedicle (mm)



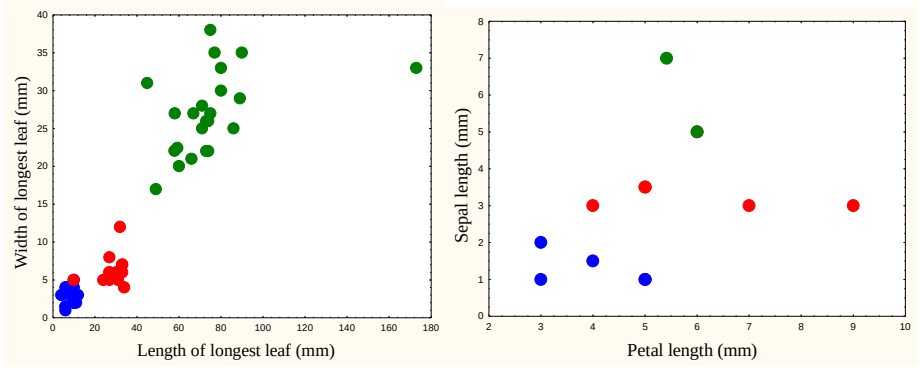
(8) Length of seeds (mm)



(9) Capsule thickness (mm)

Figure 4.3. Boxplots of herbarium data used in the character count of Wilson (1992) showing the median, upper and lower quartiles, extremes (excluding outliers), and outliers for each species.

The scattergrams in Figure 4.3 show diagrammatically the pattern of intermediacy for two traits on the x and y axis, with *P. turneri* tends to cluster between the other two taxa. (A) shows that *P. turneri* clusters between *P. divaricatum* and *P. colensoi* for leaf length x width (B) shows a scattering of values for *P. colensoi* and *P. turneri* for petal length but is intermediate for sepal length (C) shows no consistent pattern of *P. colensoi* and *P. turneri* individuals for petal length x number flowers per inflorescence (D) Shows *P. turneri* and *P. colensoi* have similar pedicel length and (E) shows *P. turneri* is generally intermediate for capsule length x thickness.



(A) (B)

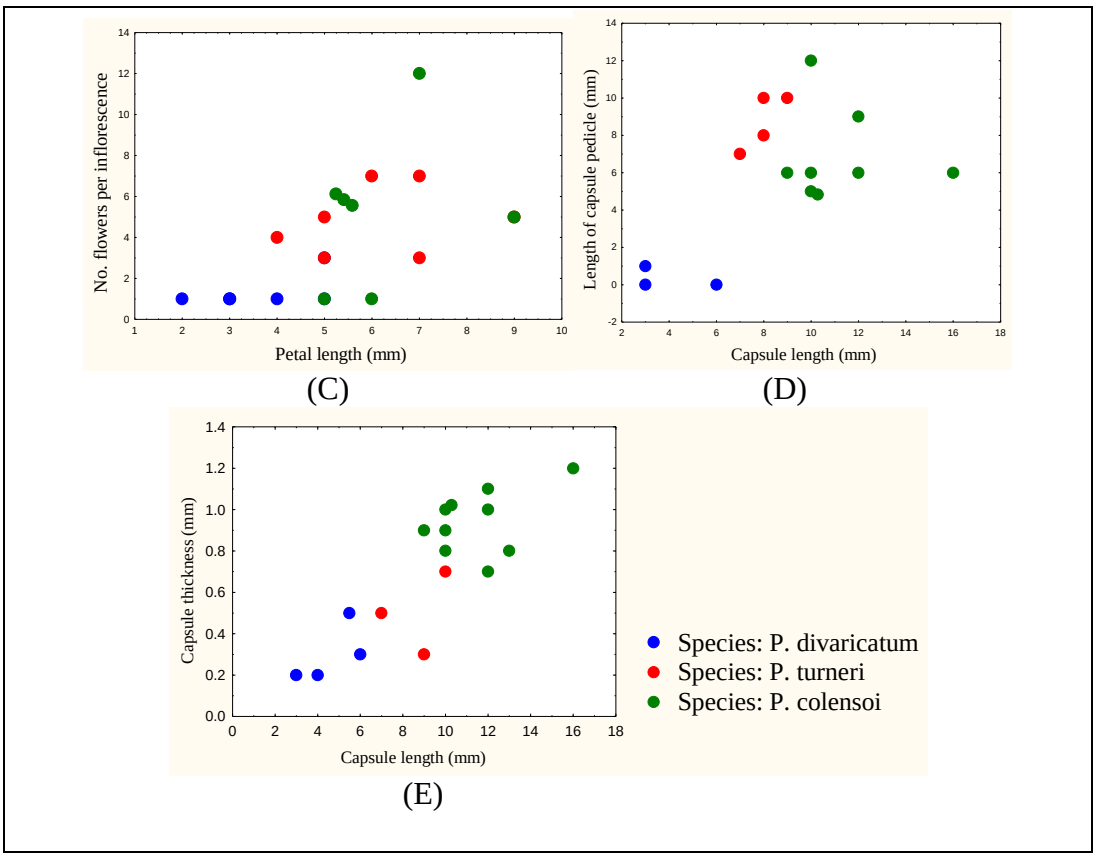


Figure 4.4. Scattergrams of character traits for the herbarium study.

4.3.1.3 Wilson (1992) character count

P. turneri is intermediate in character means for 10 out of 11 quantitative characters. This is statistically significant ($p<0.05$) and supports a hypothesis of hybridisation (see Wilson, 1992). All characters which were not found to be statistically significant between pairwise comparisons of *P. colensoi* and *P. divaricatum* were excluded. However, only two characters were significantly different between *P. turneri* and both the other taxa, while three characters were significantly different from only *P. colensoi* and three were significantly different from only *P. divaricatum*. For two continuous characters *P. turneri* had an extreme value relative to the other two species, however neither of these results were significantly different from *P. colensoi*.

Table 4.3. Wilson's (1992) character count. Means, standard deviations and results of Tukey's HSD test . * denotes data taken from Cooper (1956) and Allan (1961).

	(1) P. divaricatum	*(2) P. turneri*	*(3) P. colensoi*	Significantly different from:	Means Intermediate?
1. Length of longest leaf (mm)	8.44 ± 2.17	28.66 ± 5.94	77.17 ± 23.94	Both	Y
2.Width of longest leaf (mm)	2.88 ± 1.06	6.26 ± 1.87	28.65 ± 7.28	3	Y
3. Petiole length of longest leaf (mm)	0.66 ± 0.26	1.38 ± 0.53	9.48 ± 2.43	3	Y
4. Petal length (mm)	3.66 ± 1.21	6.14 ± 1.68	8.33 ± 2.8	Neither	Y
5. Sepal length (mm)	1.3 ± 0.44	3.5 ± 0.77	4.5 ± 1.29	1	Y
6. Length of capsule (mm)	4.93 ± 1.79	7.86 ± 1.34	11± 2.13	Both	Y
7. Length of capsule pedicel (mm)	0.33 ± 0.57	11 ± 5.19	7.14 ± 2.48	1	N
8. Length of seeds (mm)	2.25 ± 0.5	2.85 ± 0.3	2.98 ± 0.41	Neither	Y
9. Capsule thickness (mm)	0.3 ± 0.14	0.56 ± 0.19	0.93 ± 0.16	3	Y
10. Seeds per capsule*	2-6	3-10	5-31		Y
11. Maximum height (cm)*	200	900	1000		Y

10:1

4.3.1.4 Categorical characters

P. turneri has the same score as *P. divaricatum* for four out of the eight categorical characters including leaf arrangement, sepal fusion, sepal shape and capsule shape, two characters are intermediate, including venation and capsule number, and the degree of hairiness of capsules is more similar to *P. colensoi*. Figure 4.5 shows photos of capsule, floral and leaf characters of herbarium specimens.

Table 4.4: Number of intermediate categorical characters of herbarium data

	P. divaricatum	*P. turneri*	*P. colensoi*	
1. Leaf arrangement (0) Alternate (1) both alternate and fascicled (2) mainly fascicled	1	1	0	N
2. Venation (0) not netted (1) netted over some of the leaf surface (2) netted over the whole leaf surface	0	1	2	Y
3. Sepal fusion (0) not overlapping (1) slightly imbricate at base (2) imbricate at base	1	1	2	N
4. Sepal apex (0) obtuse (1) subacute (2) acute	2	2	1	N
5. Sepal shape (0) broad (1) narrow (2) very narrow/linear	1	1	0	N
6. Capsule shape (0) ovoid (1) subglobose (2) globose	1	1	2	N
7. Hairs on capsules (0) glabrous (1) sparse (2) tomentose/pilose	1	2	2	N
8. No. valves on capsules	2	2-3	3	Y
				2:6

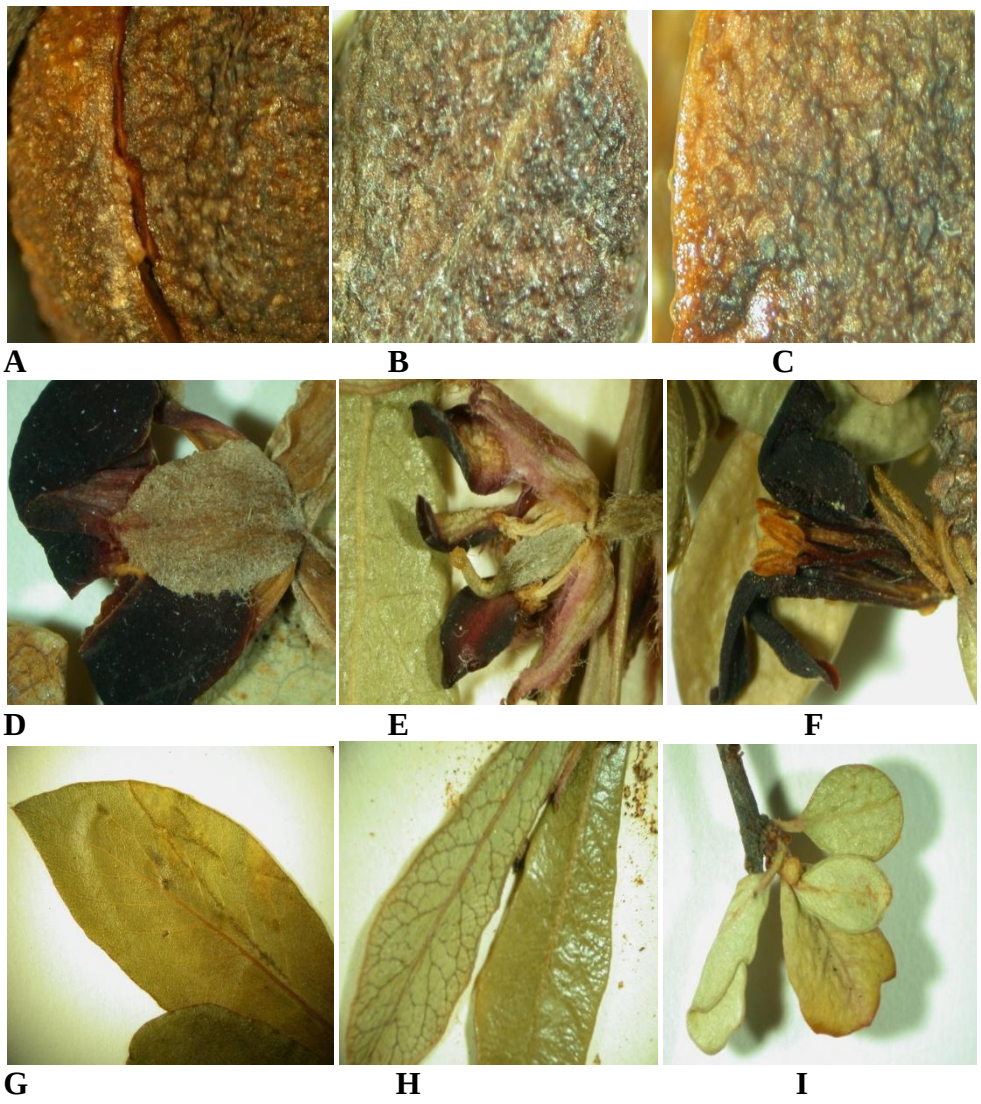


Figure 4.5. Photographs of capsules, flowers and leaves of herbarium specimens. **A, D** and **G**: *P. colensoi*, **B, E** and **H**: *P. turneri*, **C, F** and **I**: *P. divaricatum*.

4.3.1.5 Principle components analysis

The PCA diagram (Figure 4.6) shows that the first axis sufficiently resolves species into groups (leaf length) and accounts for 97.32% of the total variation found between these three characters. There is strong clustering of individuals within species with *P. turneri* appearing intermediate for all individuals on the first axis, however *P. turneri* individuals appear closer to *P. divaricatum*. *P. colensoi* appears to be the most variable with the widest range of eigenvalues.

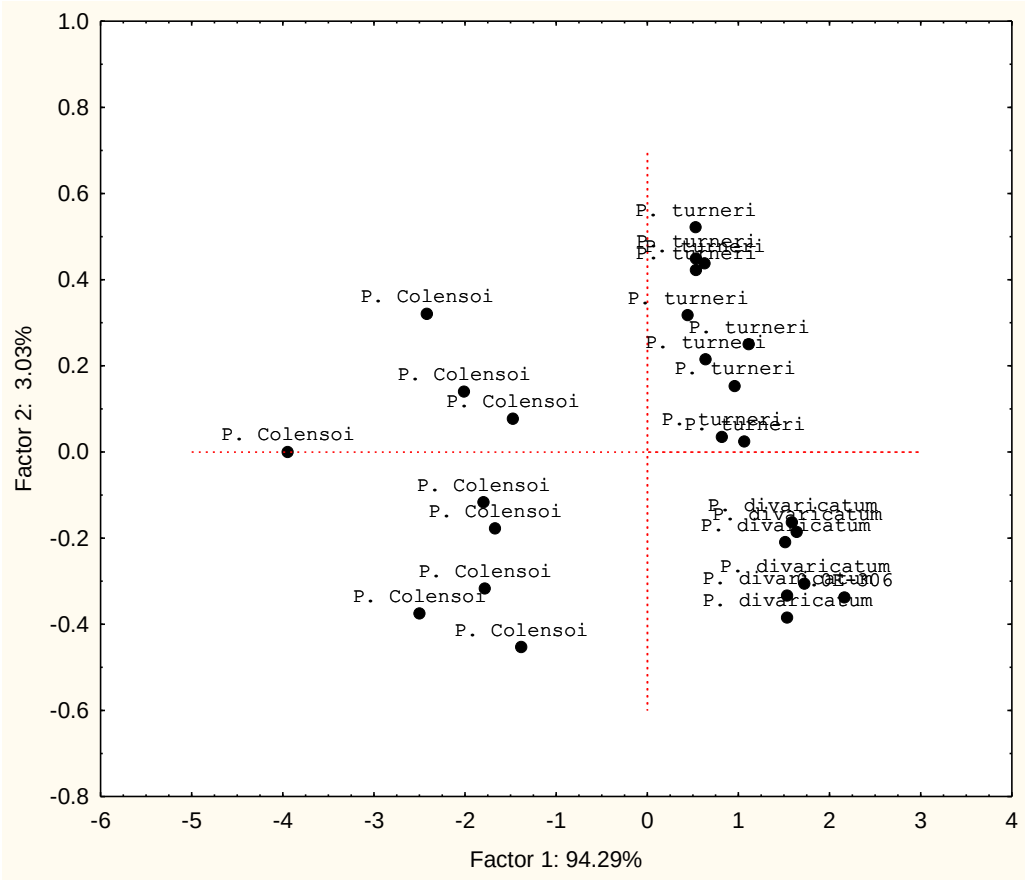


Figure 4.6. Principle components analysis of length of longest leaf, width of longest leaf and petiole length.

Table 4.5. Eigenvalues of principle components analysis of herbarium data.

Factor	Eigenvalue	% total variance	Cumulative Eigenvalue	Cumulative %
1	2.849195	94.97318	2.849195	94.973
2	0.096088	3.20293	2.945283	98.176
3	0.054717	1.82389	3.000000	100.000

4.3.2 RIPIA VALLEY STUDY

A broader range of variables were observed for the Ripia Valley site study as flowers could be dissected and characters such as length of carpels, and anther and filament length could be easily measured. Additional characters which were also observed included hairiness of pedicels, carpels and sepals which were difficult to observe on herbarium specimens. 15 out of a total of 20 continuous characters were used for the character count procedure as some characters did not have significant differences between the putative parents, including number of flowers per imfloresence, carpel length, anther length and filament length and seed number could not be easily counted with many capsules no longer containing

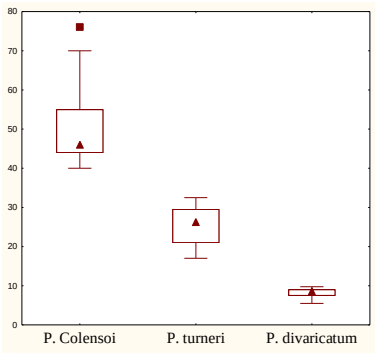
seeds. For all measurements, between two and five measurements were taken for each character depending on whether there were enough flowers, capsules etc to do so.

Table 4.6 Number of individuals measured in the Ripia Valley site study.

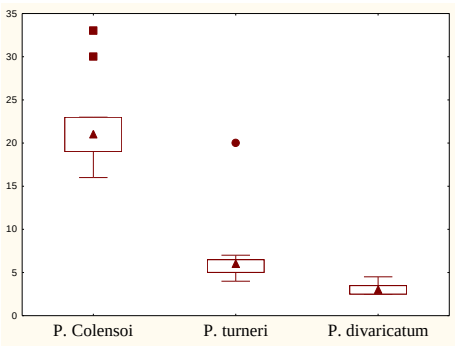
	P. colensoi	*P. divaricatum*	*P. turneri*
Length of longest leaf (mm)	10	14	10
Width of longest leaf (mm)	10	14	10
Petiole length of longest leaf (mm)	10	14	9
Petal Length (mm)	9	10	7
Petal width (mm)	9	10	7
Sepal length (mm)	8	10	6
Sepal width (mm)	5	4	7
Pedicle length (mm)	8	10	7
Anther length of male flowers (mm)	5	5	5
Filament length of male flowers (mm)	5	5	5
Gynoecium length of female flowers (mm)	3	5	3
No. flowers per inflorescence	7	10	6
Capsule length (mm)	3	10	3
Capsule thickness (mm)	3	10	3
Basal diameter (mm)	9	12	10
Height (m)	7	9	10

4.3.2.1 Data summary

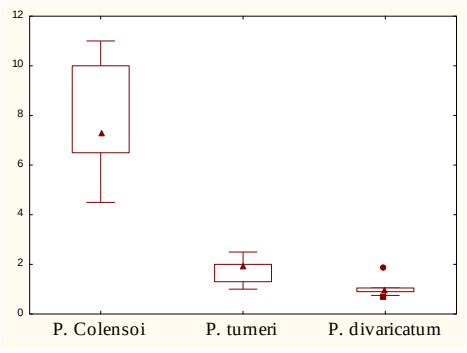
The box-plots in figure 4.7 show that for most characters, as per the herbarium study *P. divaricatum* exhibited the lowest range and few outliers (see figure 4.7). *P. turneri* has several individuals which have outlying values for leaf width, sepal width, degree of indentation, and petal length. *P. colensoi* has outlying values for width of longest leaf, length of longest leaf, pedicle length, petal length and flowers per inflorescence.



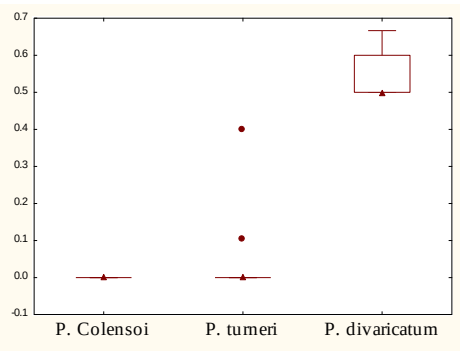
(1) Length of longest leaf



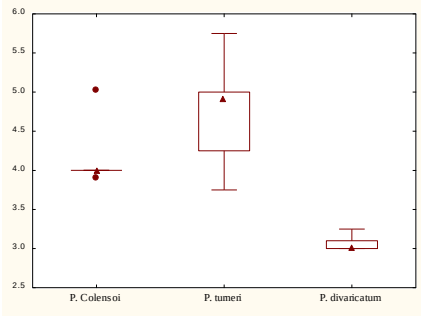
(2) Width of longest leaf



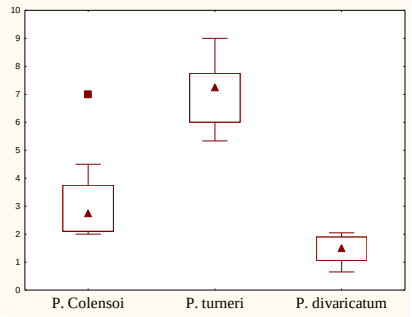
(3) Petiole length



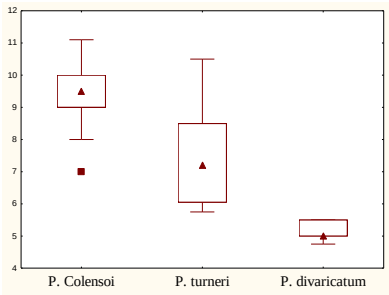
(4) Degree of indentation of leaves



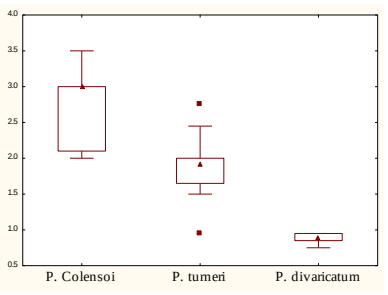
(5) Filament length (male flower)



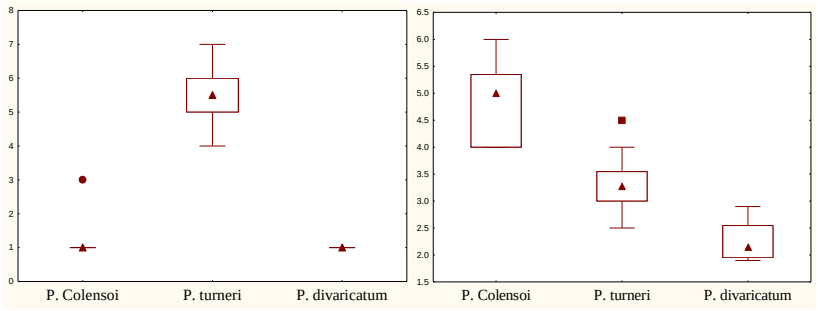
(6) Pedicle length



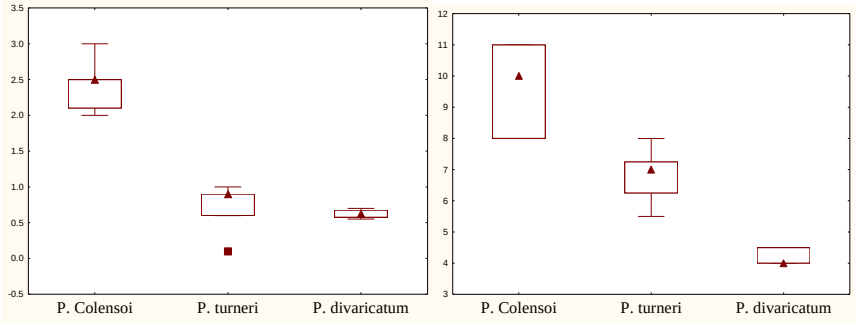
(7) Petal length



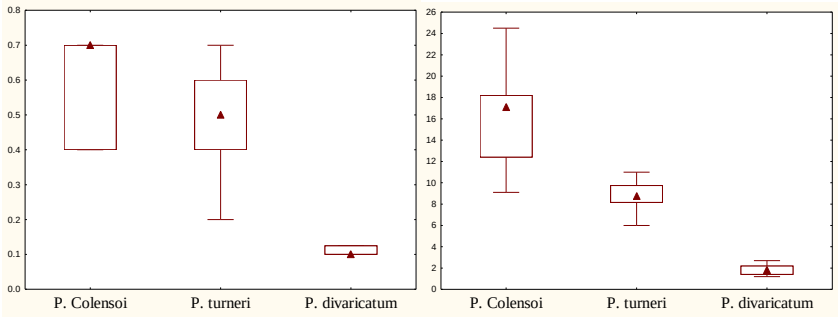
(8) Petal width



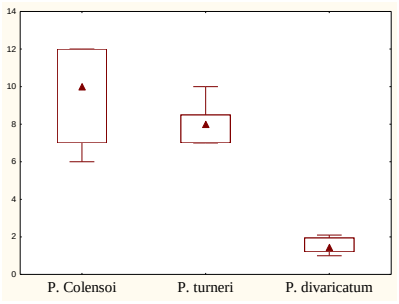
(9) Flowers per inflorescence (10) Sepal length



(11) Sepal width (12) Capsule length



(13) Capsule thickness (14) Basal diameter



(15) Height

Figure 4.7. Boxplots of data from Ripia Valley which were used in the character count of Wilson (1992) showing median, upper and lower quartiles, outliers, maximum and minimum (excluding outliers) and extremes.

The scatterplots in Figure 4.8 shows that leaf length x leaf width (A) separates between taxa, and *P. turneri* is intermediate for both characters. For petal length x petal width (B) *P. divaricatum* is the most distinct with overlap between the other two taxa, This pattern is also true for basal diameter x height (E), for which *P. turneri* overlaps with *P. colensoi*, with a number of *P. turneri* individuals attaining the same height as *P. colensoi*. For capsule length x capsule thickness (C) *P. turneri* is more similar to *P. colensoi* while for sepal length x sepal width (D) *P. turneri* is more similar to *P. divaricatum*. *P. turneri* have intermediate basal diameter but are close in height to *P. colensoi.*

4.3.2.2 Wilson's (1992) character count

A number of characters were rejected for use in the analysis as there were no significant pairwise differences between the putative parents including a number of floral characters (stigma and style length, anther and filament length). *P. turneri* is intermediate for 12 of 14 quantitative characters. This result is statistically significant ($p=<0.05$) (see Wilson, 1992) and supports a hypothesis of hybridisation. *P. turneri* differs significantly from *P. divaricatum* and *P. colensoi* only for four and five traits respectively showing no obvious resemblance to one species more than the other.

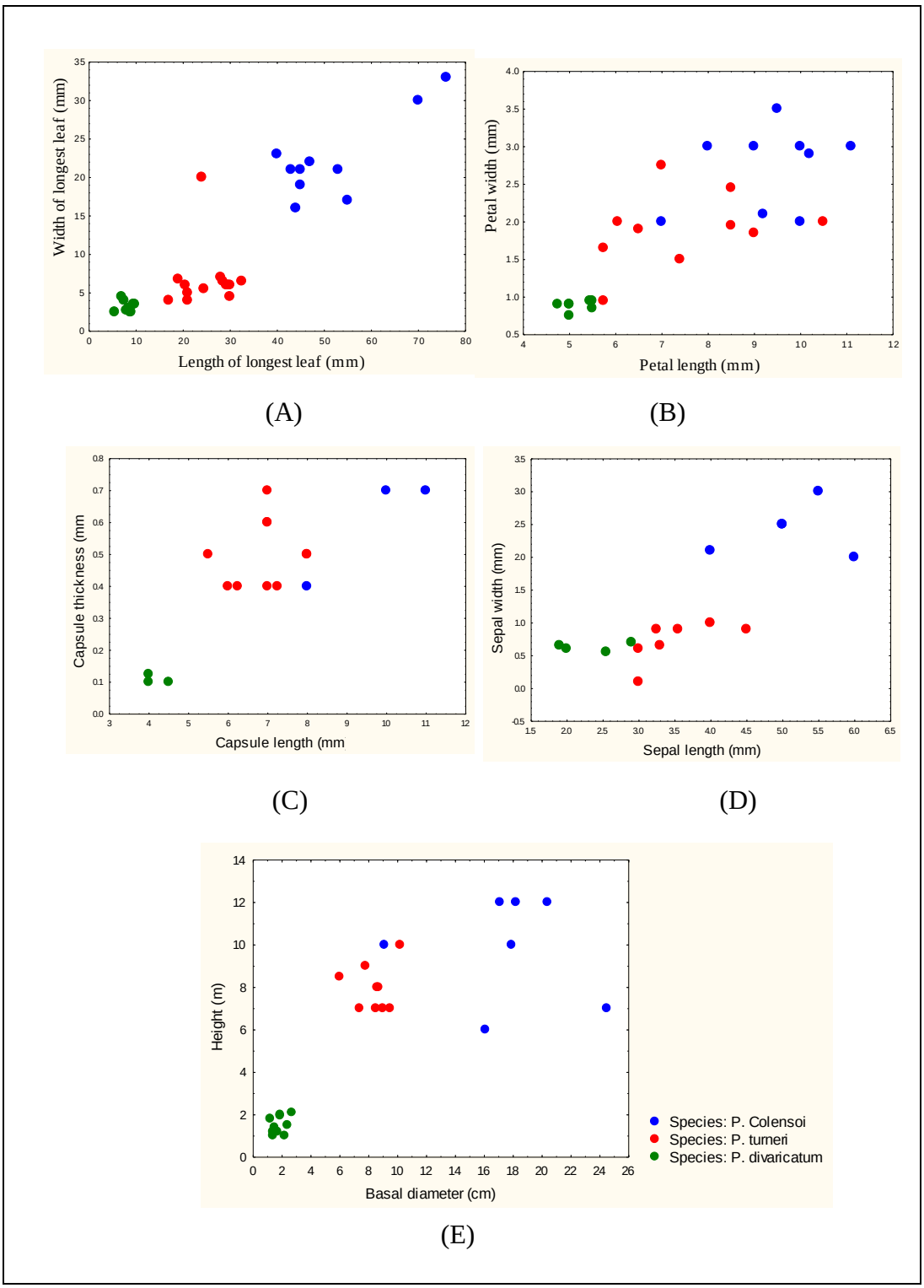


Figure 4.8. Scattergrams of character traits for the Ripia Valley study.

Table 4.7.: Wilson's (1992) character count: Means, standard deviations and results of Tukey's HSD test A * denotes data taken from Cooper (1956) and Allan (1961). All expressed in millimetres unless otherwise stated.

	(1) P. divaricatum	*(2) P. turneri*	*(3) P. colensoi*	*Significantly different from*	*Mean Intermediate?*
1. Length of longest leaf	8.22 ± 1.28	25.33 ± 4.94	28.09± 18.53	Both	Y
2. Width of longest leaf	3.17 ± 0.69	6.69 ± 3.94	22.30 ± 5.36	3	Y
3. Petiole length of longest leaf	1.02 ± 0.35	1.79 ± 0.51	7.85 ± 2.07	3	Y
4. Degree of indentation of leaves	0.54 ± 0.06	0.04 ± 0.11	0 ± 0	1	Y
5. Length of filament (male)	3.07 ± 0.1	4.73 ± 0.76	4.18 ± 0.46	1	N
6. Pedicel length	1.46 ± 0.49	7.23 ± 1.18	3.27 ± 1.71	Both	N
7. Petal length	5.17 ± 0.30	7.29 ± 1.61	9.33 ± 1.23	3	Y
8. Petal width	0.88 ± 0.07	1.89 ± 0.49	2.72 ± 0.54	3	Y
9. Sepal length	2.27 ± 0.45	3.63 ± 0.53	4.84 ± 0.76	Both	Y
10. Sepal width	0.65 ± 0.05	0.95 ± 0.07	2.42 ± 0.4	3	Y
11. Length of capsule	4.16 ± 0.29	6.75 ± 0.77	9.67 ± 1.53	Both	Y
12. Capsule thickness	0.11 ± 0.01	0.5 ± 0.11	0.6 ± 0.17	1	Y
13. Basal diameter (cm)	1.83 ± 0.48	8.82 ± 1.42	16.33 ± 4.78	Both	Y
14. Height (m)	1.51 ± 0.42	7.81 ± 1.07	9.86 ± 2.48	Both	Y

12:2

4.3.2.3 Categorical characters

Table 3.4 shows that *P. turneri* is intermediate for three out of seven categorical characters, including number of valves on capsules, leaf arrangement and hairs on carpels. For two characters, sepal fusion and surface of capsules *P. turneri* is more similar to *P. divaricatum*, and for one, hairiness of pedicels *P. turneri* is more similar to *P. colensoi*. *P. turneri* is extreme for one character, having strictly terminal flowers while *P. divaricatum* has axillary and terminal flowers, while *P. colensoi* has only axillary flowers. Figure 4.9 shows the narrow petals and sepals of *P. divaricautm*, with slightly overlapping sepals, glabrous carpels, short pedicels and small, slightly flattened capsules. Figure 4.10 illustrates the hairy carpels, narrow sepals, long pedicels, inflorescence type with large number of flowers per inflorescence, long pedicels and capsules of *P. turneri.* Figure 4.11

illustrates a *P. colensoi* flower sourced from Tongariro, and the difference in colour to that from Ripia Valley, hairy pedicels and carpels, overlapping, broad sepals and longer anthers.

Table 4.8. Number of intermediate categorical characters of Ripia Valley data.

	P. divaricatum	*P. turneri*	*P. colensoi*	
1. No. valves on capsules	2	2-3	3	Y
2. Leaf arrangement (0) alternate (1) both alternate and fascicled (3) mostly fascicled	3	1	0	Y
3. Sepal fusion (0) not fused (1) slightly imbricate at base (2) imbricate at base	0	0	1-2	N
4. Hairs on carpels (0) none (1) sparse (2) thick	0	1-2	2	Y
5. Hairs on pedicels (0) none (1) sparse (2) dense	0-1	2	2	N
6. Position of inflorescences (0) terminal only (1) terminal and axillary (2) axillary only	1	0	2	N
7. Surface of mature capsules (0) smooth (1) weakly rugose (2) rugose	1	1	1-2	N

3:4

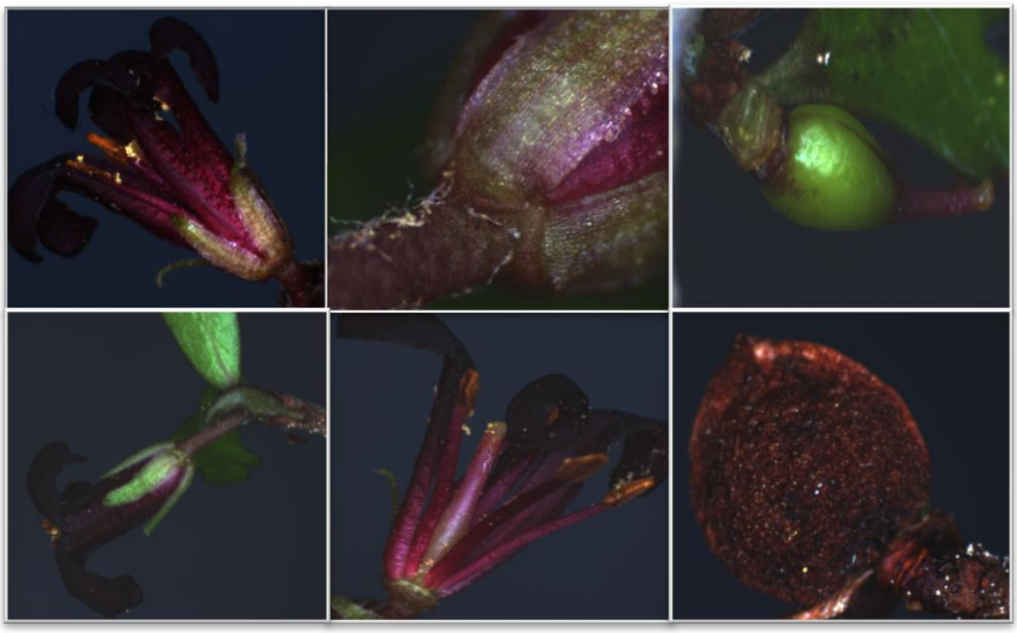


Figure 4.9. *P. divaricatum* photos**. 1 to 6 clockwise. (1)** flower, **(2)** pedicels and sepals with few hairs, **(3)** developing fruit with glabrous gynoecium, **(4)** solitary flower **(5)** glabrous gynoecium of male flower, and **(6)** capsule.

Figure 4.10. *P. turneri* flowers. (1) female flower with densely hairy gynoecium and short stamens (2) male gynoecium and androecium with hairy carpel (3) ? (4) inflorescence with many flowers with long pedicels (5) flowers of female plant (6) capsules

Figure 4.11. *P. colensoi* photos. (1) Photo of *P. colensoi* flower from outside of Ripia Valley distinctly different in colour (2) female flower with hairy carpel and short stamens (3) hairy pedicel with persistent bracts (4) male gynoecium with slender carper and long filaments and anthers (5) broad sepals (6) capsule with many seeds.

4.3.2.4 Principal Components Analysis

The PCA shows that individuals cluster together strongly within species with the first two factors explain 97.11% of the total variation. This shows that the additional characters do not add a lot of 'noise' to the data and *P. turneri* clusters towards the centre. A second PCA was conducted with floral characters only, with the first two axes accounting for 87.02% of variation, and a similar pattern. Data points for *P. turneri* and *P. colensoi* show more spread than *P. divaricatum* illustrating less variation within *P. divaricatum.*

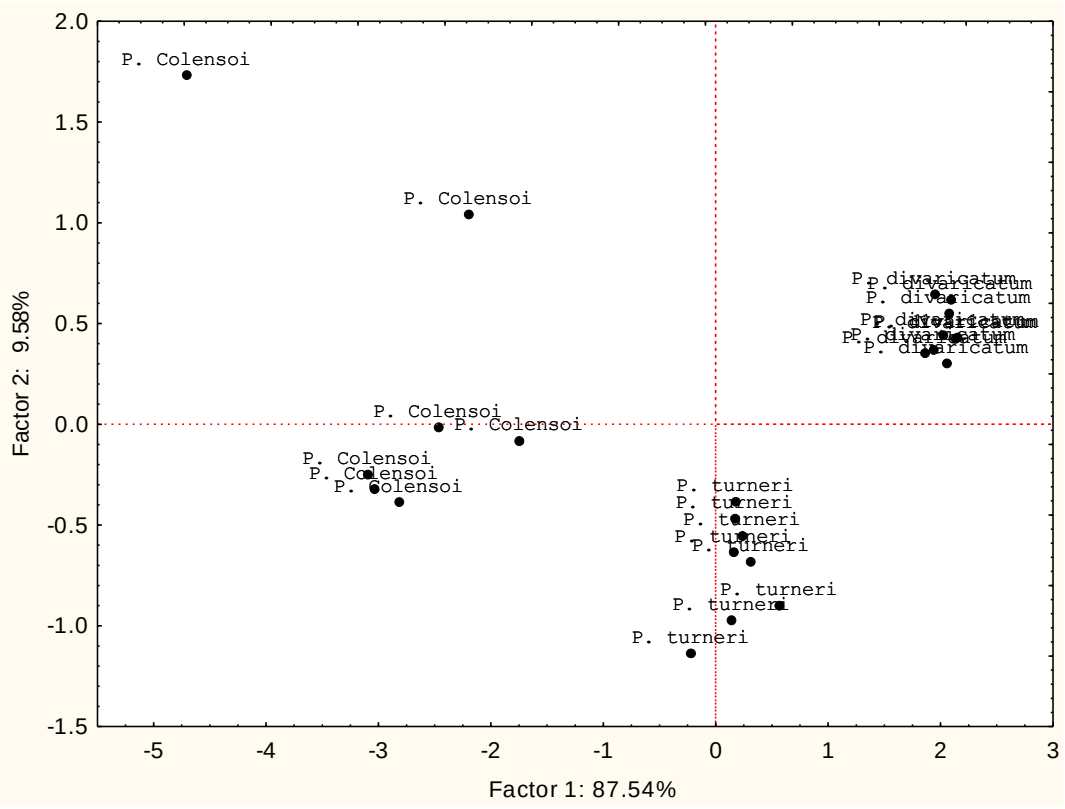


Figure 4.12. Principle component analysis of five characters including leaf length, leaf width, petiole length, basal diameter and height.

Table 4.9. Eigenvalues of the five characters used in PCA

Factor	Eigenvalue	% total variance	Cumulative eigenvalue	Cumulative %
1	4.376829	87.53658	4.376829	87.5366
2	0.478773	9.57546	4.855602	97.1120
3	0.094957	1.89915	4.950559	99.0112
4	0.038016	0.76031	4.988575	99.7715
5	0.011425	0.22850	5.000000	100.0000

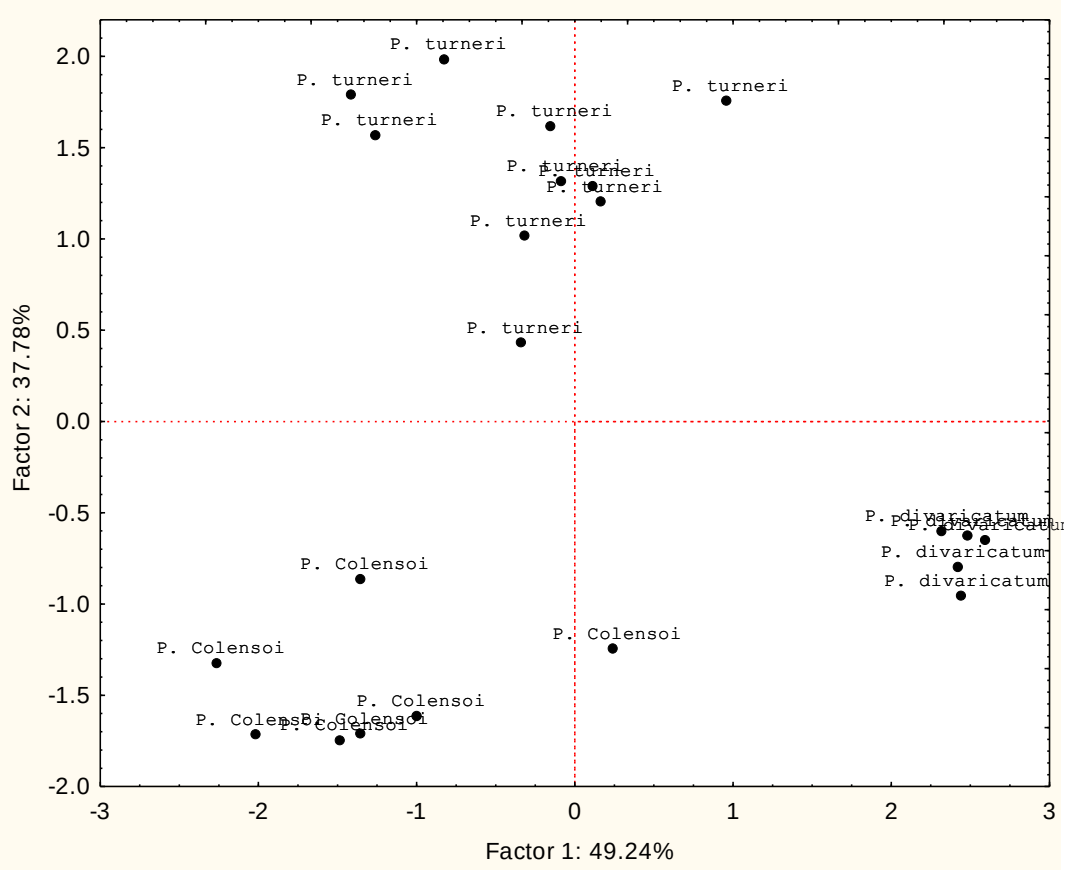


Figure 4.13. A Principle Components Analysis using five floral characters including petal length, petal width, sepal length, pedicle length and flowers per inflorescence. The first two Principle components account for 87.02% of the total variance.

Table 4.10. Eigenvalues for characters used in PCA of floral characters

Factor	Eigenvalue	% Total variance	Cumulative eigenvalue	Cumulative %
1	2.462138	49.24277	2.462138	49.2428
2	1.889185	37.78369	4.351323	87.0265
3	0.400381	8.00762	4.751704	95.0341
4	0.187264	3.74527	4.938968	98.7794
5	0.061032	1.22065	5.000000	100.0000

4.3.2.5 Hybrid Index

The traditional hybrid index of Anderson (1949) shows an intermediate peak for *P. turneri*. This method measures intermediacy across all characters, showing that overall *P. turneri* is the intermediate taxa over all eight characters. However, this method does not measure character by character intermediacy, instead it measures overall intermediacy and any individual which is morphologically similar to one species in some characteristics and similar to the other in other characteristics can cluster towards 0.5 (Wilson, 1992). The hybrid index shows that *P. colensoi* has

the largest morphological range of individuals and some individuals overall have a similar hybrid index to *P. turneri* individuals.

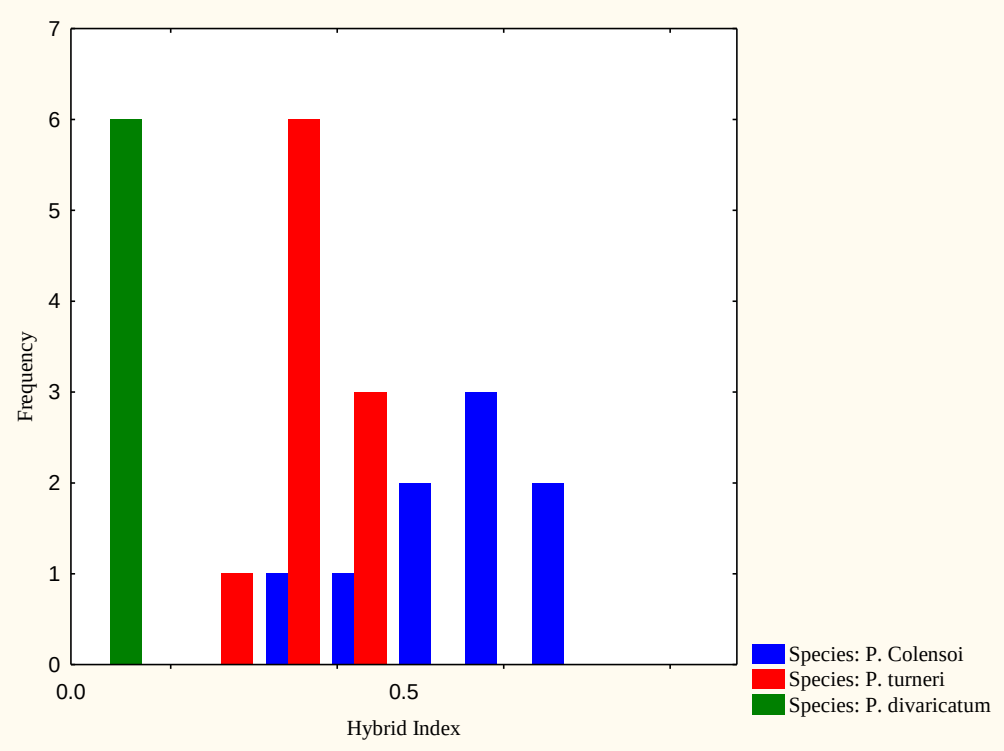


Figure 4.14. A hybrid index for eight characters including longest leaf length, longest leaf width, petiole length, sepal length, sepal width, pedicle length, basal diameter and height.

4.4 DISCUSSION

4.4.1 SAMPLE REPRESENTATION

There was not enough data to provide statistically significant results for some characters due to the small sample sizes, due to the low number of individuals on both herbarium specimens and study site specimens with either flowers or capsules. This problem is also compounded by the overlapping of ranges and outlying values for characters between taxa. This may have been resolved by using a larger number of samples for the herbarium study along with more sub-sampling within individuals to account for intra-individual variation and to reduce statistical error. However the lack of statistically significant differences between the putative parents for some characters (e.g. anther length and carpel length) may also reflect the lack of floral diversity and divergence within New Zealand *Pittosporum* (Cooper, 1956).

4.4.2 CHARACTER TRAIT REPRESENTATION

This study uses a variety of floral and vegetative characters which can be used to distinguish the putative parents. However, not all of the characters measured were found to be informative as many characters did not differ significantly between species, including hairiness of young branchlets, petioles, leaf buds, sepals, and seed shape. There were also a number of traits for which phenotypic plasticity and variation within and between populations meant taxa could not be distinguished, for example the huge variety of leaf forms on *P. divaricatum* with leaves ranging from entire to crenate to pinnatifid. A larger data set may have provided more significant differences between means for the quantitative traits measured, however ranges of many character traits in both Allan (1961) and Cooper (1956) also suggest overlap between these species in most vegetative and floral character traits (see summary in appendix 7). The Principle Components Analyses (figures 4.6, 4.12 and 4.13) show that few characters are needed to differentiate between taxa, with the majority of total variation accounted for in the first two axes. However when only using floral characters the data appears more 'noisy' with the first two axes explaining less of the total variation and all characters needed to explain the total variation (see figure 4.10 and 4.11), suggesting that floral characters do not distinguish taxa as easily as the vegetative characters used. This result is consistent with observations by Cooper (1956) who suggested that although *Pittosporum* are highly polymorphic between species in vegetative traits such as habit, leaf shape and form, floral characteristics are usually highly conserved.

4.4.3 ***HERBARIUM STUDY***

Hybrids are recognised through morphological intermediacy between the putative parental taxa, however hybrids may not be perfectly intermediate or may be extremely variable (Hardig 2000). The character count of Wilson (1992) is used to determine the proportion of character traits for which a putative hybrid is intermediate to the putative parents. The assumption of this technique is that while it is reasonable to assume that any species which has evolved through divergent evolution may be intermediate for any one trait, it is highly unlikely that divergent species would show morphological intermediacy for a large number of traits (Hardig 2000). The character count procedure produced a significant result for the

herbarium data for a wide range of characters, indicating that *P. turneri* is morphologically intermediate to *P. colensoi* and *P. divaricatum* throughout the ranges of these species, which suggests that *P. turneri* is a species of a hybrid origin. *P. turneri* has extreme values for two character traits, length of pedicle and number of flowers per inflorescence. Traits which are extreme are also expected to occur in hybrids. This often occurs directly due to transgressive segregation during hybridisation, which can affect the ecological tolerance of hybrids, and may be important in allowing the hybrid to occupy a niche which is different to that of either parent (Rieseberg 1997). Greater than 30% of characters can be found to be extreme in later generation hybrids (Rieseberg 1993).

4.4.4 RIPIA VALLEY SITE STUDY

The level of intermediacy of *P. turneri* at the Ripia Valley site shows a statistically significant result, with 12 out of 14 characters intermediate, similar to the findings of the herbarium study. This result is also consistent with a hybrid origin of *P. turneri*. Means and variation within characters that were measured were similar within the Ripia Valley populations to the results from the study of herbarium specimens. Further investigation would be needed to determine whether any individuals at this site are backcrossed with either of the putative parents, explicitly comparing genetic variation between *P. turneri* populations and populations of the putative parents where they do and do not occur in sympatry. As for the herbarium study there were some character traits for which *P. turneri* is extreme, including filament length, length of pedicle and position of inflorescences.

4.4.5 EVIDENCE FOR HYBRIDISATION IN PITTOSPORUM TURNERI

There are important considerations to make when inferring the evolutionary histories of species based on morphological studies. Some authors have entirely rejected the use of morphological characters because morphological intermediacy may not always be the result of hybridisation. The genetic basis for morphological traits is unknown, and has a non-inheritable component (Yüzbaşioğlu 2008), therefore all morphological variation may not be directly correlated to species relationships. Species which appear intermediate could be the result of convergent evolution, having been exposed to the same selection pressures (Rieseberg 1993). Morphological intermediacy could also be the result of the putative hybrid

showing plesiomorphy (the retention of ancestral characteristics), where the two putative parents are both derived from the putative hybrid (Dobzhansky 1941). Another problem with morphological studies is that later generation hybrids may deviate from the expected pattern of morphological intermediacy (Rieseberg 1993), reducing our ability to detect them. However a large number of studies have combined morphological markers with molecular methods and found that taxa identified as hybrids through molecular markers also show a distinct pattern of morphological intermediacy, and this has been found in studies of both polyploid and homoploid hybrids e.g. (Tho´ rsson 1998), (Milne 1999), (Bateman 2004), along with studies which also employ chromosome morphology e.g. (Bartoli 1998). *P. turneri* however also appears intermediate between that of the divaricating species of *Pittosporum* and the large-leaved species of the genus for capsule valve number, combining a character which is diagnostic of the divaricating species of *Pittosporum* (2-valved capsules) with a feature diagnostic of the large-leaved species (3-valved capsules) (Laing 1935). It was observed that at the Ripia Valley site study and with herbarium specimens, most *P. turneri* individuals had both 2-valved and 3-valved capsules. However because *P. turneri* also appears intermediate between other divaricating taxa and only one taxa was considered as the putative large leaved adult, there may be other taxa for which *P. turneri* exhibits overall intermediacy, including species which are now extinct. Therefore this study must be backed up by molecular work or other forms of evidence that *P. turneri* is derived from these two taxa.

4.5 CONCLUSION

This research supports the hypothesis of Godley (1985) that *P. turneri* is derived from hybridisation between a divaricating shrub and a non-divaricating tree, and that the putative parents are *P. divaricatum* and *P. colensoi.* However, this finding of morphological intermediacy must be further supported by molecular evidence as morphological intermediacy on its own does not provide proof of a hybrid origin.

CHAPTER FIVE

CROSS-POLLINATION EXPERIMENT

5.1 INTRODUCTION

Cross-pollination experiments can be used as evidence to identify hybrid species by demonstrating whether hybridisation between putative parents is possible in the wild. For example, a study by Houliston (2008) found that artificial crosses of *Phormium* species were able to produce viable, filled seed, confirming a lack of any intrinsic barriers to cross-fertilization. If cross-pollination results in the production of seed, morphological comparisons can be made between the "synthesized" hybrid and the putative natural hybrid (Godley 1985). This study investigates whether cross-pollination between *Pittosporum colensoi* and *Pittosporum divaricatum* is possible in the wild and aims to produce seedlings which can be examined to compare morphological similarities with the putative hybrid of these two species, *P. turneri.*

5.2 METHODS

Pittosporum divaricatum specimens were located at the Ripia Valley, Lochinver Station in November 2007 (GPS location: E2800102, N6240083). Ten unopened flowers on two *P. divaricatum* female plants were covered in cloth bags made of fine mesh to exclude pollinators. Flowers were not emasculated to reduce the chance of cross-pollination being unsuccessful as plants had very few, solitary flowers which were easily damaged. The site was visited each week and when flowers opened they were hand-pollinated with pollen of *P. colensoi* from a nearby location at Lochinver Station by brushing the stigma with the anther and checking using a hand lens that pollen grains had contacted the stigma. Sites were re-visited weekly for six weeks from late October through to early December 2007 to determine flowering times for *P. turneri*, *P. divaricatum* and *P. colensoi*, and to determine whether the flowering period of these species overlaps. This was done by examining 13 *P. turneri*, 13 *P. divaricatum* plants (the entire adult population) and 12 *P. colensoi* plants, and any plants which had one or more open

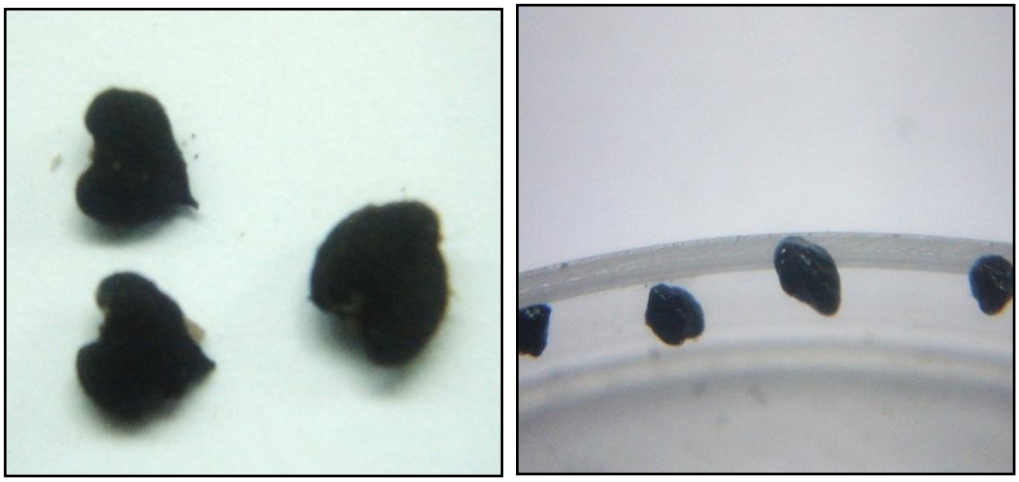
flowers visible was considered to be flowering. Half of the seeds collected from the cross-pollination experiment were sown into a mixture of 75% seed-raising mix and 25% pumice, and kept in shade-house conditions with a sheet of glass covering seed trays to regulate moisture over the winter period. The remaining half were left in damp sphagnum moss in cold storage (4 degrees) for 6 weeks then sown.

Figure 5.1. A female *P. divaricatum* with pollinator exclusion bags.

Additionally, 80 *P. divaricatum* and *P. turneri* seeds were collected to attempt to germinate seedlings from the same location and compare to any hybrid seedlings produced. They were sown in two batches of 40 seeds and treated exactly as for the hybrid seed, with one batch sown immediately and one batch left in sphagnum moss for six weeks.

5.3 RESULTS

Only four capsules were produced from cross-pollination because half of the cross-pollination experiment was lost with bags having apparently been taken by deer. Of the four capsules, 11 seeds were sown, of which only four appeared viable (see Figure 5.2B), as the other seeds were shrivelled and grain-like. All of the capsules were unusually flattened and of irregular shape as they had no or few viable seeds. However, in February 2008 ten months after sowing no hybrid seeds had germinated. Sixteen *P. turneri* seeds sown in sphagnum moss had germinated but no *P. divaricatum* seeds had germinated.



A **B**

Figure 5.2 A: Flattened, heart-shaped capsules collected from cross-pollination of *P. divaricatum* female plants. B: Photo of the four seeds from one capsule which appeared viable.

Figure 5.3. *P. turneri* seedling approximately two weeks after germination.

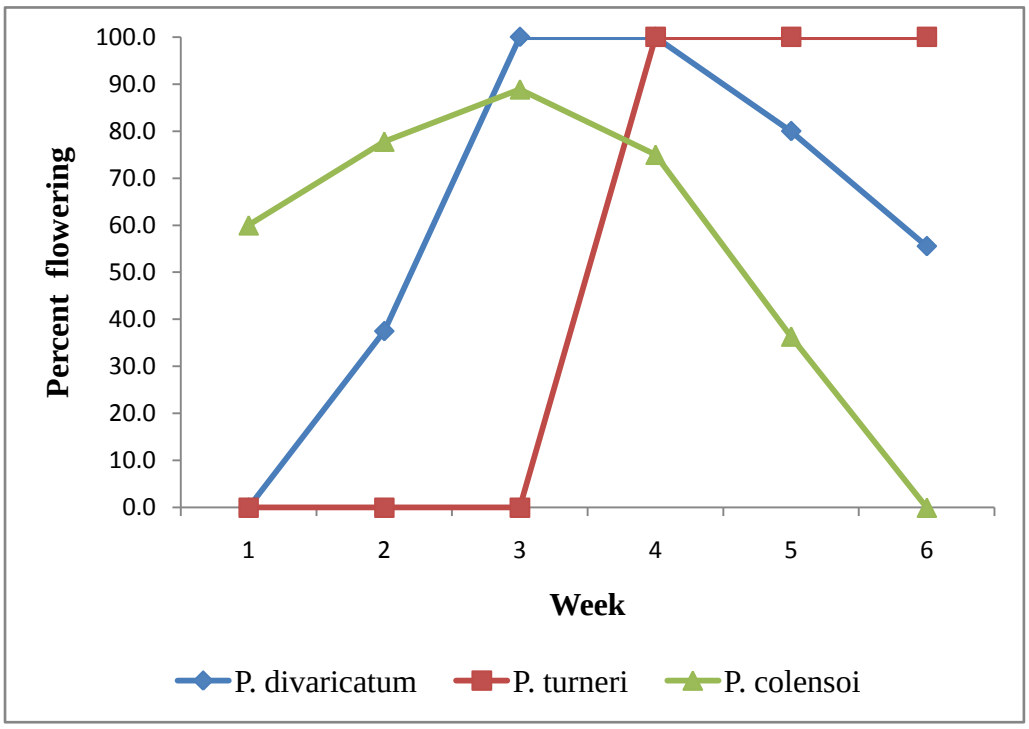


Figure 5.4. Overlap of flowering times from the last week of October-to the second week of December 2007

The trend in flowering period for each species for the 2007 flowering season (Figure 5.4) shows that all species overlap in flowering time and peak within a few weeks of each other. All of the *P. turneri* individuals were still flowering at the end of the six week period so it was not determined when flowering began to decline for this species, however the trend shows that peak flowering is later for *P. turneri* than the other two species and the proportion of individuals flowering increases more suddenly with all individuals beginning to flower in the same week.

5.4 DISCUSSION

5.4.1 *REPRODUCTIVE BARRIERS TO CROSS-POLLINATION*

In *Pittosporum* pollination is considered to be unspecialized (Webb 1999) and there are many conserved floral characters (Godley 1979), which suggests that cross-pollination may occur between closely related species if there are no intrinsic barriers to hybridisation. As no hybrid seedlings were produced in this study, the results are inconclusive in determining whether *P. colensoi* and *P. divaricatum* are inter-fertile. Only four seeds produced out of a total of four capsules collected appeared to be viable, providing a very low sample size but

indicating that cross-pollination may be possible albeit probably infrequent in the wild. There could also be many reasons why the seeds did not germinate, such as problems to do with glasshouse conditions or seed dormancy. However, pollination may also have occurred before the flowers were enclosed, or self-fertilization could have occurred, and there is no way of confirming whether the hand-pollination was successful unless the seeds germinate.

5.4.2 *OVERLAP IN FLOWERING PERIOD*

P. colensoi starts to flower before *P. divaricatum* which suggests that if *P. colensoi* and *P. divaricatum* are able to hybridise *P. divaricatum* is more likely to be the maternal parent because most female *P. colensoi* flowers will be fertilised by the time *P. divaricatum* starts to flower and this is consistent with the findings of sequencing of the maternally inherited *trnT-trnL* region of chlorolplast DNA (see Chapter Two), as *P. turneri* has the same sequence as *P. divaricatum*. However, all taxa overlap in flowering time, suggesting that if *P. turneri* is a hybrid between *P. colensoi* and *P. divaricatum* introgression may have occurred or may still still be occuring at this site. This may also be the case between *P. turneri* and *P. divaricatum*, as hybrids between these taxa have reported on the basis of morphological intermediacy (Ecroyd 1994).

5.4.3 FUTURE DIRECTIONS

Godley (1985) stressed the importance of long-term experiments where hybrids are observed from seedling stage through to maturity to identify whether the pattern of inheritance of morphological traits changes during ontogeny. Hybrid individuals between known parental species are also useful because they can be studied using molecular methods which can aid in determining how other hybrids can be recognised. Future studies could use broader-scale, statistically designed experiments, with some flowers enclosed without being cross-pollinated to determine whether selfing occurs. The level of inter-fertility between these species could then be quantified as evidence for or against the ability of these species to cross and produce hybrid offspring.

3.5 CONCLUSION

This study shows that flowering time overlaps where *P. divaricatum*, *P. colensoi* and *P. turneri* occur in sympatry, indicating that there are unlikely to be any external barriers which could prevent cross-fertilization. However no seeds from crosses between these species germinated, therefore we cannot draw any conclusions about the origin of *P. turneri* from this aspect of the study.

CHAPTER SIX

CONCLUSION

6.1 THE ORIGIN OF PITTOSPORUM TURNERI

The criteria used to support a hybrid origin of a species is an additive profile of parental nuclear markers and the organelle genome of one of one of the putative parental taxa (Wolfe 1998). This study demonstrates that *P. turneri* has an identical sequence to *P. divaricatum* for the *trn*T-*trn*L region of chloroplast DNA, indicating that the maternal parent of *P. turneri* is likely to be *P. divaricatum*. *P. turneri* was not well resolved in its placement within the New Zealand *Pittosporum* based on analyses of the nuclear ITS region, however *P. turneri* exhibits a unique ISSR profile of bands, combining the bands of *P. divaricatum* and *P. colensoi*. This suggests that *P. turneri* may be derived from hybridisation between these two species. This relationship however, needs to be confirmed by sampling these taxa more broadly with a larger number of primers, as there are inherent problems and limitations in this technique. Morphological analyses further support a hybrid origin of *P. turneri,* as this species combines a large proportion of morphological traits from both taxa, including leaf, floral, capsule and growth form characteristics. The use of allozymes and the cross-pollination experiment however did not provide any evidence for or against a hybrid origin, as too few allozyme loci were resolved, and no hybrid progeny was produced. Analysis of the ITS region using two *P. turneri* individuals from different populations exhibited variation in sequence. This illustrates that population level genetic variation is an important consideration in inferring relationships.

6.2 GODLEY'S HYPOTHESIS

This study suggests that species with a divaricating juvenile form and a non-divaricicating, arborescent form may be descended from hybridisation between a divaricating shrub and a non divaricating tree. In most cases this cannot be proven, as the parental taxa are likely to be extinct. However, the results of this study have implications on the driving forces of evolution which have led to the heteroblastic form, indicating that it may be due to hybridisation and the

associated rapid colonisation of new habitats, as a result of rapid climate change or a dynamic landscape.

6.3 RELATIONSHIPS WITHIN THE NEW ZEALAND PITTOSPORUM

The use of the *trn*T-*trn*L region of chloroplast DNA improves resolution of some closely related taxa of New Zealand *Pittosporum.* This study supports several clades not resolved using phylogenetic analysis of the ITS region alone, including a clade of *P. anomalum*, *P. patulum* and *P. virgatum*, and a clade of *P. crassifolim* and *P. huttonianum*. Several taxa are implicated as having been involved in introgression during their evolutionary history, with different placement between trees based on the *trn*T-*trn*L and ITS regions. These include *P. eugenoides*, *P. umbellatum* and *P. dallii.* The closely related species of *Pittosporum* provide an opportunity to study the process of speciation in New Zealand after recent colonization. Future studies should focus on the unresolved taxa which have conflicting signals, taking into account the variation which occurs within populations. This may also shed light on the population level processes which have led to speciation in this group. Potentially the greater understanding of the relationships between closely related species in *Pittosporum* can lead to a greater understanding of the processes that have shaped the New Zealand flora.

REFERENCES

Allan G, Clark C, Rieseberg L 1997. Distribution of parental DNA markers in Encelia virginensis (Asteraceae: Heliantheae), a diploid species of putative hybrid origin. Plant Systematics and Evolution 205: 205-221.

Allan HH 1961. Flora of New Zealand, Government Printer, Wellington.

Allendorf FW, Leary RF, Spruell P, Wenburg JK 2001. The problems with hybrids: setting conservation guidelines. Trends in Ecology and Evolution 16: 613-622.

Anderson E 1949. Introgressive hybridisation. New York, Hafner.

Anderson S 2003. The relative importance of birds and insects as pollinators of the New Zealand flora. New Zealand Journal of Ecology 27(2): 83-94.

Arnold M, Hamrick J, Bennett B 1990. Allozyme variation in Louisiana irises: A test for introgression and hybrid speciation. Heredity 65: 297-306.

Arnold ML 1997. Natural hybridization and evolution. May RM, Harvey, P.H. ed. New York, Oxford University Press.

Arnold ML, Hodges, S.A 1995. Are natural hybrids fit or unfit relative to their parents? Trends in Ecology and Evolution 10(2): 67-71.

Avise J 1994. Molecular markers, natural history and evolution. New York, Chapman and Hall.

Baldwin BG, Sanderson MJ, Porter JM 1995. The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny Annals of the Missouri Botanical Garden 82: 247-277.

Bartoli A, Tortosa, RD 1998. Intermediacy between Grindelia chiloensis and Haplopappus pectinatus (Astereae, Asteraceae). Taxon 47(2): 337-345.

Barton NH 2001. The role of hybridization in evolution. Molecular Ecology 10(3): 551-568.

Bateman R, Hollingsworth, PM 2004. Morphological and Molecular Investigation of the Parentage and Maternity of Anacamptis xalbuferensis (A. fragrans x A. robusta), a New Hybrid Orchid from Mallorca, Spain. Taxon 53(1): 43-54.

Black W 1997. Explanation of RAPDPLOT 3.0. Documentation file distributed with the RAPDPLOT program.

Blair MW, Panaud O, McCouch S 1999. Inter-simple sequence repeat (ISSR) amplificatio for analysis of microsatellite motif frequency and

fingerprinting in rice (Oryza sativa L.). Theoretical and Applied Genetics 98: 780-792.

Bond W, WG L, JM C 2004. Plant structural defences against browsing birds: a legacy of New Zealand's extinct moas Oikos 104: 500–508.

Borsche TK, Hilu W, Quandt D, Wilde V, Neinhuis C, Barthlott W 2003. Noncoding plastid trnT-trnF sequences reveal a well resolved phylogeny of basal angiosperms. Journal of Evolutionary Biology 16: 558-576.

Buerkle CA, Morris R, Asmussen M, Rieseberg L 2000. The likelihood of homoploid hybrid specation. Heredity 84: 441-451.

Burns KC, Dawson JW 2006. A morphological comparison of leaf heteroblasty between New Caledonia and New Zealand. New Zealand Journal of Botany 44: 387-396.

Burrows CJ 1994. Fruit types and seed dispersal modes of woody plants in Ahuriri Summit Bush, Port Hills, western Banks Peninsula, Canterbury, New Zealand. New Zealand Journal of Botany 32: 169-181.

Carlquist S 1974. Island Biology, Columbia University Press, New York.

Carlquist S 1981. Wood anatomy of Pittosporaceae. Allertonia 2: 355–392.

Carr GD 1995. A fully fertile intergeneric hybrid derivative from *Argyroxiphium sandwicense* spp. *macrocephalum* x *Dubautia menziessii* (Asteraceae) and its relevance to plant evolution in the Hawaiian islands. American Journal of Botany 82: 1574-1581.

Cayzer LW 1997. Revision of the systematics and biogeography of the family Pittosporaceae in Australia. Australian National University, Canberra.

Cayzer LW CM, Telford IRH 2000a. Auranticarpa, a new genus of Pittosporaceae from northern Australia. Australian. Systematic Botany 13: 903–917.

Cayzer LW CM, Telford IRH 2000b. Revision of Pittosporum (Pittosporaceae) in Australia. Australian Systematic Botany 13: 845–902.

Chandler G, Plunkett G 2004. Evolution in Apiales: nuclear and chloroplast markers together in (almost) perfect harmony. Journal of the Linnean Society 144: 123-147.

Chandler GT, Plunkett G, Pinney S, Cayzer L, Gemmill C 2007. Molecular and morphological agreement in *Pittosporaceae*: phylogenetic analysis with nuclear ITS and plastid *trnl-trnf* sequence data. Australian Systematic Botany 20: 390-401.

Clarkson BD 1988. A natural intergeneric hybrid, Celmisia gracilenta x Olearia arborescens (Compositae) from Mt Tarawera, New Zealand. New Zealand Journal of Botany 26: 325-331.

Clarkson BD, Clarkson BR 1994. Ecology of an elusive endemic shrub Pittosporum obcordatum Raoul. New Zealand Journal of Botany 32: 155-168.

Clegg M, Gaut B, Duvall M, Davis J 1993. Inferring plant evolutionary history from molecular data. New Zealand Journal of Botany 31: 307-316.

Cockayne L 1911. Observations concerning evolution derived from ecological studies in New Zealand. Transactions of the New Zealand Institute 44: 1-50.

Cockayne L 1923. Hybridism in the New Zealand Flora. New Phytologist 22(3): 105-127.

Cockayne L 1928. The Vegetation of New Zealand ed. 2. Die Vegetation der Erde 14.

Conner HE 1985. Biosystematics of higher plants in New Zealand 1965-1984. New Zealand Journal of Botany 23: 613-644.

Conte L, Cotti C, Cristofolini G 2007. Molecular evidence for hybrid origin of Quercus crenata Lam. (Fagaceae) from Q-cerris L. and Q-suber L. Plant Biosystems 141(2): 181-193.

Cooper RC 1956. The Australian and New Zealand species of Pittosporum. Annals of the Missouri Botanic Garden 43(2): 87-188.

Crawford DJ 1983. Phylogenetic and systematic inferences from electrophoretic studies. In: Tanksley SD, Orton, T.J. ed. Isozymes in plant genetics and breeding. Amsterdam, Elsevier Science Publishers.

Crawford DJ 1990. Enzyme electrophoresis and plant systematics. In: Soltis DE, and Soltis, Pamela S. ed. Isozymes in Plant Biology. London, Chapman and Hall Ltd.

Cruzan M, Arnold M 1993. Ecological and genetic assiciations in an *Iris* hybrid zone. Evolution 47(5): 1432-1445.

Day JS 1998. Light conditions and the evolution of heteroblasty (and the divaricating form) in New Zealand. New Zealand Journal of Ecology 22(1): 43-54.

de Lange P 1998. Pittosporum ellipticum subsp. serpentinum (Pittosporaceae) --a new ultramafic endemic from the Surville Cliffs, North Cape, New Zealand

New Zealand Journal of Botany 36: 389-397.

de Lange P, Norton D, Heenan P, Courtney S, Molloy B, Ogle C, Rance B, Johnson P, RA H 2004. Threatened and uncommon plants of New Zealand. New Zealand Journal of Botany 42: 45-76.

Dobzhansky T 1941. Genetics and the Origin of Species. Doebley J ed. New York, Columbia University Press.

Druce A 1977. Trees, shrubs, and lianes of New Zealand (including wild hybrids). Botany Division, Department of Scientific and Industrial Research.

Ecroyd CE 1994. Regeneration of *Pittosporum turneri* communties. Conservation advisory science notes 99.

Ellstrand NC, Schierenbeck K 2000. Hybridisation as a stimulus for the evolution of invasiveness in Plants? Proceedings of the National Academy of Sciences, USA 97: 7043-7050.

Excoffier L, Laval G, Schneider S 2005. Arlequin: An integrated software package for population genetics data analysis. Version 3.0.

Felsenstein J 1985. Confidence limits on phylogeneis: An approach using the bootstrap. Evolution 39: 783-791.

Flemming CA 1979. The geological history of New Zealand and its life, Auckland University Press, Auckland.

Fromm M, Hattemer HH 2003. Inheritance of allozymes and hybridization in two European Tilia species. Heredity 91: 337-344.

Fukuda T, Yokoyama J, Tsukaya H 2003. Phylogenetic relationships among species in the genera *Chisocheton* and *Guarea* that have unique indeterminate leaves as inferred from sequences of chloroplast DNA. International Journal of Plant Sciences 164: 13-24.

Gallez G, Gottlieb L 1982. Genetic evidence for the hybrid origin of the diploid plant Stephanomeria diegensis. Evolution 36: 1158-1167.

Gemmill C, E.C. Allan, G.J., Wagner, W.L., Zimmer, E.A. 2002. Evolution of insular pacific Pittosporum (Pittosporaceae): origin of the Hawaiian radiation. Molecular phylogenetics and evolution 22(1): 31-42.

Givnish TJ, Sytsma KJ 1997. Molecular Evolution and Adaptive Radiation, Cambridge University Press, Cambridge, U.K.

Godley EJ 1979. Leonard Cockayne and Evolution. New Zealand Journal of Botany 17: 197-215.

Godley EJ 1979. Flower biology in New Zealand. New Zealand Journal of Botany 17: 441-66.

Godley EJ 1985. Paths to Maturity. New Zealand Journal of Botany 23: 687-706.

Godwin ID, Sangduen N, Kunanuvatchaidach R, Piperidis G, Adkins SW 1997. RAPD polymorphisms among variant and phenotypically normal rice (Oryza sativa var.indica) somaclonal progenies. Plant Cell Reports 16(5): 320-324.

Goebel K 1900. Organography of plants. Pt 1. Oxford, Clarendon Press.

Gottlieb LD 1984. Genetics and morphological evolution in plants. American Naturalist 123: 681-709.

Gowda M 1951. The genus *Pittosporum* in the Sino-Indian region. Journal of the Arnold Arboretum 32: 263-343.

Grant V 1981. Plant Speciation. 1 ed, Columbia University Press, New York.

Greenwood RM, Atkinson IAE 1977. Evolution of divaricating plants in New Zealand in relation to moa browsing. Proceedings of the New Zealand Ecological Society 24: 21-33.

Gross BL, Schwarzbach AE, Rieseberg LH 2003. Origin(s) of the diploid hybrid species Helianthus deserticola (Asteraceae). Am. J. Bot. 90(12): 1708-1719.

Gross BL, Rieseberg LH 2005. The Ecological Genetics of Homoploid Hybrid Speciation. J Hered 96(3): 241-252.

Haas JE 1977. The pacific species of *Pittosporum* Banks ex Gaertn. Allertonia 1(2): 73-167.

Hansen I, Brimer L, Mølgaard P 2003. Herbivore-deterring secondary compounds in heterophyllous woody species of the Mascarene Islands. . Perspectives in Plant Ecology Evolution and Systematics 6 187-203.

Hardig TM, Brunsfeld, S.J., Fritz, RS., Morgan, M, and Orians, M 2000. Morphological and molecular evidence for hybridization and introgression in a willow (*Salix*) hybrid zone. Molecular Ecology 9: 9-24.

Hathaway LA 2001. Phylogenetic relationships of the New Zealand Pittosporum (Pittosporaceae) inferred from ITS sequences of rDNA. University of Waikato.

Heenan PB 1997. Heteroblasty in Carmichaelia, Chordospartium, Corallospartium, and Notospartium (Fabaceae - Galegeae) from New Zealand. New Zealand Journal of Botany 35: 243-249.

Heenan PB, de Lange PJ, Wilton AD 2001. Sophora (Fabaceae) in New Zealand: Taxonomy, distribution and phylogeograpy. New Zealand Journal of Botany 39(1): 17-54.

Heenan PB, Dawson MI, Redmond DN, Wagstaff SJ 2005. Relationships of the New Zealand mountain ribbonwoods (Hoheria glabrata and H. lyallii: Malvaceae), based on molecular and morphological data. New Zealand Journal of Botany 43: 527-549.

Hegarty MJ, Hiscock SJ. 2005. Hybrid speciation in plants: new insights from molecular studies. New Phytologist 165: 411-423.

Houliston GJ, Heenan PB, Smissen RD 2008. Experimental crosses in *Phormium* confirm the lack of intrinsic barriers to interspecific hybridisation and the fertility of putative wild hybrids. New Zealand Journal of Botany 46: 393-399.

Hunter RL, Markert CL 1957. Histochemical demonstration of enzymes separated by zone electrophoresis in starch gels. Science 125: 1294-1295.

Ito M, Soejima A, Ono M 1997. Allozyme diversity of *Pittosporum* (Pittosporaceae) on the Bonin (Ogasawara) Islands. Journal of Plant Research 110: 455-462.

James JK, Abbott RJ 2005. Recent, Allopatric, Homoploid Hybrid Speciation: The Origin of Senecio squalidus (Asteraceae) in the British Isles from a Hybrid Zone on Mount Etna, Sicily. Evolution 59(12): 2533-2547.

Lai Z, Gross BL, Zou Y, Andrews J, Rieseberg L, H. 2006. Microarray analysis reveals differential gene expression in hybrid sunflower species. Molecular Ecology 15: 1213-1227.

Laing RM, Gourlay HW 1935. The small-leaved species of the genus Pittosporum occurring in New Zealand with descriptions of new forms. Transactions of the Royal Society of New Zealand 65: 44-69.

Lexer C, Welch M, Raymond O, LH R 2003. The origin of ecological divergence in *Helianthus paradoxus* (Asteraceae): selection on transgressive characters in a novel hybrid habitat Evolution 57(9): 1989 - 2000.

Linn´e C 1760. Disquisitio de sexu plantarum, ab Academia Imperiali Scientiarum Petropolitana praemio ornata. Amoenitates Academicae 10: 100-131.

Maki M, Murata J 2001. Allozyme analysis of the hybrid origin of *Arisaema ehimense* (Araceae). Heredity 86: 8-93.

Mcgregor CEC, Lambert M, Greyling J, Louw H, Warnick L 2000. A comparitive assessment of DNA fingerprinting techniques (RAPD, ISSR, AFLP and SSR) in tetraploid potato (*Solanum tuberosum* L.) germplasm. Euphytica 113: 135-144.

Mallet J 2005. Hybridization as an invasion of the genome. Trends in Ecology and Evolution 20(5): 229-237.

Markert C, L. 1975. Biology of Isozymes. Bioscience 25(6): 365-368.

Markham KR, Godley EJ 1972. Chemotaxonomic studies in *Sophora* 1. An evaluation of *Sophora microphylla*. . New Zealand Journal of Botany 10: 627-640.

McCauley R, Ballard H 2002. Inferring nativity and biogeographic affinities of central and marginal populations of Froelichia floridana (Amaranthaceae)

from Inter-Simple Sequence Repeat (ISSR) Markers. Journal of the Torrey Botanical Society 129(4): 311-325.

McGlone, M.S W, C.J 1981. Selective forces influencing the evolution of divaricating plants. New Zealand Journal of Ecology 4: 20-28.

Metcalf L 1987. The Cultivation of New Zealand Trees and Shrubs. Auckland Reed Methuen.

Miller M 1997. Tools for population genetic analyses (TFPGA) 1.3: A Windows program for the analysis of allozyme and molecular population genetic data.

Milne RI, Abbott, R. J., Wolff, K, Chamberlain, D.F. 1999. Hybridization among Sympatric Species of Rhododendron (Ericaceae) in Turkey: Morphological and Molecular Evidence. American Journal of Botany 86(12): 1776-1785.

Mraz P, Chrtek J, Fehrer J, Plackova I 2005. Rare recent natural hybridization in *Hieracium*s. str. - evidence from morphology, allozymes, and chloroplast DNA. Plant Systematics and Evolution 255: 177-192.

Mummenhoff K, Linder P, Friesen N, Bowman JL, Lee J-Y, Franzke A 2004. Molecular evidence for bicontinental hybridogenous genomic constitution in Lepidium sensu stricto (Brassicaceae) species from Australia and New Zealand. Am. J. Bot. 91(2): 254-261.

Nei M 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics 89: 583-590.

Neuffer B, Jahncke P 1997. RAPD Analyses of Hybridization Events in Cardamine (Brassicaceae). Folia Geobotanica & Phytotaxonomica 32(1): 57-67.

Olmstead RG, Palmer JD 1994. Chloroplast DNA systematics: A review of methods and data analysis. American Journal of Botany 81(9): 1205-1224.

Philipson WR 1964. Habit in relation to age in New Zealand trees. Maheshwari commemorative volume, Journal of the Indian Botanical Society. Maheshwari commemorative volume 42A: 167-179.

Plunkett G, Chandler G, Lowry P, Pinney S, Sprenkle T 2004. Recent advances in understanding Apiales and a revised classification. South African Journal of Botany 70(3): 371-381.

Pole MS 1994. The New Zealand flora—entirely long-distance dispersal? Journal of Biogeography 21: 625-635.

Rakoczy-Trojanowska M, Bolibok H 2004. Characteristics and a comparison of three classes of microsatellite-based markers and their application in plants. Cellular & Molecular Biology Letters 9(2): 221-238.

Ranker T, Haufler C, Soltis P, DS S 1989. Genetic evidence for allopolyploidy in the neotropical fern *Hemionitis pinnatifida* (Adiantaceae) and the reconstruction of an ancestral genome. Systematic Botany 14: 439-447.

Rattenbury JA 1961. Cyclic hybridization as a survival mechanism in the New Zealand forest flora. Evolution 16: 348-363.

Raven PH 1973. Evolution of subalpine and alpine plant groups in New Zealand. New Zealand Journal of Botany 11: 177-200.

Rieseberg LH, Carter R, Zona S 1990. Molecular Tests of the Hypothesized Hybrid Origin of Two Diploid Helianthus Species (Asteraceae). Evolution 44(6): 1498-1511.

Rieseberg L, SM B-S, A L, DM A 1991. Phylogenetic and systematic inferences from chloroplast DNA and isozyme variation in Helianthus sect. Helianthus (Asteraceae). Systematic Botany 16: 50-76.

Rieseberg LH 1991. Homoploid reticulate evolution in Helianthus (Asteraceae): evidence from ribosomal genes. Am J Bot 78: 1218-1237.

Rieseberg LH 1997. Hybrid origins of plant species. Annual Review of Ecology and Systematics 28: 359-389.

Rieseberg LH, Carter R, Zona S 1990. Molecular tests of the hypothesized hybrid origin of two diploid *Helianthus* species (Asteraceae). Evolution 44(6): 1498-1511.

Rieseberg LH, Carney, SE 1998. Plant hybridization. New Phytologist 140: 559-624.

Rieseberg LH, Archer MA, Wayne RK, Rieseberg LH 1999. Transgressive segregation, adaptation and speciation. Heredity 83(4): 363-372.

Rieseberg LH, Baird, Stuart J. E., Gardner, Keith A. 2000. Hybridization, introgression, and linkage evolution. Plant Molecular Biology 42: 205-224.

Rieseberg LH, Ellstrand, NC 1993. What can molecular and morphological markers tell us about plant hybridization? Critical reviews in plant sciences 12(3): 213-241.

Ruas PM, Ruas CF, Rampim L, Carvalho VP, Ruas EA, Sera T 2003. Genetic relationships in Coffea species and parentage determination of interspecific hybrids using ISSR (inter-simple sequence repeat) markers. Genetics and Molecular Biology 26(3): 319-327.

Sang T, Crawford DJ, Stuessy TF 1995. Documentation of reticulate evolution in Peonies (Peonia) using internal transcribed spacer sequences of nuclear ribosomal DNA: Implications for biogeography and concerted evoution. Proc. Natl. Acad. Sci. 92: 6813-6817.

Sang T, Crawford DJ, Stuessy TF 1997. Chloroplast DNA phylogeny, reticulate evolution, and biogeography of Paeonia (Paeoniaceae). Am. J. Bot. 84(8): 1120-1136.

Seehausen O 2004. Hybridization and adaptive radiation. Trends in Ecology and Evolution 19(4): 198-207.

Shaw P 2003. Multivariate statistics for the Environmental Sciences, Hodder-Arnold.

Shaw J, Lickey EB, Beck JT, Farmer SB, Liu W, Miller J, Siripun KC, Winder CT, Schilling EE, Small RL 2005. The tortoise and the hare II: relative utility of 21 noncoding chloroplast DNA sequences for phylogenetic analysis. Am. J. Bot. 92(1): 142-166.

Soltis D, Haufler C, Darrow D, Gastony G 1983. Starch gel electrophoresis of ferns: a compilation of grinding buffers, gel and electrode buffers, and staining schedules. American Fern Journal 73: 9-27.

Soltis D, Soltis, PS 1999. Choosing an approach and an appropriate gene for phylogenetic analysis. In: Soltis DE, Soltis, Pamela S, Doyle, Jeff, J ed. Molecular systematics of plants II DNA sequencing, Kluwer academic publishers.

Soltis DE, Kuzoff RK 1995. Discordance between Nuclear and Chloroplast Phylogenies in the Heuchera Group (Saxifragaceae). Evolution 49(4): 727-742.

Taberlet P, Gielly L, Pautou G, Bouvet J 1991. Universal primers for amplification of three non-coding regions of chloroplast DNA. Plant Molecular Biology 17: 1105-1109.

Tho´ rsson ÆT, Salmela, E, Anamthawat-Jo´nsson, K 1998. Morphological, cytogenetic, and molecular evidence for introgressive hybridization in birch. The American genetic association 92: 404-408.

Ungerer MC, Baird SJE, Pan J, Rieseberg LH 1998. Rapid hybrid speciation in wild sunflowers. Proceedings of the National Academy of Sciences, USA 95: 11757-11762.

Wardle P 1963. Evolution and distribution of the New Zealand flora, as affected by quarternary climates. New Zealand Journal of Botany 1(1): 3-17.

Webb C, Lloyd D, Delph L 1999. Gender dimorphism in indigenous New Zealand seed plants. New Zealand Journal of Botany 37: 119-130.

Webb CJ, Kelly D 1993. The reproductive biology of the New Zealand flora. Trends in Ecology and Evolution 8(12): 442-447.

Weeden NF, Wendel JF 1990. Genetics of Plant Isozymes. In: Soltis DE, Soltis, Pamela S. ed. Isozymes in Plant Biology. London, Chapman and Hall Ltd.

Wichman SR, Wright SD, Cameron EK, Keeling DJ, Gardner RC 2002. Elevated genetic heterogeneity and Pleistocene climatic instability: inferences from nrDNA in New Zealand Coprosma (Rubiaceae). Journal of biogeography 29: 943-954.

Wilkinson HP 1992. Leaf anatomy of the Pittosporaceae R.Br. Botanical Journal of the Linnean Society 110: 1-59.

Wilson P 1992. On inferring hybridity from morphological intermediacy. Taxon 41(1): 11-23.

Wilson H, Galloway T 1993. Small-leaved shrubs of New Zealand. Christchurch, Manuka Press.

Wisseman V 2007. Plant evolution by means of hybridization. Systematics and Biodiversity 5(3): 243-253.

Wolfe A, WJ E 1995. Evidence of chloroplast capture and pollen-mediated gene flow in *Penstemon* sect, *Peltanthera* (Scrophulariaceae): an evaluation of diploid hybrid speciation and evidence for introgression. American Journal of Botany 80: 1082-1094.

Wolfe AD, Liston A 1998. Contributions of PCR-based methods to plant systematics and evolutionary biology. In: Soltis DE SP, Doyle JJ ed. Plant Molecular Systematics II New York, Chapman and Hall. Pp. 43-86.

Wolfe AD, Xiang, Q, Kephart, S.R 1998. Assessing hybridization in natural populations of *Penstemon* (Scrophulariaceae) using hypervariable intersimple sequence repeat (ISSR) bands. Molecular Ecology 7: 1107-1125.

Wu C-I 2001. The genic view of the process of speciation. Journal of Evolutionary Biology 14(6): 851-865.

Yüzbaşioğlu E DM, Ozcan S. 2008. Natural hybridization between Phlomis lycia D. Don P. bourgaei Boiss., (Lamiaceae) revealed by RAPD markers. Genetica 133: 13-20.

Zhou JL, Jie YC, Jiang YB, Zhong YL, Liu YH, Zhang J 2005. Development of simple sequence repeats (SSR) markers of ramie and comparison of SSR and inter-SSR marker systems. Progress in Natural Science 15(2): 137-142.

Zietkiewicz E, Rafalski A, Labuda D 1994. Genome Fingerprinting by Simple Sequence Repeat (SSR)-Anchored Polymerase Chain Reaction Amplification. Genomics 20(2): 176-183.

APPENDICES

APPENDIX 1: DNA Isolation (Ray Littler and Dick Wilkins, University of Waikato)

Homogenisation buffer:

• EDTA Ethylenediaminetetraacetic acid	20mM
• CTAB Hexadecyltrimethylammonium bromide	2 %
• PVP-40 Polyinvylpyrrolidone MW 40,000	w/v
• DIECA Diethyldithiocarbamic acid	4mM
• Tris- HCl (pH 8)	100mM
• NaCl	1.42M

Other Chemicals needed for procedure:

- 2-mercaptoethanol (3.49ul per reaction)
- RNase A (10mg/ml)
- Chloroform: Isoamyl alcohol (24:1)
- Isopropanol
- 100% ethanol
- 70% ethanol
- TE buffer

Preparation:

- Label and U.V 2x 1.5ml Microcentrifuge tubes per sample
- Collect ice and Liquid Nitrogen
- Collect Mortar and Pestles (one per sample)
- Add 500ul (X no. of samples) of Homogenisation Buffer to a15ml Conical tube, Then add 3.49ul of 2-mercaptoethanol (X no. of samples) to the conical tube and Invert to mix
- Aliquot 500ul of H. Buffer + 2-mercaptoethanol mix into the first set of U.Ved microcentrifuge tubes and sit on ice.

1: Weigh out about 0.7g (this varies with species) of frozen leaf material per sample then grind to a fine powder using Liquid Nitrogen. Scrape powder into microcentrifuge tube on ice Mix thoroughly by flicking tube (the buffer and leaf material should make a thick solution), then return to ice until all samples have been ground.

2: Vortex samples at high speed then incubate in the Thermomixer at 60°C for 10-15 minutes (more for fibrous leaves)

3: Add 500ul of Chloroform: Isoamyl alcohol (24:1), cap tube and mix vigorously using Vortex at high speed

4: Centrifuge at Max speed for 10 minutes – you should now have two distinct layers in the tube with an interface between them that may look like skin. The DNA is in the top (Aqueous) layer, Debris type plant material in the interface and proteins etc in the lower (Chloroform) layer

5: Recover DNA by gently sucking off the top layer and transferring it to a fresh 1.5ml microcentrifuge tube – Try to recover as much of the top layer without sucking up any of the interface or lower layer (NOTE: if supernatant appears cloudy or had debris left in it REPEAT steps **3 – 5**)

6: Add equal volume of Isopropanol and invert to mix, then sit samples for 10 minutes

7: Centrifuge at maximum speed for 10 minutes

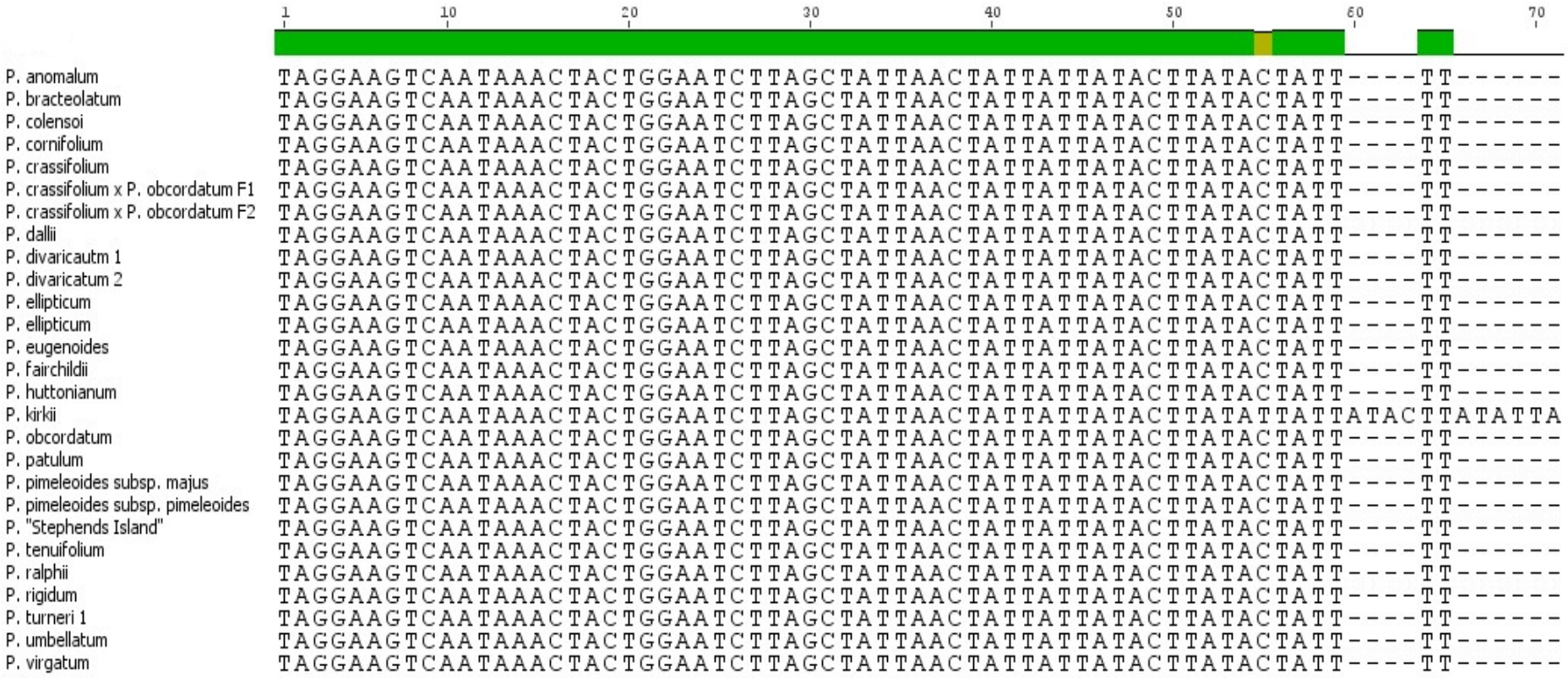
8: Locate the fine whitish pellet near the bottom of the tube, then use a fine tip pipette to remove all supernatant without disturbing pellet (which contains the DNA!)

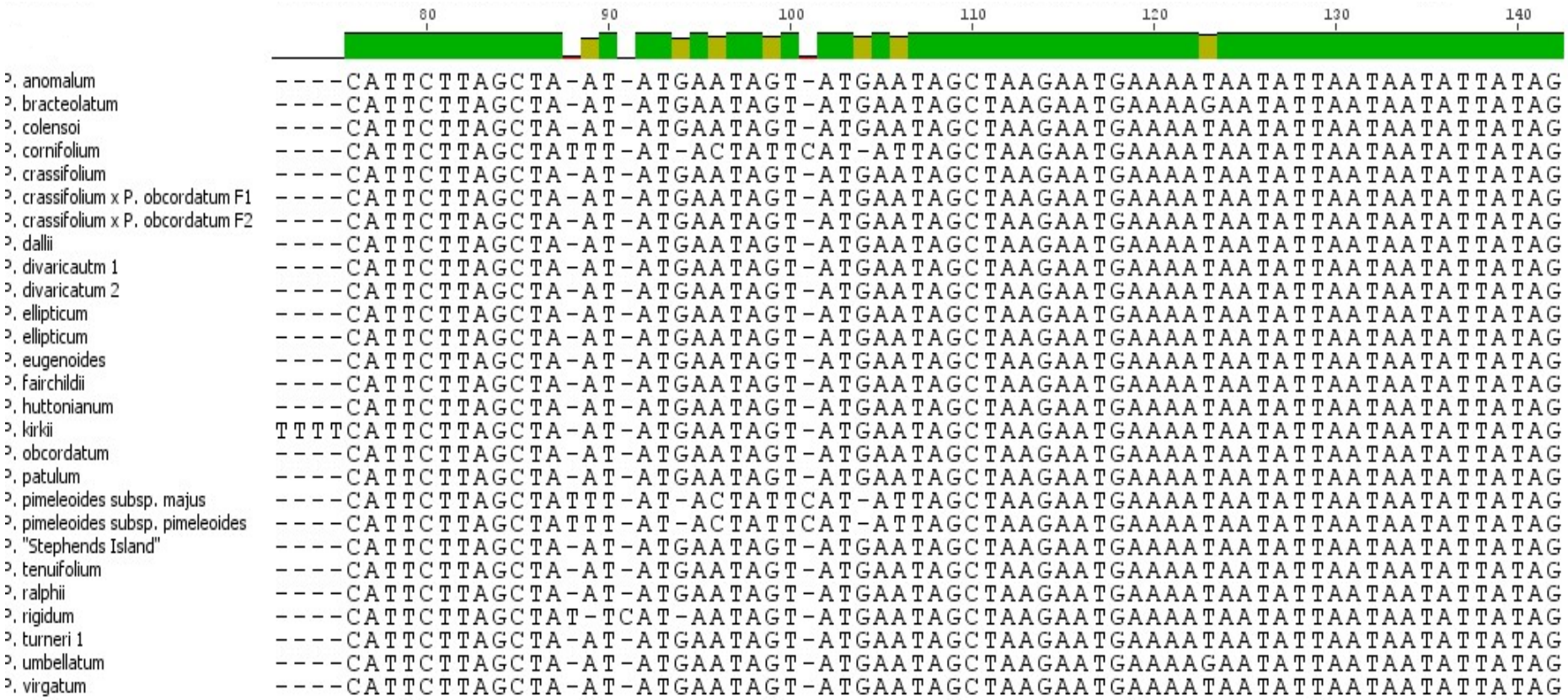
9: Add 500ul of 100% ethanol, centrifuge at maximum speed for 1 minute and again locate the pellet and remove the supernatant as above in **8**

10: Add 500ul of 70% ethanol to samples, centrifuge at maximum speed for 1 minute – again locate pellet and remove supernatant as in **8** – Re-spin at maximum briefly to bring liquid from sides down – remove excess liquid without disturbing pellet

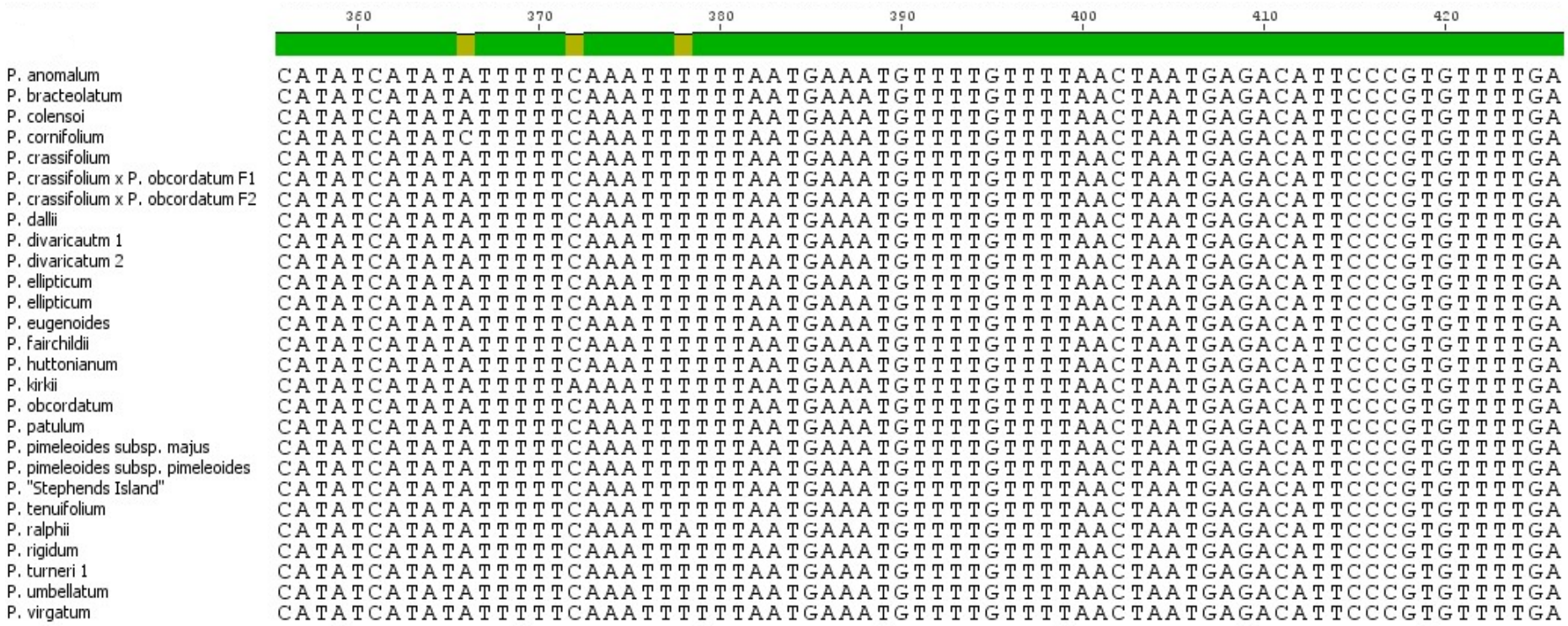
11: Sit on bench for 5 mins to dry off any ethanol. Then add 50ul of TE buffer – Shake tube in thermomixer at 37 °C for 10-20 minutes to resuspend DNA.

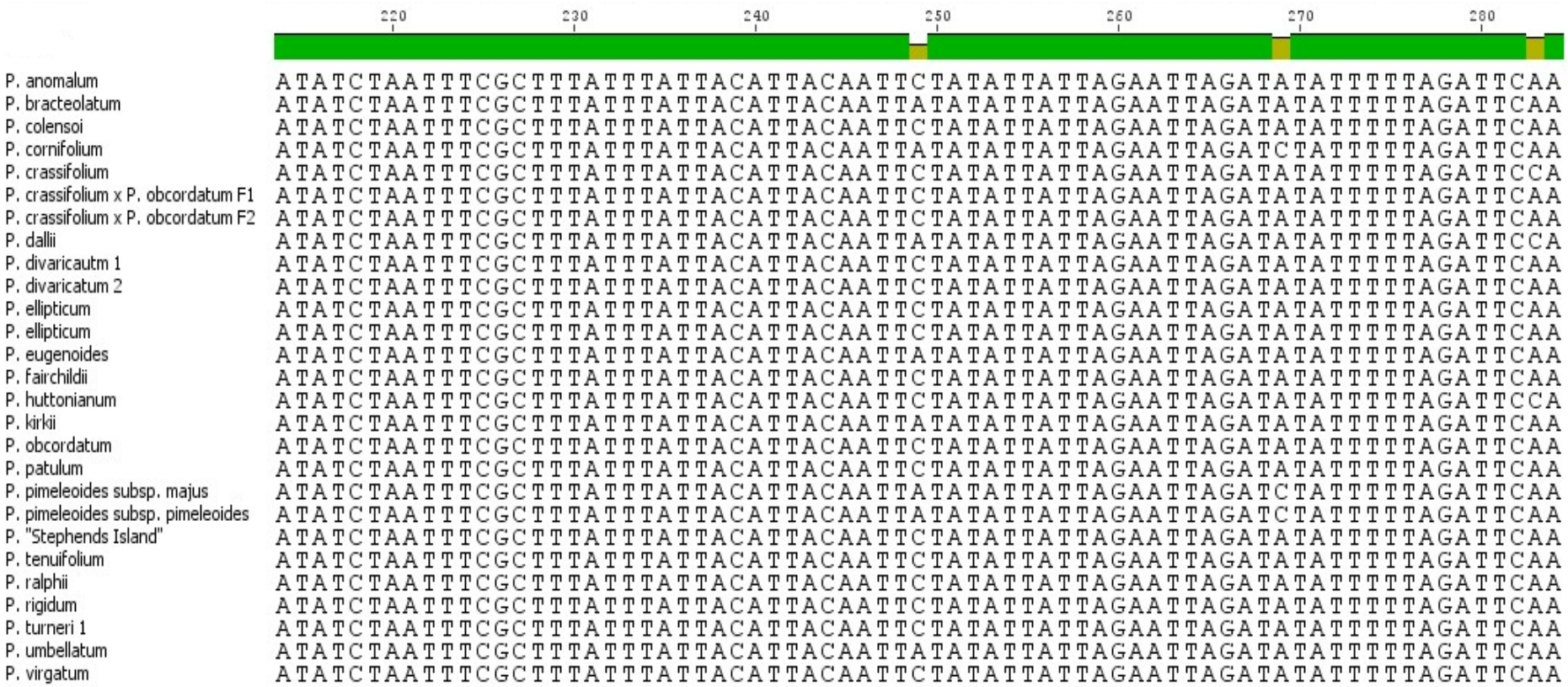
APPENDIX 2: Aligned data matrix for the *trnT-trnL* region

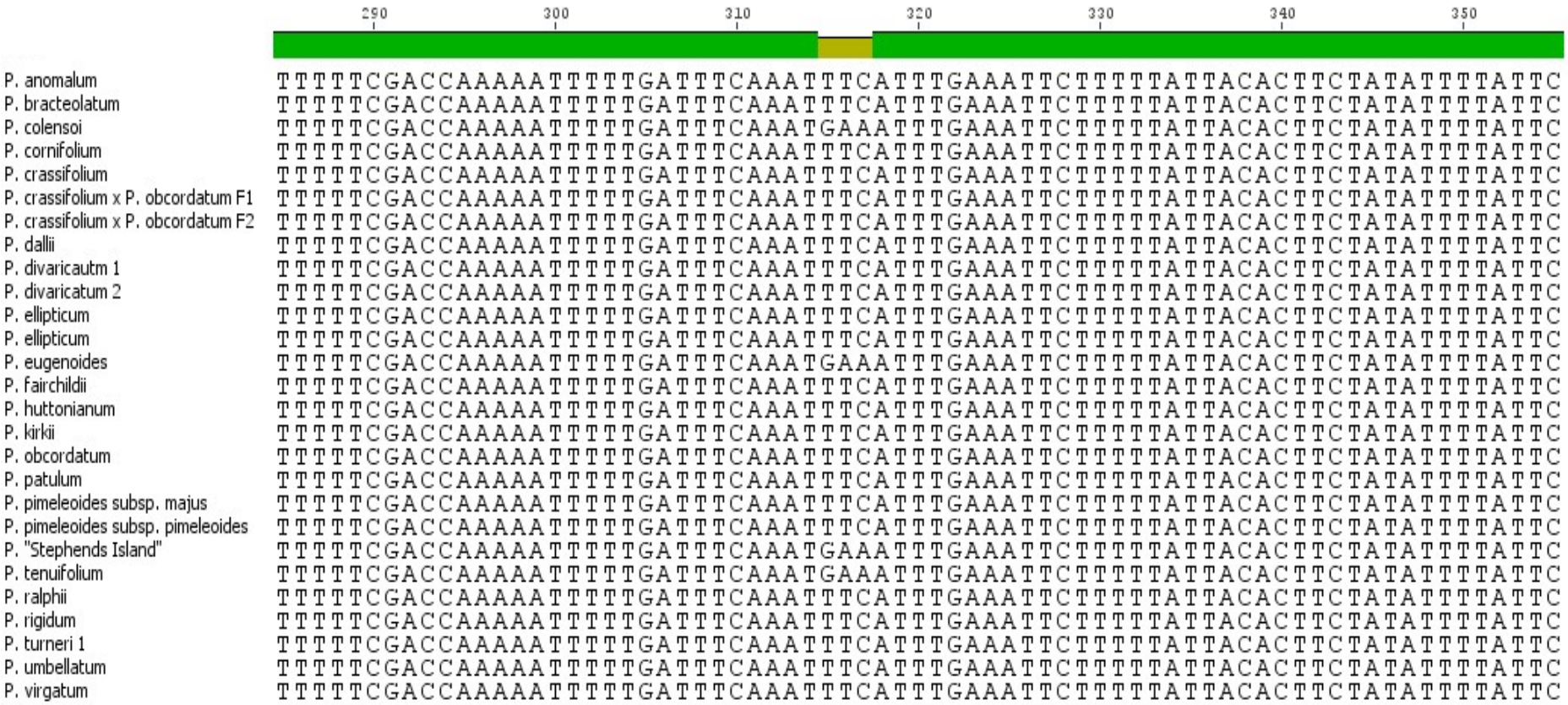


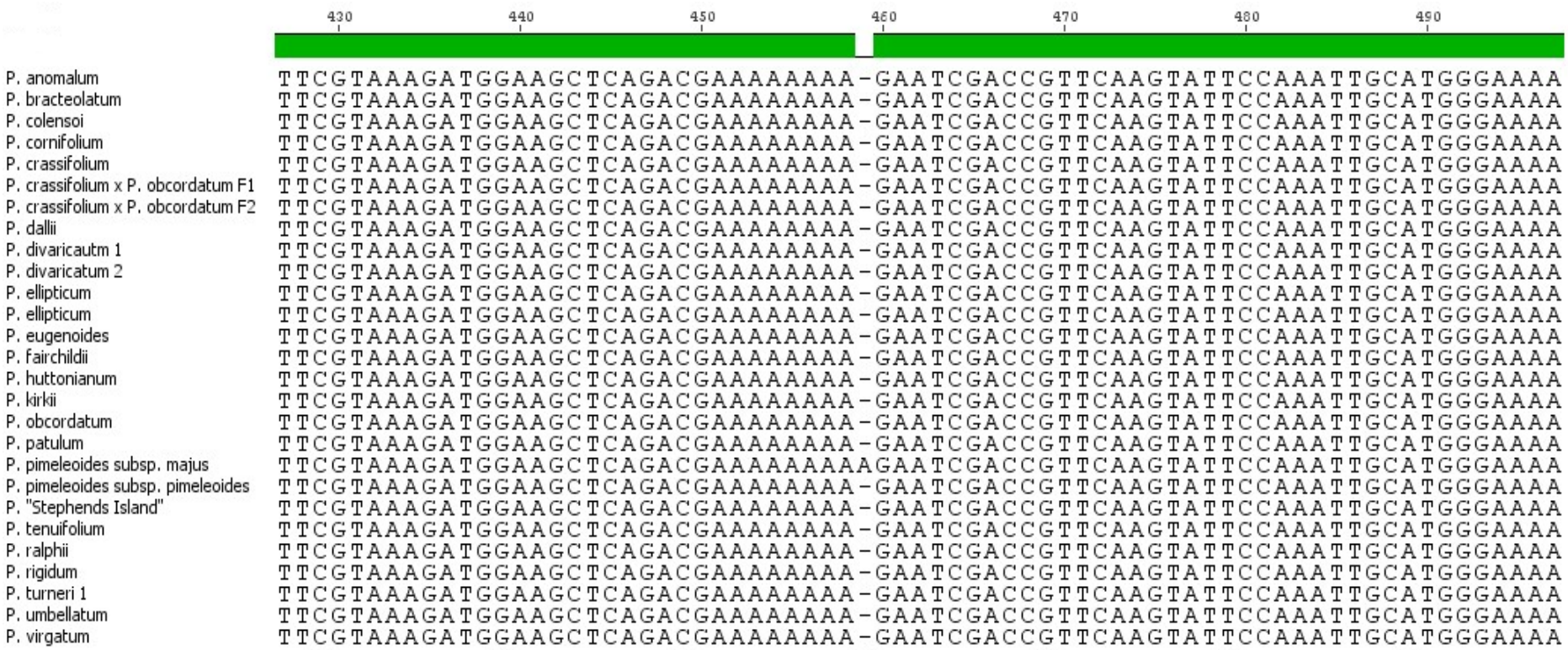

80
90
100
110
120
130
140
P. anomalum ----CATTCTTAGCTA-AT-ATGAATAGT-ATGAATAGCTAAGAATGAAAATAATATTAATAATATTATAG
P. bracteolatum ----CATTCTTAGCTA-AT-ATGAATAGT-ATGAATAGCTAAGAATGAAAAGAATATTAATAATATTATAG
P. colensoi ----CATTCTTAGCTA-AT-ATGAATAGT-ATGAATAGCTAAGAATGAAAATAATATTAATAATATTATAG
P. cornifolium ----CATTCTTAGCTATTT-AT-ACTATTCAT-ATTAGCTAAGAATGAAAATAATATTAATAATATTATAG
P. crassifolium ----CATTCTTAGCTA-AT-ATGAATAGT-ATGAATAGCTAAGAATGAAAATAATATTAATAATATTATAG
P. crassifolium x P. obcordatum F1 ----CATTCTTAGCTA-AT-ATGAATAGT-ATGAATAGCTAAGAATGAAAATAATATTAATAATATTATAG
P. crassifolium x P. obcordatum F2 ----CATTCTTAGCTA-AT-ATGAATAGT-ATGAATAGCTAAGAATGAAAATAATATTAATAATATTATAG
P. dallii ----CATTCTTAGCTA-AT-ATGAATAGT-ATGAATAGCTAAGAATGAAAATAATATTAATAATATTATAG
P. divaricautm 1 ----CATTCTTAGCTA-AT-ATGAATAGT-ATGAATAGCTAAGAATGAAAATAATATTAATAATATTATAG
P. divaricatum 2 ----CATTCTTAGCTA-AT-ATGAATAGT-ATGAATAGCTAAGAATGAAAATAATATTAATAATATTATAG
P. ellipticum ----CATTCTTAGCTA-AT-ATGAATAGT-ATGAATAGCTAAGAATGAAAATAATATTAATAATATTATAG
P. ellipticum ----CATTCTTAGCTA-AT-ATGAATAGT-ATGAATAGCTAAGAATGAAAATAATATTAATAATATTATAG
P. eugenoides ----CATTCTTAGCTA-AT-ATGAATAGT-ATGAATAGCTAAGAATGAAAATAATATTAATAATATTATAG
P. fairchildii ----CATTCTTAGCTA-AT-ATGAATAGT-ATGAATAGCTAAGAATGAAAATAATATTAATAATATTATAG
P. huttonianum ----CATTCTTAGCTA-AT-ATGAATAGT-ATGAATAGCTAAGAATGAAAATAATATTAATAATATTATAG
P. kirkii TTTTCATTCTTAGCTA-AT-ATGAATAGT-ATGAATAGCTAAGAATGAAAATAATATTAATAATATTATAG
P. obcordatum ----CATTCTTAGCTA-AT-ATGAATAGT-ATGAATAGCTAAGAATGAAAATAATATTAATAATATTATAG
P. patulum ----CATTCTTAGCTA-AT-ATGAATAGT-ATGAATAGCTAAGAATGAAAATAATATTAATAATATTATAG
P. pimeleoides subsp. majus ----CATTCTTAGCTATTT-AT-ACTATTCAT-ATTAGCTAAGAATGAAAATAATATTAATAATATTATAG
P. pimeleoides subsp. pimeleoides ----CATTCTTAGCTATTT-AT-ACTATTCAT-ATTAGCTAAGAATGAAAATAATATTAATAATATTATAG
P. "Stephends Island" ----CATTCTTAGCTA-AT-ATGAATAGT-ATGAATAGCTAAGAATGAAAATAATATTAATAATATTATAG
P. tenuifolium ----CATTCTTAGCTA-AT-ATGAATAGT-ATGAATAGCTAAGAATGAAAATAATATTAATAATATTATAG
P. ralphii ----CATTCTTAGCTA-AT-ATGAATAGT-ATGAATAGCTAAGAATGAAAATAATATTAATAATATTATAG
P. rigidum ----CATTCTTAGCTAT-TCAT-AATAGT-ATGAATAGCTAAGAATGAAAATAATATTAATAATATTATAG
P. turneri 1 ----CATTCTTAGCTA-AT-ATGAATAGT-ATGAATAGCTAAGAATGAAAATAATATTAATAATATTATAG
P. umbellatum ----CATTCTTAGCTA-AT-ATGAATAGT-ATGAATAGCTAAGAATGAAAAGAATATTAATAATATTATAG
P. virgatum ----CATTCTTAGCTA-AT-ATGAATAGT-ATGAATAGCTAAGAATGAAAATAATATTAATAATATTATAG

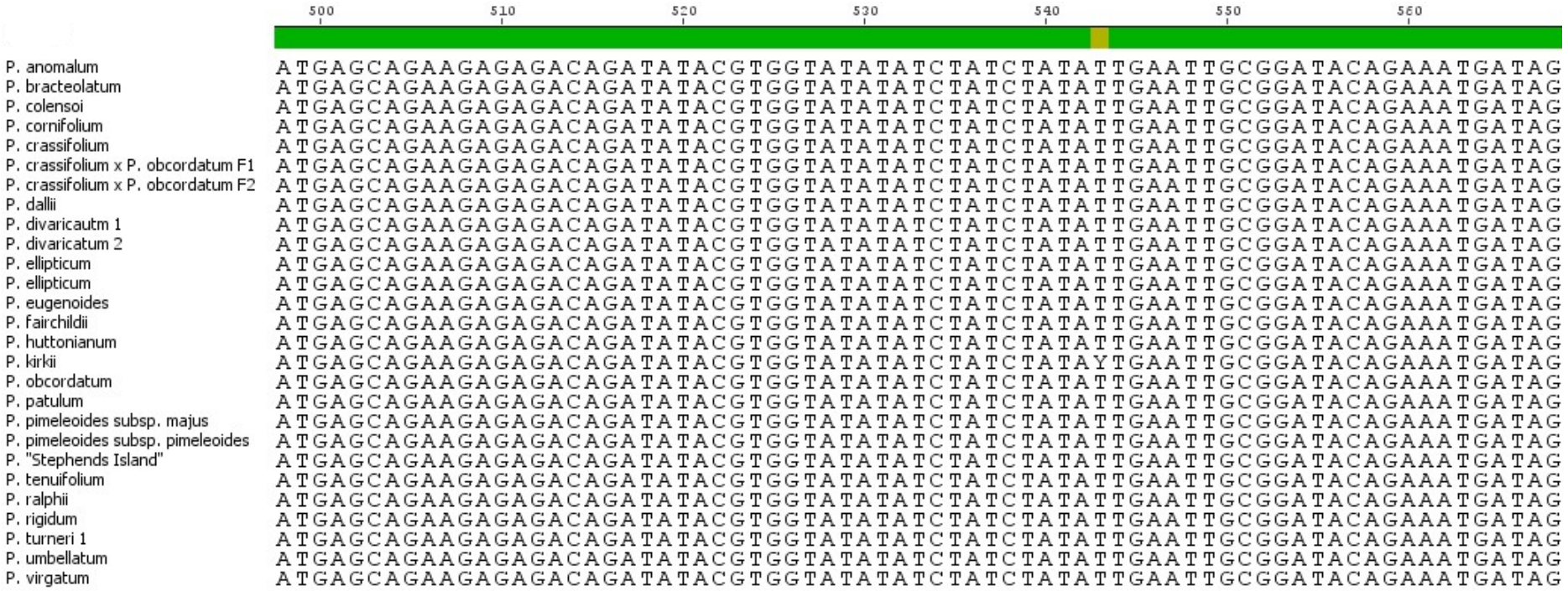

150
160
170
180
190
200
210
P. anomalum AATTGAAAATCAATATTTTCAATACTAATACTTCAATACTAATACTATAGAATATAACGATGAATCTAGCG
P. bracteolatum AATTGAAAATAAATATTTTCAATACTAATACTTCAATACTAATACTATAGAATATAACGATGAATCTAGCG
P. colensoi AATTGAAAATAAATATTTTCAATACTAATACTTCAATACTAATACTATAGAATATAACGATGAATCTAGCG
P. cornifolium AATTGAAAATAAATAGTTTCAATACTAATACTTCAATACTAATACTATAGAATATAACGATGAATCTAGCG
P. crassifolium AATTGAAAATAAATATTTTCAATACTAATACTTCAATACTAATACTATAGAATATAACGATGAATCTAGCG
P. crassifolium x P. obcordatum F1 AATTGAAAATAAATATTTTCAATACTAATACTTCAATACTAATACTATAGAATATAACGATGAATCTAGCG
P. crassifolium x P. obcordatum F2 AATTGAAAATAAATATTTTCAATACTAATACTTCAATACTAATACTATAGAATATAACGATGAATCTAGCG
P. dallii AATTGAAAATAAATATTTTCAATACTAATACTTCAATACTAATACTATAGAATATAACGATGAATCTAGCG
P. divaricautm 1 AATTGAAAATATTGATTTTCAATACTAATACTTCAATACTAATACTATAGAATATAACGATGAATCTAGCG
P. divaricatum 2 AATTGAAAATATTGATTTTCAATACTAATACTTCAATACTAATACTATAGAATATAACGATGAATCTAGCG
P. ellipticum AATTGAAAATAAATATTTTCAATACTAATACTTCAATACTAATACTATAGAATATAACGATGAATCTAGCG
P. ellipticum AATTGAAAATAAATATTTTCAATACTAATACTTCAATACTAATACTATAGAATATAACGATGAATCTAGCG
P. eugenoides AATTGAAAATATTTATTTTCAATACTAATACTTCAATACTAATACTATAGAATATAACGATGAATCTAGCG
P. fairchildii AATTGAAAATAAATATTTTCAATACTAATACTTCAATACTAATACTATAGAATATAACGATGAATCTAGCG
P. huttonianum AATTGAAAATAAATATTTTCAATACTAATACTTCAATACTAATACTATAGAATATAACGATGAATCTAGCG
P. kirkii AATTGAAAATCAATATTTTCAATACTAATACTTCAATACTAATACTATAGAATATAACGATGAATCTAGCG
P. obcordatum AATTGAAAATAAATATTTTCAATACTAATACTTCAATACTAATACTATAGAATATAACGATGAATCTAGCG
P. patulum AATTGAAAATCAATATTTTCAATACTAATACTTCAATACTAATACTATAGAATATAACGATGAATCTAGCG
P. pimeleoides subsp. majus AATTGAAAATAAATAGTTTCAATACTAATACTTCAATACTAATACTATAGAATATAACGATGAATCTAGCG
P. pimeleoides subsp. pimeleoides AATTGAAAATAAATAGTTTCAATACTAATACTTCAATACTAATACTATAGAATATAACGATGAATCTAGCG
P. "Stephends Island" AATTGAAAATAAATATTTTCAATACTAATACTTCAATACTAATACTATAGAATATAACGATGAATCTAGCG
P. tenuifolium AATTGAAAATAAATATTTTCAATACTAATACTTCAATACTAATACTATAGAATATAACGATGAATCTAGCG
P. ralphii AATTGAAAATAAATATTTTCAATACTAATACTTCAATACTAATACTATAGAATATAACGATGAATCTAGCG
P. rigidum AATTGAAAATATTGATTTTCAATACTAATACTTCAATACTAATACTATAGAATATAACGATGAATCTAGCG
P. turneri 1 AATTGAAAATATTGATTTTCAATACTAATACTTCAATACTAATACTATAGAATATAACGATGAATCTAGCG
P. umbellatum AATTGAAAATAAATATTTTCAATACTAATACTTCAATACTAATACTATAGAATATAACGATGAATCTAGCG
P. virgatum AATTGAAAATCWWKATTTTCAATACTAATACTTCAATACTAATACTATAGAATATAACGATGAATCTAGCG

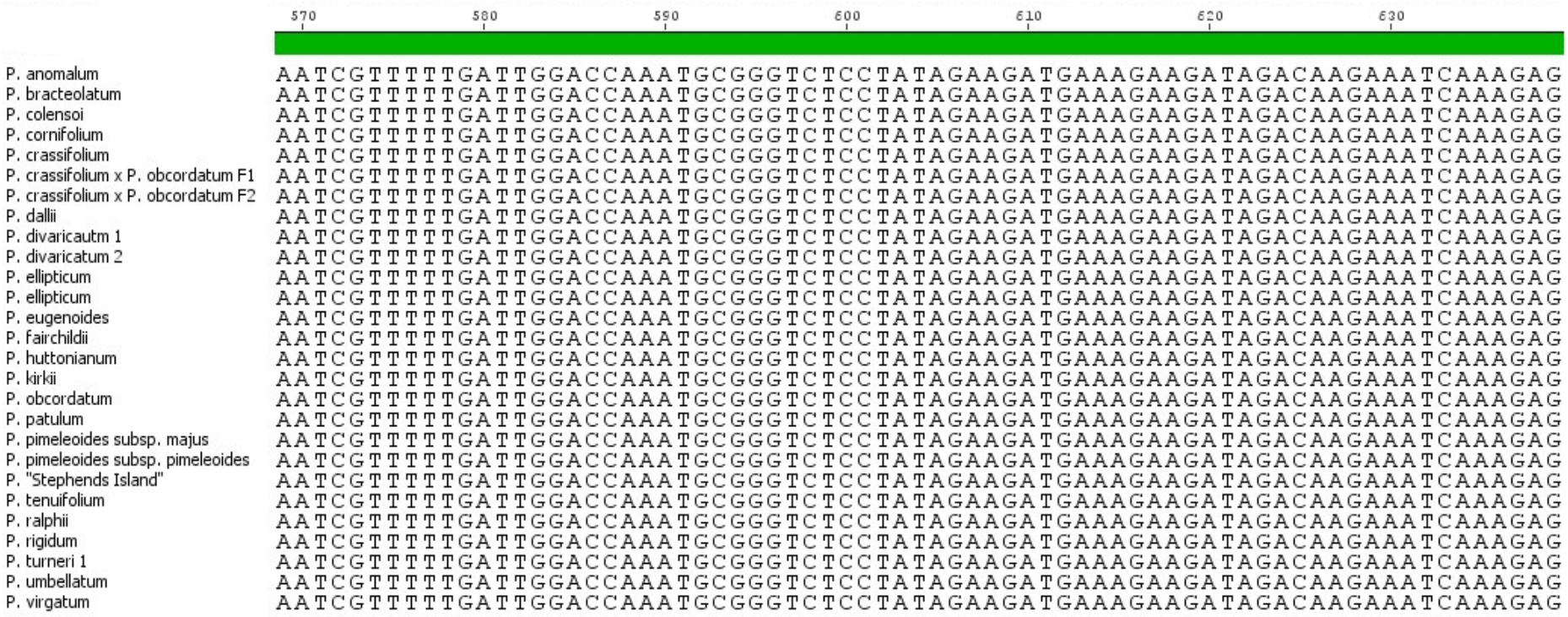

360
370
380
390
400
410
420
P. anomalum CATATCATATATTTTTCAAATTTTTTAATGAAATGTTTTGTTTTAACTAATGAGACATTCCCGTGTTTTGA
P. bracteolatum CATATCATATATTTTTCAAATTTTTTAATGAAATGTTTTGTTTTAACTAATGAGACATTCCCGTGTTTTGA
P. colensoi CATATCATATATTTTTCAAATTTTTTAATGAAATGTTTTGTTTTAACTAATGAGACATTCCCGTGTTTTGA
P. cornifolium CATATCATATCTTTTTCAAATTTTTTAATGAAATGTTTTGTTTTAACTAATGAGACATTCCCGTGTTTTGA
P. crassifolium CATATCATATATTTTTCAAATTTTTTAATGAAATGTTTTGTTTTAACTAATGAGACATTCCCGTGTTTTGA
P. crassifolium x P. obcordatum F1 CATATCATATATTTTTCAAATTTTTTAATGAAATGTTTTGTTTTAACTAATGAGACATTCCCGTGTTTTGA
P. crassifolium x P. obcordatum F2 CATATCATATATTTTTCAAATTTTTTAATGAAATGTTTTGTTTTAACTAATGAGACATTCCCGTGTTTTGA
P. dallii CATATCATATATTTTTCAAATTTTTTAATGAAATGTTTTGTTTTAACTAATGAGACATTCCCGTGTTTTGA
P. divaricautm 1 CATATCATATATTTTTCAAATTTTTTAATGAAATGTTTTGTTTTAACTAATGAGACATTCCCGTGTTTTGA
P. divaricatum 2 CATATCATATATTTTTCAAATTTTTTAATGAAATGTTTTGTTTTAACTAATGAGACATTCCCGTGTTTTGA
P. ellipticum CATATCATATATTTTTCAAATTTTTTAATGAAATGTTTTGTTTTAACTAATGAGACATTCCCGTGTTTTGA
P. ellipticum CATATCATATATTTTTCAAATTTTTTAATGAAATGTTTTGTTTTAACTAATGAGACATTCCCGTGTTTTGA
P. eugenoides CATATCATATATTTTTCAAATTTTTTAATGAAATGTTTTGTTTTAACTAATGAGACATTCCCGTGTTTTGA
P. fairchildii CATATCATATATTTTTCAAATTTTTTAATGAAATGTTTTGTTTTAACTAATGAGACATTCCCGTGTTTTGA
P. huttonianum CATATCATATATTTTTCAAATTTTTTAATGAAATGTTTTGTTTTAACTAATGAGACATTCCCGTGTTTTGA
P. kirkii CATATCATATATTTTTAAAATTTTTTAATGAAATGTTTTGTTTTAACTAATGAGACATTCCCGTGTTTTGA
P. obcordatum CATATCATATATTTTTCAAATTTTTTAATGAAATGTTTTGTTTTAACTAATGAGACATTCCCGTGTTTTGA
P. patulum CATATCATATATTTTTCAAATTTTTTAATGAAATGTTTTGTTTTAACTAATGAGACATTCCCGTGTTTTGA
P. pimeleoides subsp. majus CATATCATATATTTTTCAAATTTTTTAATGAAATGTTTTGTTTTAACTAATGAGACATTCCCGTGTTTTGA
P. pimeleoides subsp. pimeleoides CATATCATATATTTTTCAAATTTTTTAATGAAATGTTTTGTTTTAACTAATGAGACATTCCCGTGTTTTGA
P. "Stephends Island" CATATCATATATTTTTCAAATTTTTTAATGAAATGTTTTGTTTTAACTAATGAGACATTCCCGTGTTTTGA
P. tenuifolium CATATCATATATTTTTCAAATTTTTTAATGAAATGTTTTGTTTTAACTAATGAGACATTCCCGTGTTTTGA
P. ralphii CATATCATATATTTTTCAAATTATTTAATGAAATGTTTTGTTTTAACTAATGAGACATTCCCGTGTTTTGA
P. rigidum CATATCATATATTTTTCAAATTTTTTAATGAAATGTTTTGTTTTAACTAATGAGACATTCCCGTGTTTTGA
P. turneri 1 CATATCATATATTTTTCAAATTTTTTAATGAAATGTTTTGTTTTAACTAATGAGACATTCCCGTGTTTTGA
P. umbellatum CATATCATATATTTTTCAAATTTTTTAATGAAATGTTTTGTTTTAACTAATGAGACATTCCCGTGTTTTGA
P. virgatum CATATCATATATTTTTCAAATTTTTTAATGAAATGTTTTGTTTTAACTAATGAGACATTCCCGTGTTTTGA


220
230
240
250
260
270
280
P. anomalum ATATCTAATTTCGCTTTATTTATTACATTACAATTCTATATTATTAGAATTAGATATATTTTTAGATTCAA
P. bracteolatum ATATCTAATTTCGCTTTATTTATTACATTACAATTATATATTATTAGAATTAGATATATTTTTAGATTCAA
P. colensoi ATATCTAATTTCGCTTTATTTATTACATTACAATTCTATATTATTAGAATTAGATATATTTTTAGATTCAA
P. cornifolium ATATCTAATTTCGCTTTATTTATTACATTACAATTATATATTATTAGAATTAGATCTATTTTTAGATTCAA
P. crassifolium ATATCTAATTTCGCTTTATTTATTACATTACAATTCTATATTATTAGAATTAGATATATTTTTAGATTCCA
P. crassifolium x P. obcordatum F1 ATATCTAATTTCGCTTTATTTATTACATTACAATTCTATATTATTAGAATTAGATATATTTTTAGATTCAA
P. crassifolium x P. obcordatum F2 ATATCTAATTTCGCTTTATTTATTACATTACAATTCTATATTATTAGAATTAGATATATTTTTAGATTCAA
P. dallii ATATCTAATTTCGCTTTATTTATTACATTACAATTATATATTATTAGAATTAGATATATTTTTAGATTCCA
P. divaricautm 1 ATATCTAATTTCGCTTTATTTATTACATTACAATTCTATATTATTAGAATTAGATATATTTTTAGATTCAA
P. divaricatum 2 ATATCTAATTTCGCTTTATTTATTACATTACAATTCTATATTATTAGAATTAGATATATTTTTAGATTCAA
P. ellipticum ATATCTAATTTCGCTTTATTTATTACATTACAATTCTATATTATTAGAATTAGATATATTTTTAGATTCAA
P. ellipticum ATATCTAATTTCGCTTTATTTATTACATTACAATTCTATATTATTAGAATTAGATATATTTTTAGATTCAA
P. eugenoides ATATCTAATTTCGCTTTATTTATTACATTACAATTATATATTATTAGAATTAGATATATTTTTAGATTCAA
P. fairchildii ATATCTAATTTCGCTTTATTTATTACATTACAATTCTATATTATTAGAATTAGATATATTTTTAGATTCAA
P. huttonianum ATATCTAATTTCGCTTTATTTATTACATTACAATTCTATATTATTAGAATTAGATATATTTTTAGATTCCA
P. kirkii ATATCTAATTTCGCTTTATTTATTACATTACAATTATATATTATTAGAATTAGATATATTTTTAGATTCAA
P. obcordatum ATATCTAATTTCGCTTTATTTATTACATTACAATTCTATATTATTAGAATTAGATATATTTTTAGATTCAA
P. patulum ATATCTAATTTCGCTTTATTTATTACATTACAATTCTATATTATTAGAATTAGATATATTTTTAGATTCAA
P. pimeleoides subsp. majus ATATCTAATTTCGCTTTATTTATTACATTACAATTATATATTATTAGAATTAGATCTATTTTTAGATTCAA
P. pimeleoides subsp. pimeleoides ATATCTAATTTCGCTTTATTTATTACATTACAATTATATATTATTAGAATTAGATCTATTTTTAGATTCAA
P. "Stephends Island" ATATCTAATTTCGCTTTATTTATTACATTACAATTCTATATTATTAGAATTAGATATATTTTTAGATTCAA
P. tenuifolium ATATCTAATTTCGCTTTATTTATTACATTACAATTCTATATTATTAGAATTAGATATATTTTTAGATTCAA
P. ralphii ATATCTAATTTCGCTTTATTTATTACATTACAATTCTATATTATTAGAATTAGATATATTTTTAGATTCAA
P. rigidum ATATCTAATTTCGCTTTATTTATTACATTACAATTCTATATTATTAGAATTAGATATATTTTTAGATTCAA
P. turneri 1 ATATCTAATTTCGCTTTATTTATTACATTACAATTCTATATTATTAGAATTAGATATATTTTTAGATTCAA
P. umbellatum ATATCTAATTTCGCTTTATTTATTACATTACAATTATATATTATTAGAATTAGATATATTTTTAGATTCAA
P. virgatum ATATCTAATTTCGCTTTATTTATTACATTACAATTCTATATTATTAGAATTAGATATATTTTTAGATTCAA


290
300
310
320
330
340
350
P. anomalum TTTTTCGACCAAAAATTTTTGATTTCAAATTTCATTTGAAATTCTTTTTATTACACTTCTATATTTTATTC
P. bracteolatum TTTTTCGACCAAAAATTTTTGATTTCAAATTTCATTTGAAATTCTTTTTATTACACTTCTATATTTTATTC
P. colensoi TTTTTCGACCAAAAATTTTTGATTTCAAATGAAATTTGAAATTCTTTTTATTACACTTCTATATTTTATTC
P. cornifolium TTTTTCGACCAAAAATTTTTGATTTCAAATTTCATTTGAAATTCTTTTTATTACACTTCTATATTTTATTC
P. crassifolium TTTTTCGACCAAAAATTTTTGATTTCAAATTTCATTTGAAATTCTTTTTATTACACTTCTATATTTTATTC
P. crassifolium x P. obcordatum F1 TTTTTCGACCAAAAATTTTTGATTTCAAATTTCATTTGAAATTCTTTTTATTACACTTCTATATTTTATTC
P. crassifolium x P. obcordatum F2 TTTTTCGACCAAAAATTTTTGATTTCAAATTTCATTTGAAATTCTTTTTATTACACTTCTATATTTTATTC
P. dallii TTTTTCGACCAAAAATTTTTGATTTCAAATTTCATTTGAAATTCTTTTTATTACACTTCTATATTTTATTC
P. divaricautm 1 TTTTTCGACCAAAAATTTTTGATTTCAAATTTCATTTGAAATTCTTTTTATTACACTTCTATATTTTATTC
P. divaricatum 2 TTTTTCGACCAAAAATTTTTGATTTCAAATTTCATTTGAAATTCTTTTTATTACACTTCTATATTTTATTC
P. ellipticum TTTTTCGACCAAAAATTTTTGATTTCAAATTTCATTTGAAATTCTTTTTATTACACTTCTATATTTTATTC
P. ellipticum TTTTTCGACCAAAAATTTTTGATTTCAAATTTCATTTGAAATTCTTTTTATTACACTTCTATATTTTATTC
P. eugenoides TTTTTCGACCAAAAATTTTTGATTTCAAATGAAATTTGAAATTCTTTTTATTACACTTCTATATTTTATTC
P. fairchildii TTTTTCGACCAAAAATTTTTGATTTCAAATTTCATTTGAAATTCTTTTTATTACACTTCTATATTTTATTC
P. huttonianum TTTTTCGACCAAAAATTTTTGATTTCAAATTTCATTTGAAATTCTTTTTATTACACTTCTATATTTTATTC
P. kirkii TTTTTCGACCAAAAATTTTTGATTTCAAATTTCATTTGAAATTCTTTTTATTACACTTCTATATTTTATTC
P. obcordatum TTTTTCGACCAAAAATTTTTGATTTCAAATTTCATTTGAAATTCTTTTTATTACACTTCTATATTTTATTC
P. patulum TTTTTCGACCAAAAATTTTTGATTTCAAATTTCATTTGAAATTCTTTTTATTACACTTCTATATTTTATTC
P. pimeleoides subsp. majus TTTTTCGACCAAAAATTTTTGATTTCAAATTTCATTTGAAATTCTTTTTATTACACTTCTATATTTTATTC
P. pimeleoides subsp. pimeleoides TTTTTCGACCAAAAATTTTTGATTTCAAATTTCATTTGAAATTCTTTTTATTACACTTCTATATTTTATTC
P. "Stephends Island" TTTTTCGACCAAAAATTTTTGATTTCAAATGAAATTTGAAATTCTTTTTATTACACTTCTATATTTTATTC
P. tenuifolium TTTTTCGACCAAAAATTTTTGATTTCAAATGAAATTTGAAATTCTTTTTATTACACTTCTATATTTTATTC
P. ralphii TTTTTCGACCAAAAATTTTTGATTTCAAATTTCATTTGAAATTCTTTTTATTACACTTCTATATTTTATTC
P. rigidum TTTTTCGACCAAAAATTTTTGATTTCAAATTTCATTTGAAATTCTTTTTATTACACTTCTATATTTTATTC
P. turneri 1 TTTTTCGACCAAAAATTTTTGATTTCAAATTTCATTTGAAATTCTTTTTATTACACTTCTATATTTTATTC
P. umbellatum TTTTTCGACCAAAAATTTTTGATTTCAAATTTCATTTGAAATTCTTTTTATTACACTTCTATATTTTATTC
P. virgatum TTTTTCGACCAAAAATTTTTGATTTCAAATTTCATTTGAAATTCTTTTTATTACACTTCTATATTTTATTC

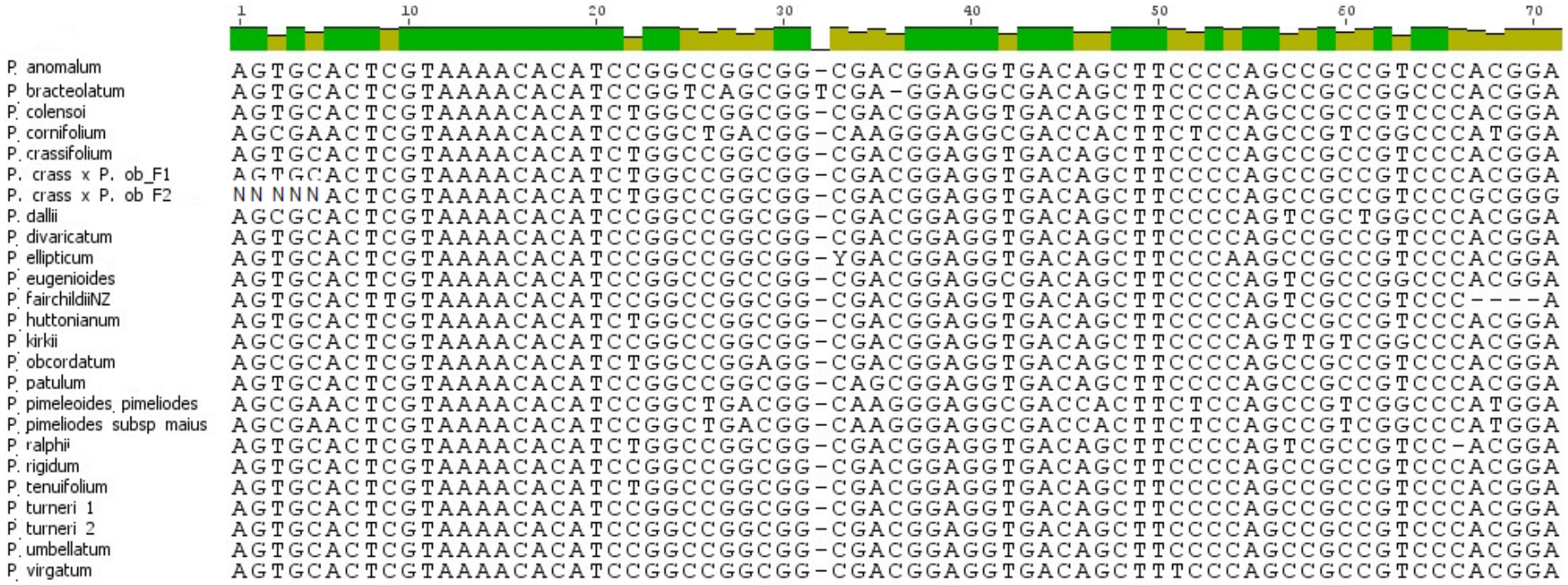

430
440
450
460
470
480
490
P. anomalum TTCGTAAAGATGGAAGCTCAGACGAAAAAAAA-GAATCGACCGTTCAAGTATTCCAAATTGCATGGGAAAA
P. bracteolatum TTCGTAAAGATGGAAGCTCAGACGAAAAAAAA-GAATCGACCGTTCAAGTATTCCAAATTGCATGGGAAAA
P. colensoi TTCGTAAAGATGGAAGCTCAGACGAAAAAAAA-GAATCGACCGTTCAAGTATTCCAAATTGCATGGGAAAA
P. cornifolium TTCGTAAAGATGGAAGCTCAGACGAAAAAAAA-GAATCGACCGTTCAAGTATTCCAAATTGCATGGGAAAA
P. crassifolium TTCGTAAAGATGGAAGCTCAGACGAAAAAAAA-GAATCGACCGTTCAAGTATTCCAAATTGCATGGGAAAA
P. crassifolium x P. obcordatum F1 TTCGTAAAGATGGAAGCTCAGACGAAAAAAAA-GAATCGACCGTTCAAGTATTCCAAATTGCATGGGAAAA
P. crassifolium x P. obcordatum F2 TTCGTAAAGATGGAAGCTCAGACGAAAAAAAA-GAATCGACCGTTCAAGTATTCCAAATTGCATGGGAAAA
P. dallii TTCGTAAAGATGGAAGCTCAGACGAAAAAAAA-GAATCGACCGTTCAAGTATTCCAAATTGCATGGGAAAA
P. divaricautm 1 TTCGTAAAGATGGAAGCTCAGACGAAAAAAAA-GAATCGACCGTTCAAGTATTCCAAATTGCATGGGAAAA
P. divaricatum 2 TTCGTAAAGATGGAAGCTCAGACGAAAAAAAA-GAATCGACCGTTCAAGTATTCCAAATTGCATGGGAAAA
P. ellipticum TTCGTAAAGATGGAAGCTCAGACGAAAAAAAA-GAATCGACCGTTCAAGTATTCCAAATTGCATGGGAAAA
P. ellipticum TTCGTAAAGATGGAAGCTCAGACGAAAAAAAA-GAATCGACCGTTCAAGTATTCCAAATTGCATGGGAAAA
P. eugenoides TTCGTAAAGATGGAAGCTCAGACGAAAAAAAA-GAATCGACCGTTCAAGTATTCCAAATTGCATGGGAAAA
P. fairchildii TTCGTAAAGATGGAAGCTCAGACGAAAAAAAA-GAATCGACCGTTCAAGTATTCCAAATTGCATGGGAAAA
P. huttonianum TTCGTAAAGATGGAAGCTCAGACGAAAAAAAA-GAATCGACCGTTCAAGTATTCCAAATTGCATGGGAAAA
P. kirkii TTCGTAAAGATGGAAGCTCAGACGAAAAAAAA-GAATCGACCGTTCAAGTATTCCAAATTGCATGGGAAAA
P. obcordatum TTCGTAAAGATGGAAGCTCAGACGAAAAAAAA-GAATCGACCGTTCAAGTATTCCAAATTGCATGGGAAAA
P. patulum TTCGTAAAGATGGAAGCTCAGACGAAAAAAAA-GAATCGACCGTTCAAGTATTCCAAATTGCATGGGAAAA
P. pimeleoides subsp. majus TTCGTAAAGATGGAAGCTCAGACGAAAAAAAAAGAATCGACCGTTCAAGTATTCCAAATTGCATGGGAAAA
P. pimeleoides subsp. pimeleoides TTCGTAAAGATGGAAGCTCAGACGAAAAAAAA-GAATCGACCGTTCAAGTATTCCAAATTGCATGGGAAAA
P. "Stephends Island" TTCGTAAAGATGGAAGCTCAGACGAAAAAAAA-GAATCGACCGTTCAAGTATTCCAAATTGCATGGGAAAA
P. tenuifolium TTCGTAAAGATGGAAGCTCAGACGAAAAAAAA-GAATCGACCGTTCAAGTATTCCAAATTGCATGGGAAAA
P. ralphii TTCGTAAAGATGGAAGCTCAGACGAAAAAAAA-GAATCGACCGTTCAAGTATTCCAAATTGCATGGGAAAA
P. rigidum TTCGTAAAGATGGAAGCTCAGACGAAAAAAAA-GAATCGACCGTTCAAGTATTCCAAATTGCATGGGAAAA
P. turneri 1 TTCGTAAAGATGGAAGCTCAGACGAAAAAAAA-GAATCGACCGTTCAAGTATTCCAAATTGCATGGGAAAA
P. umbellatum TTCGTAAAGATGGAAGCTCAGACGAAAAAAAA-GAATCGACCGTTCAAGTATTCCAAATTGCATGGGAAAA
P. virgatum TTCGTAAAGATGGAAGCTCAGACGAAAAAAAA-GAATCGACCGTTCAAGTATTCCAAATTGCATGGGAAAA

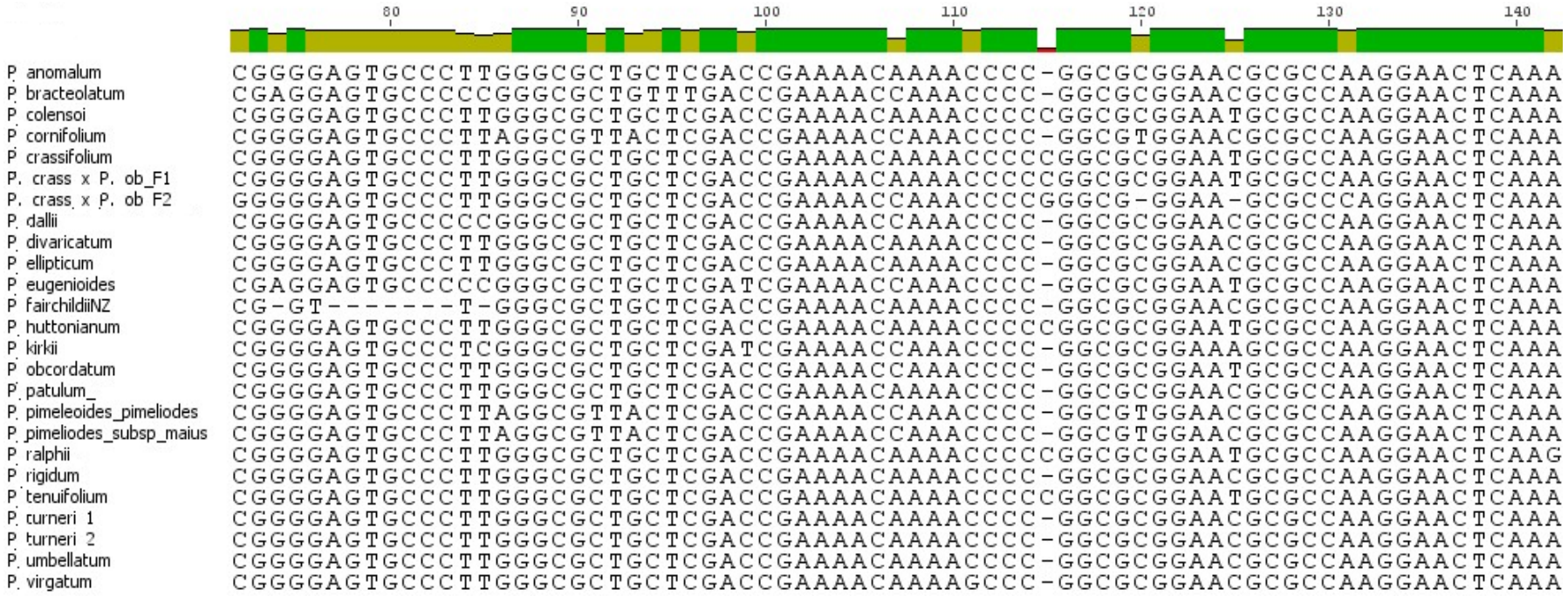

500
510
520
530
540
550
560
P. anomalum ATGAGCAGAAGAGAGACAGATATACGTGGTATATATCTATCTATATTGAATTGCGGATACAGAAATGATAG
P. bracteolatum ATGAGCAGAAGAGAGACAGATATACGTGGTATATATCTATCTATATTGAATTGCGGATACAGAAATGATAG
P. colensoi ATGAGCAGAAGAGAGACAGATATACGTGGTATATATCTATCTATATTGAATTGCGGATACAGAAATGATAG
P. cornifolium ATGAGCAGAAGAGAGACAGATATACGTGGTATATATCTATCTATATTGAATTGCGGATACAGAAATGATAG
P. crassifolium ATGAGCAGAAGAGAGACAGATATACGTGGTATATATCTATCTATATTGAATTGCGGATACAGAAATGATAG
P. crassifolium x P. obcordatum F1 ATGAGCAGAAGAGAGACAGATATACGTGGTATATATCTATCTATATTGAATTGCGGATACAGAAATGATAG
P. crassifolium x P. obcordatum F2 ATGAGCAGAAGAGAGACAGATATACGTGGTATATATCTATCTATATTGAATTGCGGATACAGAAATGATAG
P. dallii ATGAGCAGAAGAGAGACAGATATACGTGGTATATATCTATCTATATTGAATTGCGGATACAGAAATGATAG
P. divaricautm 1 ATGAGCAGAAGAGAGACAGATATACGTGGTATATATCTATCTATATTGAATTGCGGATACAGAAATGATAG
P. divaricatum 2 ATGAGCAGAAGAGAGACAGATATACGTGGTATATATCTATCTATATTGAATTGCGGATACAGAAATGATAG
P. ellipticum ATGAGCAGAAGAGAGACAGATATACGTGGTATATATCTATCTATATTGAATTGCGGATACAGAAATGATAG
P. ellipticum ATGAGCAGAAGAGAGACAGATATACGTGGTATATATCTATCTATATTGAATTGCGGATACAGAAATGATAG
P. eugenoides ATGAGCAGAAGAGAGACAGATATACGTGGTATATATCTATCTATATTGAATTGCGGATACAGAAATGATAG
P. fairchildii ATGAGCAGAAGAGAGACAGATATACGTGGTATATATCTATCTATATTGAATTGCGGATACAGAAATGATAG
P. huttonianum ATGAGCAGAAGAGAGACAGATATACGTGGTATATATCTATCTATATTGAATTGCGGATACAGAAATGATAG
P. kirkii ATGAGCAGAAGAGAGACAGATATACGTGGTATATATCTATCTATAYTGAATTGCGGATACAGAAATGATAG
P. obcordatum ATGAGCAGAAGAGAGACAGATATACGTGGTATATATCTATCTATATTGAATTGCGGATACAGAAATGATAG
P. patulum ATGAGCAGAAGAGAGACAGATATACGTGGTATATATCTATCTATATTGAATTGCGGATACAGAAATGATAG
P. pimeleoides subsp. majus ATGAGCAGAAGAGAGACAGATATACGTGGTATATATCTATCTATATTGAATTGCGGATACAGAAATGATAG
P. pimeleoides subsp. pimeleoides ATGAGCAGAAGAGAGACAGATATACGTGGTATATATCTATCTATATTGAATTGCGGATACAGAAATGATAG
P. "Stephends Island" ATGAGCAGAAGAGAGACAGATATACGTGGTATATATCTATCTATATTGAATTGCGGATACAGAAATGATAG
P. tenuifolium ATGAGCAGAAGAGAGACAGATATACGTGGTATATATCTATCTATATTGAATTGCGGATACAGAAATGATAG
P. ralphii ATGAGCAGAAGAGAGACAGATATACGTGGTATATATCTATCTATATTGAATTGCGGATACAGAAATGATAG
P. rigidum ATGAGCAGAAGAGAGACAGATATACGTGGTATATATCTATCTATATTGAATTGCGGATACAGAAATGATAG
P. turneri 1 ATGAGCAGAAGAGAGACAGATATACGTGGTATATATCTATCTATATTGAATTGCGGATACAGAAATGATAG
P. umbellatum ATGAGCAGAAGAGAGACAGATATACGTGGTATATATCTATCTATATTGAATTGCGGATACAGAAATGATAG
P. virgatum ATGAGCAGAAGAGAGACAGATATACGTGGTATATATCTATCTATATTGAATTGCGGATACAGAAATGATAG

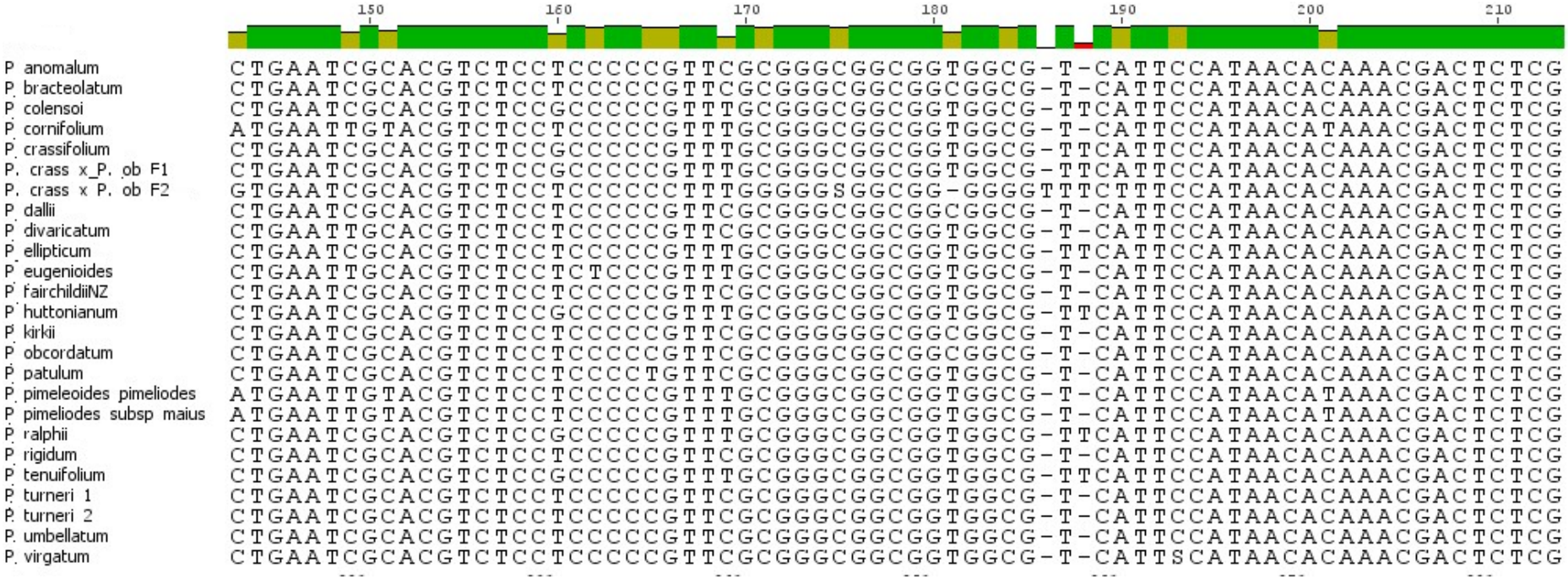

570
580
590
600
610
620
630
P. anomalum AATCGTTTTTGATTGGACCAAATGCGGGTCTCCTATAGAAGATGAAAGAAGATAGACAAGAAATCAAAGAG
P. bracteolatum AATCGTTTTTGATTGGACCAAATGCGGGTCTCCTATAGAAGATGAAAGAAGATAGACAAGAAATCAAAGAG
P. colensoi AATCGTTTTTGATTGGACCAAATGCGGGTCTCCTATAGAAGATGAAAGAAGATAGACAAGAAATCAAAGAG
P. cornifolium AATCGTTTTTGATTGGACCAAATGCGGGTCTCCTATAGAAGATGAAAGAAGATAGACAAGAAATCAAAGAG
P. crassifolium AATCGTTTTTGATTGGACCAAATGCGGGTCTCCTATAGAAGATGAAAGAAGATAGACAAGAAATCAAAGAG
P. crassifolium x P. obcordatum F1 AATCGTTTTTGATTGGACCAAATGCGGGTCTCCTATAGAAGATGAAAGAAGATAGACAAGAAATCAAAGAG
P. crassifolium x P. obcordatum F2 AATCGTTTTTGATTGGACCAAATGCGGGTCTCCTATAGAAGATGAAAGAAGATAGACAAGAAATCAAAGAG
P. dallii AATCGTTTTTGATTGGACCAAATGCGGGTCTCCTATAGAAGATGAAAGAAGATAGACAAGAAATCAAAGAG
P. divaricautm 1 AATCGTTTTTGATTGGACCAAATGCGGGTCTCCTATAGAAGATGAAAGAAGATAGACAAGAAATCAAAGAG
P. divaricatum 2 AATCGTTTTTGATTGGACCAAATGCGGGTCTCCTATAGAAGATGAAAGAAGATAGACAAGAAATCAAAGAG
P. ellipticum AATCGTTTTTGATTGGACCAAATGCGGGTCTCCTATAGAAGATGAAAGAAGATAGACAAGAAATCAAAGAG
P. ellipticum AATCGTTTTTGATTGGACCAAATGCGGGTCTCCTATAGAAGATGAAAGAAGATAGACAAGAAATCAAAGAG
P. eugenoides AATCGTTTTTGATTGGACCAAATGCGGGTCTCCTATAGAAGATGAAAGAAGATAGACAAGAAATCAAAGAG
P. fairchildii AATCGTTTTTGATTGGACCAAATGCGGGTCTCCTATAGAAGATGAAAGAAGATAGACAAGAAATCAAAGAG
P. huttonianum AATCGTTTTTGATTGGACCAAATGCGGGTCTCCTATAGAAGATGAAAGAAGATAGACAAGAAATCAAAGAG
P. kirkii AATCGTTTTTGATTGGACCAAATGCGGGTCTCCTATAGAAGATGAAAGAAGATAGACAAGAAATCAAAGAG
P. obcordatum AATCGTTTTTGATTGGACCAAATGCGGGTCTCCTATAGAAGATGAAAGAAGATAGACAAGAAATCAAAGAG
P. patulum AATCGTTTTTGATTGGACCAAATGCGGGTCTCCTATAGAAGATGAAAGAAGATAGACAAGAAATCAAAGAG
P. pimeleoides subsp. majus AATCGTTTTTGATTGGACCAAATGCGGGTCTCCTATAGAAGATGAAAGAAGATAGACAAGAAATCAAAGAG
P. pimeleoides subsp. pimeleoides AATCGTTTTTGATTGGACCAAATGCGGGTCTCCTATAGAAGATGAAAGAAGATAGACAAGAAATCAAAGAG
P. "Stephends Island" AATCGTTTTTGATTGGACCAAATGCGGGTCTCCTATAGAAGATGAAAGAAGATAGACAAGAAATCAAAGAG
P. tenuifolium AATCGTTTTTGATTGGACCAAATGCGGGTCTCCTATAGAAGATGAAAGAAGATAGACAAGAAATCAAAGAG
P. ralphii AATCGTTTTTGATTGGACCAAATGCGGGTCTCCTATAGAAGATGAAAGAAGATAGACAAGAAATCAAAGAG
P. rigidum AATCGTTTTTGATTGGACCAAATGCGGGTCTCCTATAGAAGATGAAAGAAGATAGACAAGAAATCAAAGAG
P. turneri 1 AATCGTTTTTGATTGGACCAAATGCGGGTCTCCTATAGAAGATGAAAGAAGATAGACAAGAAATCAAAGAG
P. umbellatum AATCGTTTTTGATTGGACCAAATGCGGGTCTCCTATAGAAGATGAAAGAAGATAGACAAGAAATCAAAGAG
P. virgatum AATCGTTTTTGATTGGACCAAATGCGGGTCTCCTATAGAAGATGAAAGAAGATAGACAAGAAATCAAAGAG


540
650
660
670
680
690
700
708
P. anomalum GAGAAAACTTTTTCCAGATAGGAATCGGTATCTAATGAATTCAACAGTTCCAGTATAAATAAA-CGAAA
P. bracteolatum GAGAAAACTTTTTCCAGAT-NNAATCGGTATCTAATGAATTCAACAGTTCCAGTATAAATAAA-CGAAA
P. colensoi GAGAAAACTTTTTCCAGATAGGAATCGGTATCTAATGAATTCAACAGTTCCAGTATAAATAAA-CGAAA
P. cornifolium GAGAAAACTTTTTCCAGATAGGAATCGGTATCTAATGAATTCAACAGTTCCAGTATAAATAAA-CGAAA
P. crassifolium GAGAAAACTTTTTCCAGATAGGAATCGGTATCTAATGAATTCAACAGTTCCAGTATAAATAAA-CGAAA
P. crassifolium x P. obcordatum F1 GAGAAAACTTTTTCCAGATAGGAATCGGTATCTAATGAATTCAACAGTTCCAGTATAAATAAA-CGAAA
P. crassifolium x P. obcordatum F2 GAGAAAACTTTTTCCAGATAGGAATCGGTATCTAATGAATTCAACAGTTCCAGTATAAATAAA-CGAAA
P. dallii GAGAAAACTTTTTCCAGATAGGAATCGGTATCTAATGAATTCAACAGTTCCAGTATAAATAAA-CGAAA
P. divaricautm 1 GAGAAAACTTTTTCCAGATAGGAATCGGTATCTAATGAATTCAACAGTTCCAGTATAAATAAA-CGAAA
P. divaricatum 2 GAGAAAACTTTTTCCAGATAGGAATCGGTATCTAATGAATTCAACAGTTCCAGTATAAATAAA-CGAAA
P. ellipticum GAGAAAACTTTTTCCAGATAGGAATCGGTATCTAATGAATTCAACAGTTCCAGTATAAATAAA-CGAAA
P. ellipticum GAGAAAACTTTTTCCAGATAGGAATCGGTATCTAATGAATTCAACAGTTCCAGTATAAATAAA-CGAAA
P. eugenoides GAGAAAACTTTTTCCAGATAGGAATCGGTATCTAATGAATTCAACAGTTCCAGTATAAATAAA-CGAAA
P. fairchildii GAGAAAACTTTTTCCAGATAGGAATCGGTATCTAATGAATTCAACAGTTCCAGTATAAATAAA-CGAAA
P. huttonianum GAGAAAACTTTTTCCAGATAGGAATCGGTATCTAATGAATTCAACAGTTCCAGTATAAATAAA-CGAAA
P. kirkii GAGAAAACTTTTTCCAGATAGGAATCGGTATCTAATGAATTCAACAGTTCCAGTATAAATAAA-CGAAA
P. obcordatum GAGAAAACTTTTTCCAGATAGGAATCGGTATCTAATGAATTCAACAGTTCCAGTATAAATAAA-CGAAA
P. patulum GAGAAAACTTTTTCCAGATAGGAATCGGTATCTAATGAATTCAACAGTTCCAGTATAAATAAA-CGAAA
P. pimeleoides subsp. majus GAGAAAACTTTTTCCAGATAGGAATCGGTATCTAATGAATTCAACAGTTCCAGTATAAATAAA-CGAAA
P. pimeleoides subsp. pimeleoides GAGAAAACTTTTTCCAGATAGGAATCGGTATCTAATGAATTCAACAGTTCCAGTATAAATAAA-CGAAA
P. "Stephends Island" GAGAAAACTTTTTCCAGATAGGAATCGGTATCTAATGAATTCAACAGTTCCAGTATAAATAAA-CGAAA
P. tenuifolium GAGAAAACTTTTTCCAGATAGGAATCGGTATCTAATGAATTCAACAGTTCCAGTATAAATAAA-CGAAA
P. ralphii GAGAAAACTTTTTCCAGATAGGAATCGGTATCTAATGAATTCAACAGTTCCAGTATAAATAAA-CGAAA
P. rigidum GAGAAAACTTTTTCCAGATAGGAATCGGTATCTAATGAATTCAACAGTTCCAGTATAAATAAAACGAAA
P. turneri 1 GAGAAAACTTTTTCCAGATAGGAATCGGTATCTAATGAATTCAACAGTTCCAGTATAAATAAA-CGAAA
P. umbellatum GAGAAAACTTTTTCCAGATAGGAATCGGTATCTAATGAATTCAACAGTTCCAGTATAAATAAA-CGAAA
P. virgatum GAGAAAACTTTTTCCAGATAGGAATCGGTATCTAATGAATTCAACAGTTCCAGTATAAATAAA-CGAAA

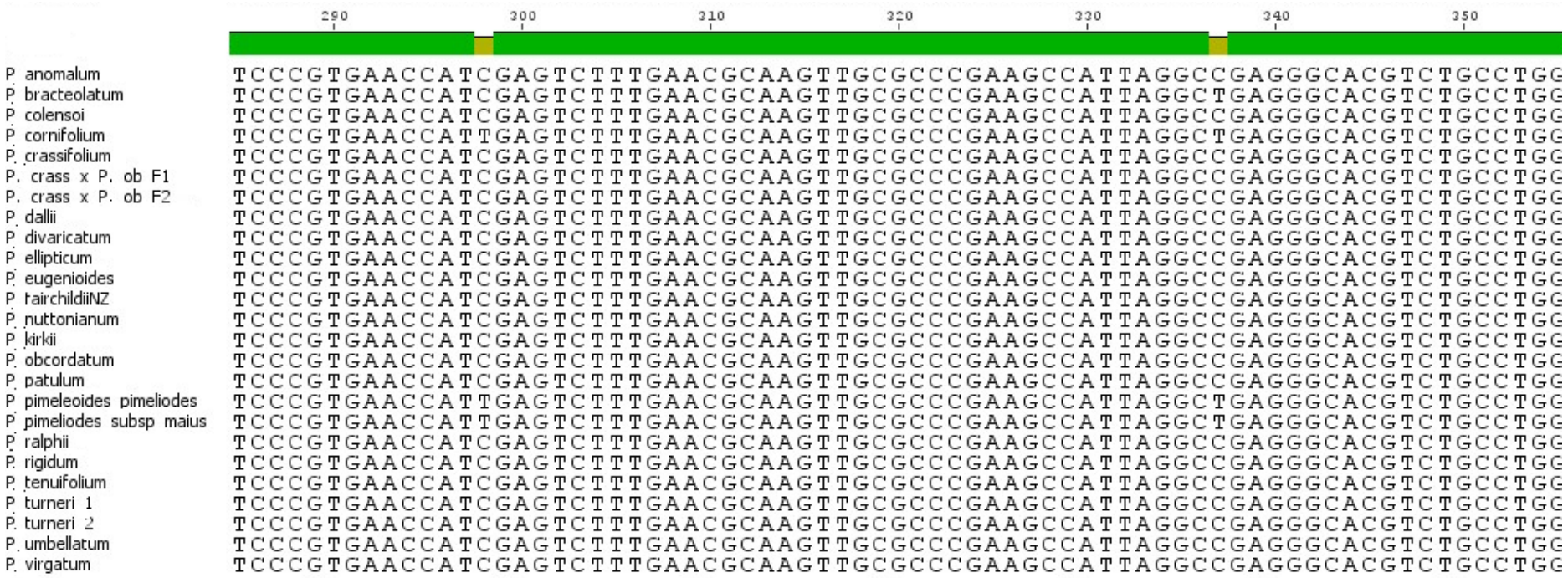
APPENDIX 3: Aligned matrix of the ITS region

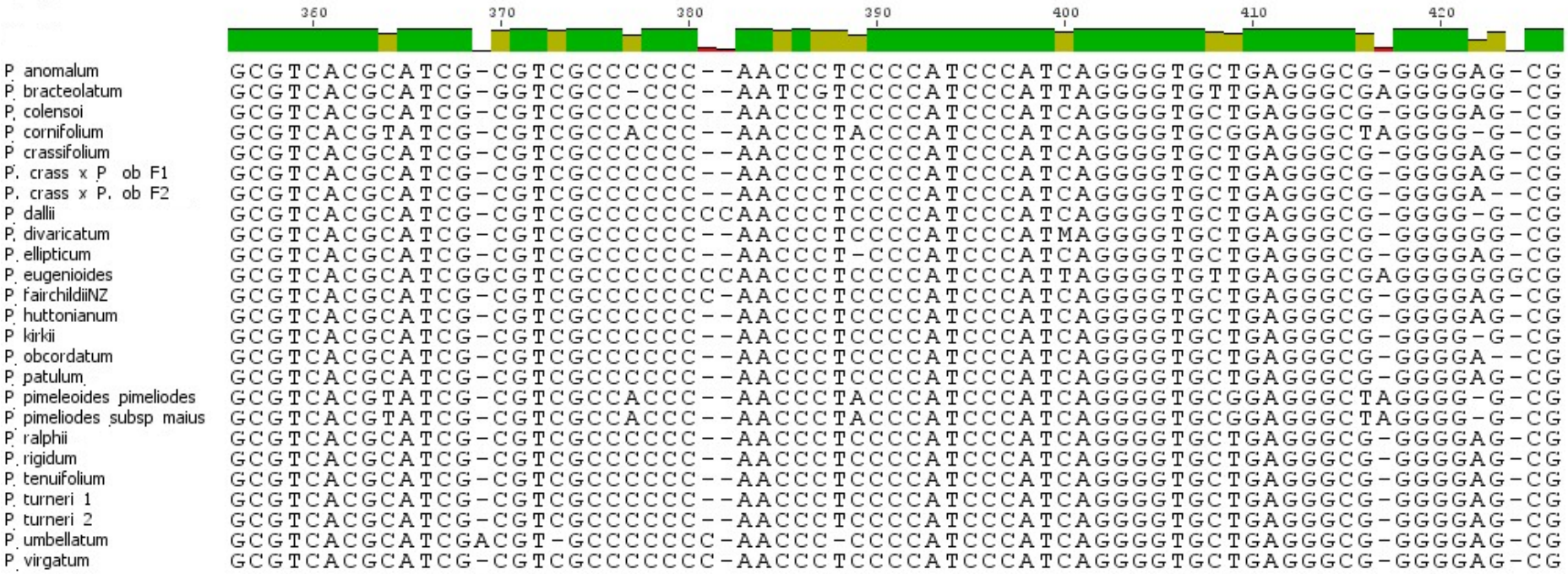


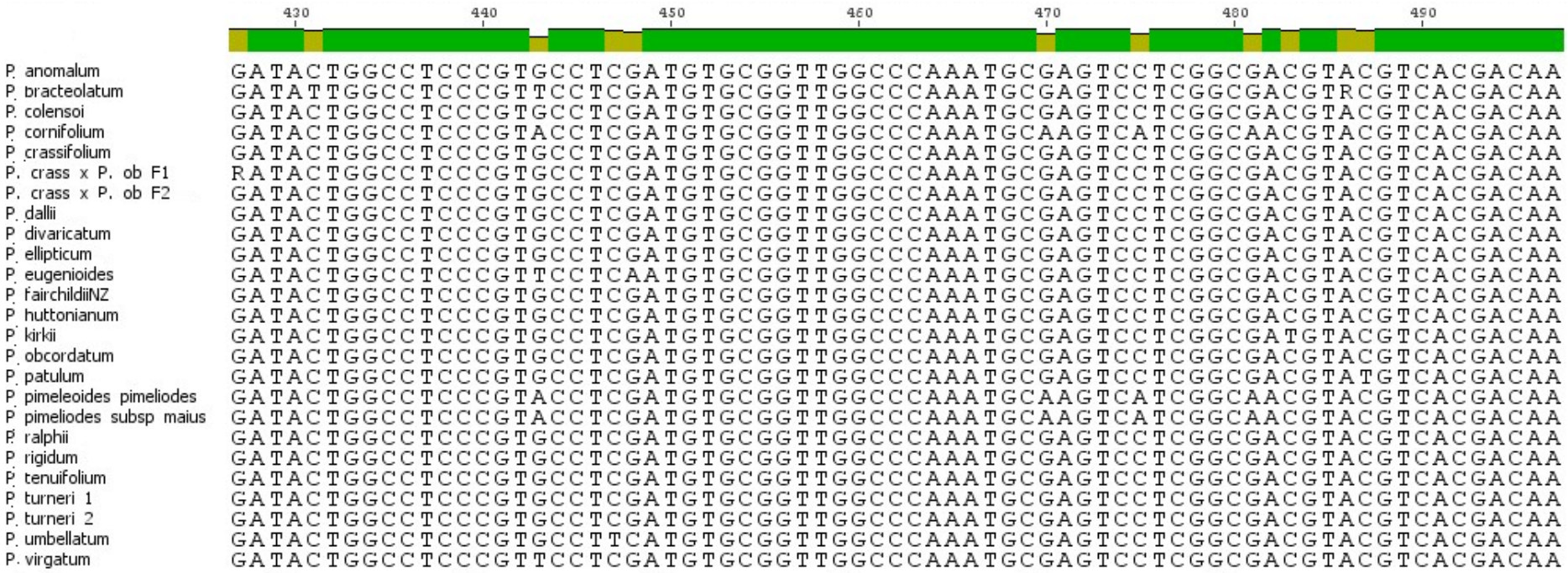

80
90
100
110
120
130
140
P. anomalum CGGGGAGTGCCCTTGGGCGCTGCTCGACCGAAAACAAAACCCC-GGCGCGGAACGCGCCAAGGAACTCAAA
P. bracteolatum CGAGGAGTGCCCCCGGGCGCTGTTTGACCGAAAACCAAACCCC-GGCGCGGAACGCGCCAAGGAACTCAAA
P. colensoi CGGGGAGTGCCCTTGGGCGCTGCTCGACCGAAAACAAAACCCCCGGCGCGGAATGCGCCAAGGAACTCAAA
P. cornifolium CGGGGAGTGCCCTTAGGCGTTACTCGACCGAAAACCAAACCCC-GGCGTGGAACGCGCCAAGGAACTCAAA
P. crassifolium CGGGGAGTGCCCTTGGGCGCTGCTCGACCGAAAACAAAACCCCCGGCGCGGAATGCGCCAAGGAACTCAAA
P. crass x P. ob_F1 CGGGGAGTGCCCTTGGGCGCTGCTCGACCGAAAACAAAACCCCCGGCGCGGAATGCGCCAAGGAACTCAAA
P. crass x P. ob F2 GGGGGAGTGCCCTTGGGCGCTGCTCGACCGAAAACCAAACCCCGGGCG-GGAA-GCGCCCAGGAACTCAAA
P. dallii CGGGGAGTGCCCCCGGGCGCTGCTCGACCGAAAACCAAACCCC-GGCGCGGAACGCGCCAAGGAACTCAAA
P. divaricatum CGGGGAGTGCCCTTGGGCGCTGCTCGACCGAAAACAAAACCCC-GGCGCGGAACGCGCCAAGGAACTCAAA
P. ellipticum CGGGGAGTGCCCTTGGGCGCTGCTCGACCGAAAACAAAACCCC-GGCGCGGAACGCGCCAAGGAACTCAAA
P. eugenioides CGAGGAGTGCCCCCGGGCGCTGCTCGATCGAAAACCAAACCCC-GGCGCGGAATGCGCCAAGGAACTCAAA
P. fairchildiiNZ CG-GT-------T-GGGCGCTGCTCGACCGAAAACAAAACCCC-GGCGCGGAACGCGCCAAGGAACTCAAA
P. huttonianum CGGGGAGTGCCCTTGGGCGCTGCTCGACCGAAAACAAAACCCCCGGCGCGGAATGCGCCAAGGAACTCAAA
P. kirkii CGGGGAGTGCCCTCGGGCGCTGCTCGATCGAAAACCAAACCCC-GGCGCGGAAAGCGCCAAGGAACTCAAA
P. obcordatum CGGGGAGTGCCCTTGGGCGCTGCTCGACCGAAAACCAAACCCC-GGCGCGGAATGCGCCAAGGAACTCAAA
P. patulum_ CGGGGAGTGCCCTTGGGCGCTGCTCGACCGAAAACAAAACCCC-GGCGCGGAACGCGCCAAGGAACTCAAA
P. pimeleoides_pimeliodes CGGGGAGTGCCCTTAGGCGTTACTCGACCGAAAACCAAACCCC-GGCGTGGAACGCGCCAAGGAACTCAAA
P. pimeliodes_subsp_maius CGGGGAGTGCCCTTAGGCGTTACTCGACCGAAAACCAAACCCC-GGCGTGGAACGCGCCAAGGAACTCAAA
P. ralphii CGGGGAGTGCCCTTGGGCGCTGCTCGACCGAAAACAAAACCCCCGGCGCGGAATGCGCCAAGGAACTCAAG
P. rigidum CGGGGAGTGCCCTTGGGCGCTGCTCGACCGAAAACAAAACCCC-GGCGCGGAACGCGCCAAGGAACTCAAA
P. tenuifolium CGGGGAGTGCCCTTGGGCGCTGCTCGACCGAAAACAAAACCCCCGGCGCGGAATGCGCCAAGGAACTCAAA
P. turneri 1 CGGGGAGTGCCCTTGGGCGCTGCTCGACCGAAAACAAAACCCC-GGCGCGGAACGCGCCAAGGAACTCAAA
P. turneri 2 CGGGGAGTGCCCTTGGGCGCTGCTCGACCGAAAACAAAACCCC-GGCGCGGAACGCGCCAAGGAACTCAAA
P. umbellatum CGGGGAGTGCCCTTGGGCGCTGCTCGACCGAAAACAAAACCCC-GGCGCGGAACGCGCCAAGGAACTCAAA
P. virgatum CGGGGAGTGCCCTTGGGCGCTGCTCGACCGAAAACAAAAGCCC-GGCGCGGAACGCGCCAAGGAACTCAAA

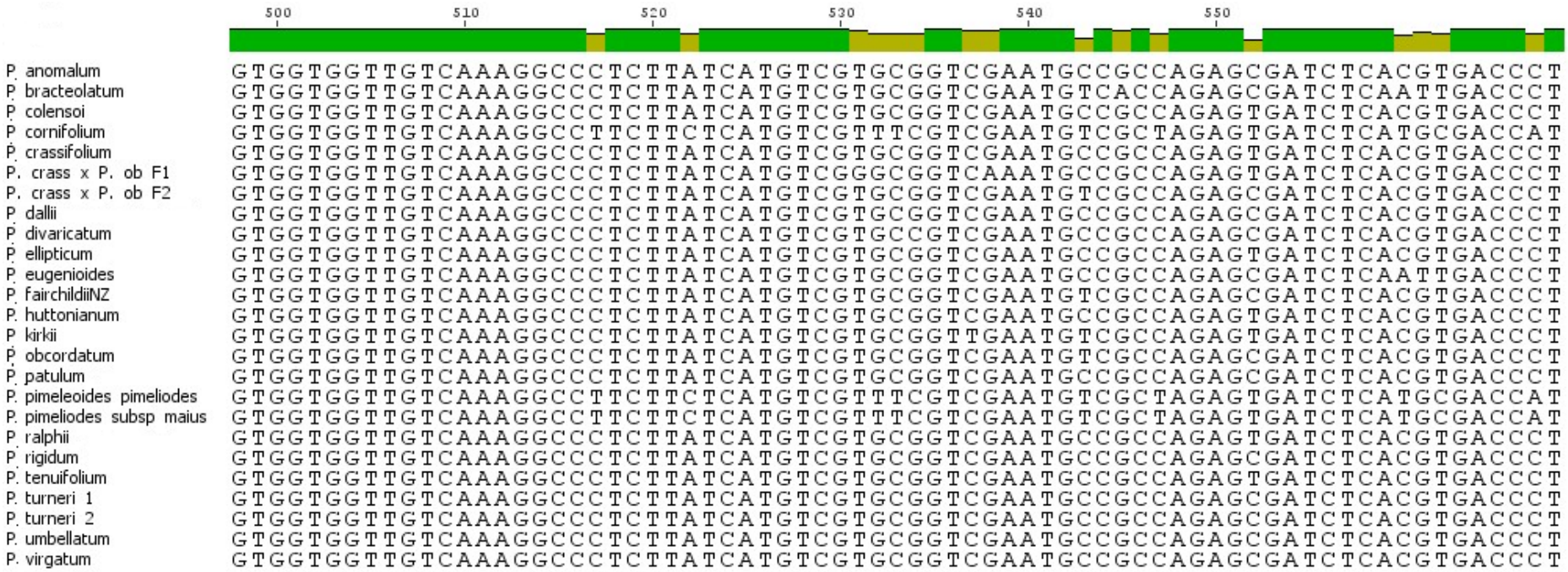

150
160
170
180
190
200
210
P. anomalum CTGAATCGCACGTCTCCTCCCCCCGTTCGCGGGCGGCGGTGGCG-T-CATTCCATAACACAAACGACTCTCG
P. bracteolatum CTGAATCGCACGTCTCCTCCCCCCGTTCGCGGGCGGCGGCGGCG-T-CATTCCATAACACAAACGACTCTCG
P. colensoi CTGAATCGCACGTCTCCGCCCCCCGTTTGCGGGCGGCGGTGGCG-TTCATTCCATAACACAAACGACTCTCG
P. cornifolium ATGAATTGTACGTCTCCTCCCCCCGTTTGCGGGCGGCGGTGGCG-T-CATTCCATAACATAAACGACTCTCG
P. crassifolium CTGAATCGCACGTCTCCGCCCCCCGTTTGCGGGCGGCGGTGGCG-TTCATTCCATAACACAAACGACTCTCG
P. crass x_P. ob F1 CTGAATCGCACGTCTCCGCCCCCCGTTTGCGGGCGGCGGTGGCG-TTCATTCCATAACACAAACGACTCTCG
P. crass x P. ob F2 GTGAATCGCACGTCTCCTCCCCCCCTTTGGGGGGSGGCGG-GGGGTTTCTTTCCATAACACAAACGACTCTCG
P. dallii CTGAATCGCACGTCTCCTCCCCCCGTTCGCGGGCGGCGGCGGCG-T-CATTCCATAACACAAACGACTCTCG
P. divaricatum CTGAATTGCACGTCTCCTCCCCCCGTTCGCGGGCGGCGGTGGCG-T-CATTCCATAACACAAACGACTCTCG
P. ellipticum CTGAATCGCACGTCTCCTCCCCCCGTTTGCGGGCGGCGGTGGCG-TTCATTCCATAACACAAACGACTCTCG
P. eugenioides CTGAATTGCACGTCTCCTCTCCCCGTTTGCGGGCGGCGGTGGCG-T-CATTCCATAACACAAACGACTCTCG
P. fairchildiiNZ CTGAATCGCACGTCTCCTCCCCCCGTTCGCGGGCGGCGGTGGCG-T-CATTCCATAACACAAACGACTCTCG
P. huttonianum CTGAATCGCACGTCTCCGCCCCCCGTTTGCGGGCGGCGGTGGCG-TTCATTCCATAACACAAACGACTCTCG
P. kirkii CTGAATCGCACGTCTCCTCCCCCCGTTCGCGGGGGGCGGCGGCG-T-CATTCCATAACACAAACGACTCTCG
P. obcordatum CTGAATCGCACGTCTCCTCCCCCCGTTCGCGGGCGGCGGCGGCG-T-CATTCCATAACACAAACGACTCTCG
P. patulum CTGAATCGCACGTCTCCTCCCCTGTTCGCGGGCGGCGGTGGCG-T-CATTCCATAACACAAACGACTCTCG
P. pimeleoides pimeliodes ATGAATTGTACGTCTCCTCCCCCCGTTTGCGGGCGGCGGTGGCG-T-CATTCCATAACATAAACGACTCTCG
P. pimeliodes subsp maius ATGAATTGTACGTCTCCTCCCCCCGTTTGCGGGCGGCGGTGGCG-T-CATTCCATAACATAAACGACTCTCG
P. ralphii CTGAATCGCACGTCTCCGCCCCCCGTTTGCGGGCGGCGGTGGCG-TTCATTCCATAACACAAACGACTCTCG
P. rigidum CTGAATCGCACGTCTCCTCCCCCCGTTCGCGGGCGGCGGTGGCG-T-CATTCCATAACACAAACGACTCTCG
P. tenuifolium CTGAATCGCACGTCTCCGCCCCCCGTTTGCGGGCGGCGGTGGCG-TTCATTCCATAACACAAACGACTCTCG
P. turneri 1 CTGAATCGCACGTCTCCTCCCCCCGTTCGCGGGCGGCGGTGGCG-T-CATTCCATAACACAAACGACTCTCG
P. turneri 2 CTGAATCGCACGTCTCCTCCCCCCGTTCGCGGGCGGCGGTGGCG-T-CATTCCATAACACAAACGACTCTCG
P. umbellatum CTGAATCGCACGTCTCCTCCCCCCGTTCGCGGGCGGCGGTGGCG-T-CATTCCATAACACAAACGACTCTCG
P. virgatum CTGAATCGCACGTCTCCTCCCCCCGTTCGCGGGCGGCGGTGGCG-T-CATTSCATAACACAAACGACTCTCG


220
230
240
250
260
270
280
P. anomalum GCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAA
P. bracteolatum GCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAA
P. colensoi GCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAA
P. cornifolium GCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAA
P. crassifolium GCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAA
P. crass x P. ob F1 GCAACGGATATCTCGGCTCTCGCATCGATGAAAAACGTAGCAAAATGCGATACTTGGTGTGAATTGCAGAA
P. crass x P. ob F2 GCAACGGATATCTCGGCTCTCGCATCGATGAAAAACGTAGCGAAATGCGATACTTGGTGGGAATTCCAGAA
P. dallii GCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAA
P. divaricatum GCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAA
P. ellipticum GCAACGGATATCTCGGCTCTCGCATCGATGAAAAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAA
P. eugenioides GCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAA
P. fairchildiiNZ GCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAA
P. huttonianum GCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAA
P. kirkii GCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAA
P. obcordatum GCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAA
P. patulum GCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAA
P. pimeleoides pimeliodes GCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAA
P. pimeliodes subsp maius GCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAA
P. ralphii GCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAA
P. rigidum GCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAA
P. tenuifolium GCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAA
P. turneri 1 GCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAA
P. turneri 2 GCAACGGATTTCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAA
P. umbellatum GCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAA
P. virgatum GCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAA


290
300
310
320
330
340
350
P. anomalum TCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCATTAGGCCGAGGGCACGTCTGCCTGG
P. bracteolatum TCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCATTAGGCTGAGGGCACGTCTGCCTGG
P. colensoi TCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCATTAGGCCGAGGGCACGTCTGCCTGG
P. cornifolium TCCCGTGAACCATTGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCATTAGGCTGAGGGCACGTCTGCCTGG
P. crassifolium TCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCATTAGGCCGAGGGCACGTCTGCCTGG
P. crass x P. ob F1 TCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCATTAGGCCGAGGGCACGTCTGCCTGG
P. crass x P. ob F2 TCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCATTAGGCCGAGGGCACGTCTGCCTGG
P. dallii TCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCATTAGGCCGAGGGCACGTCTGCCTGG
P. divaricatum TCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCATTAGGCCGAGGGCACGTCTGCCTGG
P. ellipticum TCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCATTAGGCCGAGGGCACGTCTGCCTGG
P. eugenioides TCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCATTAGGCCGAGGGCACGTCTGCCTGG
P. fairchildiiNZ TCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCATTAGGCCGAGGGCACGTCTGCCTGG
P. nuttonianum TCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCATTAGGCCGAGGGCACGTCTGCCTGG
P. kirkii TCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCATTAGGCCGAGGGCACGTCTGCCTGG
P. obcordatum TCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCATTAGGCCGAGGGCACGTCTGCCTGG
P. patulum TCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCATTAGGCCGAGGGCACGTCTGCCTGG
P. pimeleoides pimeliodes TCCCGTGAACCATTGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCATTAGGCTGAGGGCACGTCTGCCTGG
P. pimeliodes subsp maius TCCCGTGAACCATTGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCATTAGGCTGAGGGCACGTCTGCCTGG
P. ralphii TCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCATTAGGCCGAGGGCACGTCTGCCTGG
P. rigidum TCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCATTAGGCCGAGGGCACGTCTGCCTGG
P. tenuifolium TCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCATTAGGCCGAGGGCACGTCTGCCTGG
P. turneri 1 TCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCATTAGGCCGAGGGCACGTCTGCCTGG
P. turneri 2 TCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCATTAGGCCGAGGGCACGTCTGCCTGG
P. umbellatum TCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCATTAGGCCGAGGGCACGTCTGCCTGG
P. virgatum TCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCATTAGGCCGAGGGCACGTCTGCCTGG


360
370
380
390
400
410
420
P. anomalum GCGTCACGCATCG-CGTCGCCCCCC--AACCCTCCCCATCCCATCAGGGGTGCTGAGGGCG-GGGGAG-CG
P. bracteolatum GCGTCACGCATCG-GGTCGCC-CCC--AATCGTCCCCATCCCATTAGGGGTGTTGAGGGCGAGGGGGG-CG
P. colensoi GCGTCACGCATCG-CGTCGCCCCCC--AACCCTCCCCATCCCATCAGGGGTGCTGAGGGCG-GGGGAG-CG
P. cornifolium GCGTCACGTATCG-CGTCGCCACCC--AACCCTACCCATCCCATCAGGGGTGCGGAGGGCTAGGGG-G-CG
P. crassifolium GCGTCACGCATCG-CGTCGCCCCCC--AACCCTCCCCATCCCATCAGGGGTGCTGAGGGCG-GGGGAG-CG
P. crass x P ob F1 GCGTCACGCATCG-CGTCGCCCCCC--AACCCTCCCCATCCCATCAGGGGTGCTGAGGGCG-GGGGAG-CG
P. crass x P. ob F2 GCGTCACGCATCG-CGTCGCCCCCC--AACCCTCCCCATCCCATCAGGGGTGCTGAGGGCG-GGGGA--CG
P. dallii GCGTCACGCATCG-CGTCGCCCCCCCCAACCCTCCCCATCCCATCAGGGGTGCTGAGGGCG-GGGG-G-CG
P. divaricatum GCGTCACGCATCG-CGTCGCCCCCC--AACCCTCCCCATCCCATMAGGGGTGCTGAGGGCG-GGGGGG-CG
P. ellipticum GCGTCACGCATCG-CGTCGCCCCCC--AACCCT-CCCATCCCATCAGGGGTGCTGAGGGCG-GGGGAG-CG
P. eugenioides GCGTCACGCATCGGCGTCGCCCCCCCCAACCCTCCCCATCCCATTAGGGGTGTTGAGGGCGAGGGGGGGCG
P. fairchildiiNZ GCGTCACGCATCG-CGTCGCCCCCCC-AACCCTCCCCATCCCATCAGGGGTGCTGAGGGCG-GGGGAG-CG
P. huttonianum GCGTCACGCATCG-CGTCGCCCCCC--AACCCTCCCCATCCCATCAGGGGTGCTGAGGGCG-GGGGAG-CG
P kirkii GCGTCACGCATCG-CGTCGCCCCCC--AACCCTCCCCATCCCATCAGGGGTGCTGAGGGCG-GGGG-G-CG
P. obcordatum GCGTCACGCATCG-CGTCGCCCCCC--AACCCTCCCCATCCCATCAGGGGTGCTGAGGGCG-GGGGA--CG
P. patulum GCGTCACGCATCG-CGTCGCCCCCC--AACCCTCCCCATCCCATCAGGGGTGCTGAGGGCG-GGGGAG-CG
P. pimeleoides pimeliodes GCGTCACGTATCG-CGTCGCCACCC--AACCCTACCCATCCCATCAGGGGTGCGGAGGGCTAGGGG-G-CG
P. pimeliodes subsp maius GCGTCACGTATCG-CGTCGCCACCC--AACCCTACCCATCCCATCAGGGGTGCGGAGGGCTAGGGG-G-CG
P. ralphii GCGTCACGCATCG-CGTCGCCCCCC--AACCCTCCCCATCCCATCAGGGGTGCTGAGGGCG-GGGGAG-CG
P. rigidum GCGTCACGCATCG-CGTCGCCCCCC--AACCCTCCCCATCCCATCAGGGGTGCTGAGGGCG-GGGGAG-CG
P. tenuifolium GCGTCACGCATCG-CGTCGCCCCCC--AACCCTCCCCATCCCATCAGGGGTGCTGAGGGCG-GGGGAG-CG
P. turneri 1 GCGTCACGCATCG-CGTCGCCCCCC--AACCCTCCCCATCCCATCAGGGGTGCTGAGGGCG-GGGGAG-CG
P. turneri 2 GCGTCACGCATCG-CGTCGCCCCCC--AACCCTCCCCATCCCATCAGGGGTGCTGAGGGCG-GGGGAG-CG
P. umbellatum GCGTCACGCATCGACGT-GCCCCCCCC-AACCC-CCCCATCCCATCAGGGGTGCTGAGGGCG-GGGGAG-CG
P. virgatum GCGTCACGCATCG-CGTCGCCCCCCCC-AACCCTCCCCATCCCATCAGGGGTGCTGAGGGCG-GGGGAG-CG


430
440
450
460
470
480
490
P. anomalum GATACTGGCCTCCCCGTGCCTCGATGTGCGGTTGGCCCAAATGCGAGTCCTCGGCGACGTACGTCACGACAA
P. bracteolatum GATATTGGCCTCCCCGTTCCTCGATGTGCGGTTGGCCCAAATGCGAGTCCTCGGCGACGTRCGTCACGACAA
P. colensoi GATACTGGCCTCCCCGTGCCTCGATGTGCGGTTGGCCCAAATGCGAGTCCTCGGCGACGTACGTCACGACAA
P. cornifolium GATACTGGCCTCCCCGTACCTCGATGTGCGGTTGGCCCAAATGCAAGTCATCGGCAACGTACGTCACGACAA
P. crassifolium GATACTGGCCTCCCCGTGCCTCGATGTGCGGTTGGCCCAAATGCGAGTCCTCGGCGACGTACGTCACGACAA
P. crass x P. ob F1 RATACTGGCCTCCCCGTGCCTCGATGTGCGGTTGGCCCAAATGCGAGTCCTCGGCGACGTACGTCACGACAA
P. crass x P. ob F2 GATACTGGCCTCCCCGTGCCTCGATGTGCGGTTGGCCCAAATGCGAGTCCTCGGCGACGTACGTCACGACAA
P. dallii GATACTGGCCTCCCCGTGCCTCGATGTGCGGTTGGCCCAAATGCGAGTCCTCGGCGACGTACGTCACGACAA
P. divaricatum GATACTGGCCTCCCCGTGCCTCGATGTGCGGTTGGCCCAAATGCGAGTCCTCGGCGACGTACGTCACGACAA
P. ellipticum GATACTGGCCTCCCCGTGCCTCGATGTGCGGTTGGCCCAAATGCGAGTCCTCGGCGACGTACGTCACGACAA
P. eugenioides GATACTGGCCTCCCCGTTCCTCAATGTGCGGTTGGCCCAAATGCGAGTCCTCGGCGACGTACGTCACGACAA
P. fairchildiiNZ GATACTGGCCTCCCCGTGCCTCGATGTGCGGTTGGCCCAAATGCGAGTCCTCGGCGACGTACGTCACGACAA
P. huttonianum GATACTGGCCTCCCCGTGCCTCGATGTGCGGTTGGCCCAAATGCGAGTCCTCGGCGACGTACGTCACGACAA
P. kirkii GATACTGGCCTCCCCGTGCCTCGATGTGCGGTTGGCCCAAATGCGAGTCCTCGGCGATGTACGTCACGACAA
P. obcordatum GATACTGGCCTCCCCGTGCCTCGATGTGCGGTTGGCCCAAATGCGAGTCCTCGGCGACGTACGTCACGACAA
P. patulum GATACTGGCCTCCCCGTGCCTCGATGTGCGGTTGGCCCAAATGCGAGTCCTCGGCGACGTATGTCACGACAA
P. pimeleoides pimeliodes GATACTGGCCTCCCCGTACCTCGATGTGCGGTTGGCCCAAATGCAAGTCATCGGCAACGTACGTCACGACAA
P. pimeliodes subsp maius GATACTGGCCTCCCCGTACCTCGATGTGCGGTTGGCCCAAATGCAAGTCATCGGCAACGTACGTCACGACAA
P. ralphii GATACTGGCCTCCCCGTGCCTCGATGTGCGGTTGGCCCAAATGCGAGTCCTCGGCGACGTACGTCACGACAA
P. rigidum GATACTGGCCTCCCCGTGCCTCGATGTGCGGTTGGCCCAAATGCGAGTCCTCGGCGACGTACGTCACGACAA
P. tenuifolium GATACTGGCCTCCCCGTGCCTCGATGTGCGGTTGGCCCAAATGCGAGTCCTCGGCGACGTACGTCACGACAA
P. turneri 1 GATACTGGCCTCCCCGTGCCTCGATGTGCGGTTGGCCCAAATGCGAGTCCTCGGCGACGTACGTCACGACAA
P. turneri 2 GATACTGGCCTCCCCGTGCCTCGATGTGCGGTTGGCCCAAATGCGAGTCCTCGGCGACGTACGTCACGACAA
P. umbellatum GATACTGGCCTCCCCGTGCCTTCATGTGCGGTTGGCCCAAATGCGAGTCCTCGGCGACGTACGTCACGACAA
P. virgatum GATACTGGCCTCCCCGTTCCTCGATGTGCGGTTGGCCCAAATGCGAGTCCTCGGCGACGTACGTCACGACAA


500
510
520
530
540
550
P. anomalum GTGGTGGTTGTCAAAGGCCCTCTTATCATGTCGTGCGGTCGAATGCCGCCAGAGCGATCTCACGTGACCCT
P. bracteolatum GTGGTGGTTGTCAAAGGCCCTCTTATCATGTCGTGCGGTCGAATGTCACCAGAGCGATCTCAATTGACCCT
P. colensoi GTGGTGGTTGTCAAAGGCCCTCTTATCATGTCGTGCGGTCGAATGCCGCCAGAGTGATCTCACGTGACCCT
P. cornifolium GTGGTGGTTGTCAAAGGCCTTCTTCTCATGTCGTTTCGTCGAATGTCGCTAGAGTGATCTCATGCGACCAT
P. crassifolium GTGGTGGTTGTCAAAGGCCCTCTTATCATGTCGTGCGGTCGAATGCCGCCAGAGTGATCTCACGTGACCCT
P. crass x P. ob F1 GTGGTGGTTGTCAAAGGCCCTCTTATCATGTCGGGCGGTCAAATGCCGCCAGAGTGATCTCACGTGACCCT
P. crass x P. ob F2 GTGGTGGTTGTCAAAGGCCCTCTTATCATGTCGTGCGGTCGAATGTCGCCAGAGCGATCTCACGTGACCCT
P. dallii GTGGTGGTTGTCAAAGGCCCTCTTATCATGTCGTGCGGTCGAATGCCGCCAGAGCGATCTCACGTGACCCT
P. divaricatum GTGGTGGTTGTCAAAGGCCCTCTTATCATGTCGTGCCGTCGAATGCCGCCAGAGCGATCTCACGTGACCCT
P. ellipticum GTGGTGGTTGTCAAAGGCCCTCTTATCATGTCGTGCGGTCGAATGCCGCCAGAGTGATCTCACGTGACCCT
P. eugenioides GTGGTGGTTGTCAAAGGCCCTCTTATCATGTCGTGCGGTCGAATGCCGCCAGAGCGATCTCAATTGACCCT
P. fairchildiiNZ GTGGTGGTTGTCAAAGGCCCTCTTATCATGTCGTGCGGTCGAATGTCGCCAGAGCGATCTCACGTGACCCT
P. huttonianum GTGGTGGTTGTCAAAGGCCCTCTTATCATGTCGTGCGGTCGAATGCCGCCAGAGTGATCTCACGTGACCCT
P. kirkii GTGGTGGTTGTCAAAGGCCCTCTTATCATGTCGTGCGGTTGAATGTCGCCAGAGTGATCTCACGTGACCCT
P. obcordatum GTGGTGGTTGTCAAAGGCCCTCTTATCATGTCGTGCGGTCGAATGTCGCCAGAGCGATCTCACGTGACCCT
P. patulum GTGGTGGTTGTCAAAGGCCCTCTTATCATGTCGTGCGGTCGAATGCCGCCAGAGCGATCTCACGTGACCCT
P. pimeleoides pimeliodes GTGGTGGTTGTCAAAGGCCTTCTTCTCATGTCGTTTCGTCGAATGTCGCTAGAGTGATCTCATGCGACCAT
P. pimeliodes subsp maius GTGGTGGTTGTCAAAGGCCTTCTTCTCATGTCGTTTCGTCGAATGTCGCTAGAGTGATCTCATGCGACCAT
P. ralphii GTGGTGGTTGTCAAAGGCCCTCTTATCATGTCGTGCGGTCGAATGTCGCCAGAGTGATCTCACGTGACCCT
P. rigidum GTGGTGGTTGTCAAAGGCCCTCTTATCATGTCGTGCGGTCGAATGCCGCCAGAGCGATCTCACGTGACCCT
P. tenuifolium GTGGTGGTTGTCAAAGGCCCTCTTATCATGTCGTGCGGTCGAATGCCGCCAGAGTGATCTCACGTGACCCT
P. turneri 1 GTGGTGGTTGTCAAAGGCCCTCTTATCATGTCGTGCGGTCGAATGCCGCCAGAGCGATCTCACGTGACCCT
P. turneri 2 GTGGTGGTTGTCAAAGGCCCTCTTATCATGTCGTGCGGTCGAATGCCGCCAGAGCGATCTCACGTGACCCT
P. umbellatum GTGGTGGTTGTCAAAGGCCCTCTTATCATGTCGTGCGGTCGAATGCCGCCAGAGCGATCTCACGTGACCCT
P. virgatum GTGGTGGTTGTCAAAGGCCCTCTTATCATGTCGTGCGGTCGAATGCCGCCAGAGCGATCTCACGTGACCCT

APPENDIX 4: Stain and buffer combinations trialled for allozymes. Question marks show that bands were faint but appeared polymorphic.

Stain	TC8	System 6	MC	HC	System 8	Polymorphic?
MDH		no	yes			no
ADH					yes	yes
PGM				yes		no
IDH				no	yes	no
PGI					yes	yes
LAP						
6PGD	yes	no	no	no	no	yes
ALD					yes	no
G6PDH			no		no	yes?
ME		yes		no	no	no
SKDH					yes	yes?
HK	y/n				no	yes?
EST (FE)					no	
DIA					no	

APPENDIX 5 : Allozyme data matrix. 01 represents an individual homozygous for allele 1, 02 represents an individual homozygous for allele 2, 12 represents a heterozygous individual (prepared for analyses using TFPGA).

P. divaricatum 1	01,01,01
P. divaricatum 2	02,01,01
P. divaricatum 3	02,01,01
P. divaricatum 4	01,01,01
P. divaricatum 5	01,01,01
P. turneri 1	01,01,01
P. turneri 2	01,01,01
P. turneri 3	01,01,01
P. turneri 4	01,01,01
P. colensoi 1	12,01,01
P. colensoi 2	01,01,01
P. colensoi 3	01,01,01
P. colensoi 4	02,01,01
P. colensoi 5	01,01,01
P. colensoi 6	01,01,01
P. colensoi 7	01,01,01

APPENDIX SIX: ISSR data matrix. Band absent = 0, band present = 1.

P. cornifoliim	11101111001010011011000110110001001010000 0
P.divaricatum 1	01100100111000111001001101111010111001010 0
P. divaricatum 2	01110100111000111001011101111010111001010 0
P. divaricatum 3	01100100111000111101001101111010111000010 0
P. turneri 1	01110110101101111001101111111111011100111 11
P. turneri 2	01110110101101111001101111111111011100111 11
P. turneri 3	01110110101101111001101111111111011110111 11
P. colensoi 1	01101100001100111001001111111010111000100 1
P. colensoi 2	01101100001100111001001111111010111001100 1
P. colensoi 3	0110110000111??????????????1110101110001001

APPENDIX 7: Herbarium specimens used in the morphology study.

Specimens are from the Allan Herbarium at Landcare Research (NZCHAR), New Zealand Forest Research Institute (NZFRI) or University of Waikato Herbarium (WAIK).

Accession number	Taxa	Location
NZCHAR649433	*P. divaricatum*	Ripia valley, N.I
NZCHAR 231693	*P. divaricatum*	North Canterbury, S.I
NZCHAR 254997	*P. divaricatum*	Hooker Valley, S.I
NZCHAR 537259	*P. divaricatum*	Marlborough, S.I
NZCHAR511862A	*P. divaricatum*	Canterbury, S.I
NZCHAR 312083	*P. divaricatum*	North-West Nelson,S.I
NZCHAR 415966	*P. divaricatum*	Nelson, S.I
NZCHAR 221440	*P. divaricatum*	Kaimanawa Forest, N.I
NZCHAR 112704	*P. divaricatum*	Tongariro National Park, N.I
NZCHAR 469735	*P. divaricatum*	Wairarapa, N.I
NZCHAR 469445	*P. divaricatum*	Ripia valley, N.I
NZCHAR 420048	*P. divaricatum*	Otago, S.I
NZCHAR 568971	*P. divaricatum*	Mt Algidus, S.I
NZCHAR 467437	*P. divaricatum*	Canterbury, S.I
NZCHAR 112704	*P. divaricatum*	?
NZCHAR 244221	*P. divaricatum*	?
NZCHAR 467792	*P. divaricatum*	North-West Nelson, S.I
NZCHAR 519197	*P. divaricatum*	Canterbury, S.I
NZCHAR511862B	*P. divaricatum*	Canterbury, S.I
NZFRI17003	*P. divaricatum*	Ripia valley, N.I
NZCHAR 319061	*P. divaricatum*	Canterbury,S.I
NZCHAR 537252	*P. colensoi*	Ohakune, N.I
NZCHAR 535718	*P. colensoi*	Bruce rd, Ohakune, N.I
NZCHAR 537254	*P. colensoi*	Ohakune, N.I
NZCHAR 537253	*P. colensoi*	Ohakune, N.I
NZCHAR 247615	*P. colensoi*	Fiordland, S.I
NZCHAR 445818	*P. colensoi*	Waitomo, Waikato, N.I
NZCHAR 238650	*P. colensoi*	Peketi forest, Northland, N.I
NZCHAR 153065	*P. colensoi*	Catlins Forest, Otago, S.I
NZCHAR 505709	*P. colensoi*	Lake Wakatipu, Otago, S.I
NZCHAR 417329	*P. colensoi*	South Otago, S.I
NZCHAR 419184	*P. colensoi*	Karamea bluffs, S.I
NZCHAR 166793	*P. colensoi*	Franz Joseph, Westland, S.I
NZCHAR 296048	*P. colensoi*	Waimarino, Waikato, N.I
NZCHAR 192450	*P. colensoi*	Wanganui river, N.I
NZCHAR 296096	*P. colensoi*	Auckland (cultivated), N.I
NZCHAR 537251	*P. colensoi*	Taupo, N.I
NZCHAR 117284	*P. colensoi*	Tongariro, N.I
NZCHAR 206903	*P. colensoi*	Rotorua, N.I
NZCHAR 394772	*P. colensoi*	Nelson, N.I

NZCHAR 218779	*P. colensoi*	Southland, N.I
NZCHAR 243555	*P. colensoi*	Manapouri, Fiordland, S.I
NZCHAR 537255	*P. colensoi*	Esk, N.I
NZCHAR 535687	*P. colensoi*	Wairere Stream, Salmond Track, N.I
NZCHAR 368554	*P. colensoi*	Kererutahi, Opotiki, N.I
NZCHAR 537250	*P. colensoi*	Taupo, N.I
NZCHAR 332093	*P. colensoi*	Ohakune, N.I
NZCHAR 329258	*P. colensoi*	Bruce rd, Ohakune, N.I
NZCHAR 326424	*P. colensoi*	Ohakune, N.I
WAIK19706	*P. turneri*	Waitihi saddle scenic Reserve, N.I
WAIK19710	*P. turneri*	Waitihi saddle scenic Reserve, N.I
WAIK12539	*P. turneri*	Whenuakura plains, Pureora, N.I
WAIK21183	*P. turneri*	Ripia Valley, N.I
WAIK14636B	*P. turneri*	Pureora, N.I
WAIK12840	*P. turneri*	Kuratau, N.I
NZFRI18575	*P. turneri*	Ripia valley, N.I
NZFRI17962	*P. turneri*	Whenuakura plains, N.I
NZFRI18884	*P. turneri*	Kuratau river, N.I
NZFRI117961	*P. turneri*	Whenuakura plains, N.I
NZFRI24470	*P. turneri*	Pureora, Waimiha Stream, N.I
NZFRI8249	*P. turneri*	Maraeroa Rd, Pureora, N.I
NZFRI18576	*P. turneri*	Ripia valley, N.I
NZFRI18577	*P. turneri*	?

APPENDIX 8: Morphological differences between *P. colensoi, P. turneri* and *P. divaricatum*. (From Allan (1961) and Cooper (1956).

	P. colensoi	***P. turneri***	***P. divaricatum***	***P. rigidum***
Adult leaf length	40-100 mm (Allan), 39-122 mm (Cooper)	30-50 mm (Allan), 10-40 mm (Cooper)	4-10 mm (Cooper)	8-10 mm (Allan), 5-24 mm (Cooper)
Adult leaf width	20-50 mm (Allan), 11-47 (Cooper)	5-10 mm (Allan), 6-12 mm (Cooper)	2-7 mm (Cooper)	5-8 mm (Allan), 3-13 mm (Cooper)
Adult leaf shape	Elliptic to lanceolate to obovate-oblong us. Acute (Allan), Lanceolate-oblong - obovate-oblong, acute to shorly acuminate (Cooper)	Obovate-narrow obovate cuneately narrowed to slender petiole (Allan), obovate – oblacelolate, obtuse – acute at apex, attenuate at base (Cooper)	Obovate- elliptic (Allan), linear-oblanceolate to linear-oblong or ovate (Cooper)	Elliptic-obovate to broadly elliptic (Allan), elliptic-oblong, occ oblanceolate – obovate, rarely lanceolate , obtuse – subacute at apex, obtuse at base (Cooper)
Adult leaf margin	Flat (Allan), Entire, usually flat, (Cooper)	Entire to irregularly toothed or pinnatifid (Allan), entire or obscurely crenate (Cooper)	Dimorphic (a) entire to subentire (b) shallowly or deeply lobed or toothed (Allan)	Entire to obscurely sinuate-dentate (Allan), Entire, rarely toothed (Cooper)
Juvenile leaf shape	As adult	Obovate to narrow-obovate to linear , entire to irregularly toothed or pinnatifid (Allan), orbicular, obovate, or linear, entire or variously lobed and parted (Cooper)	Narrow-lanceolate – obovate (Allan), Oblong, obovate to lanceolate or almost linear, margins with 1-several lobes or teeth on either side, occasionally crenate (Cooper)	Entire-irregularly lobed or rarely pinnatifid (Allan), obovate, oblanceolate, or elliptic-oblong (Cooper)
Petiole length	3-12mm (Cooper)	1-2 mm (Allan), 0.5-2.5 mm (Cooper)	1 mm (Cooper)	2 mm (Allan), 1-3 mm (Cooper)
Juvenile leaf length	As adult (40-100)	2-15 mm (Cooper), 3-20 mm (Allan)	2 cm (Allan), 6-9 mm (Cooper)	5-13 mm (Cooper)
Juvenile leaf width	As adult (20-50)	2-5 mm (Allan), 1-5 mm (Cooper)	1-4 mm (Cooper)	3-10 mm (Cooper)

Leaf arrangement of adult	Alternate (Allan) (Cooper)	Alternate (Cooper)	Alternate on young branchlets, later restricted to the tips of arrested branchlets (Cooper)	Alternate fascicles on short arrested branchlets (Allan),
Leaf texture of juvenile and adult	Coriacious (Allan)	Coriacious (Allan), tomentose when young, glabrate when older (Cooper)	Submembrenous when juvenile, Coriacious as adult (Cooper)	Coriacious (Allan)
Hairs on leaves	Tomentose when young, soon glabrate (Cooper)	Tomentulose when young, soon glabrate (Cooper)	Glabrous (Cooper)	Glabrate (Cooper)
Leaf colour above	Dark green (Allan)	Brownish green (Cooper)	Green (Cooper)	Dark green (Cooper)
Secondary veins	6-12 per side (Cooper) includes P. ten	10-13 per side (Cooper)	Obscure (Cooper)	Usually obscure (Cooper)
costa	Sunken above, raised beneath (Cooper)	Obscure above, raised beneath (Cooper)	Immersed above, sunken beneath (Cooper)	Raised or immersed above, raised beneath (Cooper)
Pedical length	Sessile or pedicels up to 1 cm (Cooper)	1cm (Allan), 1-3 mm (Cooper)	Minute or sessile flowers (cooper)	0.5-4 mm (Cooper)
Petal colour	Dark-very dark red (Allan) Dark purple, maroon, pink or white (Cooper)	Light red (Allan) Pink or purple (Cooper)	Very dark red (Allan) Purple (Cooper)	Very dark red (Allan), “dingy purple” (Cooper)
Petal length	8-16mm (Cooper)	6-9mm (Cooper)	4-6mm (Cooper)	8-12mm (Cooper)
Position of flowers	Axillary, solitary or occ. in few flowered cymes (Allan) Auxillary 1-3 solitary or fascicled, occasionally terminal (Cooper)	Terminal, 4-10 flowered (Allan), 4-12 fascicled (Cooper)	Terminal, on short arrested branchlets and subtended by fascicles of leaves or strictly terminal (Allan) solitary (Cooper)	Axillary, solitary (Allan), terminal or Axillary, solitary (Cooper)
Sepal length	3-7 mm (Cooper)	3.5-5 mm (Cooper)	2 mm (Allan), 1.5-2.5 mm (Cooper)	3.5-6 mm (Cooper)
Type of hairs on sepals	Pubescent (Allan)	Ciliolate, tomentose (Cooper)	Ciliolate (Allan)	Ciliolate (Allan), Sparsely ciliolate with

				scattered hairs (Cooper)
Sepal shape	Broad-oblong (Allan), slightly imbricate at base, ovate – oblong, subacute – obtuse (Cooper)	Slightly imbricate at base, lanceolate, acute to acuminate (Cooper)	Lanceolate, acute (Cooper)	Ovate, obtuse – acute (Allan), lanceolate-oblong , not imbricate (Cooper)
Style length (female)	1.6-2 mm (Cooper)	2 mm (Cooper)	1-1.5 mm	2.5 mm (Cooper)
Ovaries (female)	4 mm (Cooper)	2.5-3.5 mm (Cooper)	1-2.2 mm (Cooper)	2-2.5 mm (Cooper)
Stigma shape	Capitate or truncate (Cooper)	Weakly capitate–truncate (Cooper)	Capitate-truncate (Cooper)	Capitate-truncate (Cooper)
Stamen length (male)	6-8mm (Cooper)	4-5.5 mm (cooper)	1.5-2.6 mm (Cooper)	4-6.5 mm (Cooper)
Anther length (male)	2-3 mm (Cooper)	1.5-2 mm (Cooper)	0.5-1.4 mm	4.6-1.8 mm (Cooper)
Capsules diameter	12 mm (Allan)	7 mm (Allan), 5-8mm (Cooper)	5-8 mm (Allan), 6mm (Cooper)	8-10 mm (Cooper)
Valve number	3, rarely 2 or 4 (Cooper)	2 (Allan) 2 rarely 3 (Cooper)	2 (Cooper)	2 (Cooper)
Valve thickness/ shape	1-1.5mm thick, coriaceous, convex in transcerse section with a placenta raised and fused at the base, bearing peg-like or flattened funicles from the base to above the middle (Cooper)	Convex or sometimes sulcate in transverse section <1mm thick, coriacious, with a conspicuous placenta bearing several pairs of short stout funicles between the base and the middle (Cooper)	<1mm thick, coriaceous with a slightly thickened placenta bearing 1-2 pairs of peg-like funicles near the middle (Cooper)	Convex in transverse section, <1mm thick, coriacious (Cooper)
Seeds	5-31 black, irregular (Cooper),	3-10 (Cooper)	2-6 (Allan), 1-6 (Cooper)	3-9 (Cooper)
Capsule shape	Globose (Allan)	Subglobose (Allan) Subcompressed (Allan) globose, apiculate (Cooper)	Subglobose (Allan) (Cooper) Subcompressed-ovoid (Allan)	Subglobose, apiculate (Cooper)
Type of hairs on capsules	Tomentose, glabrate	? (Allan) ?	Pilose when young (Allan),	Tomentose, glabrate

	(Cooper)	(Cooper)	glabrous (Cooper)	(Cooper)
Capsule texture	Woody, thick, rugulose valves (Allan), weakly rugose (Cooper)	Woody valves (Allan), slightly rugose (Cooper)	Granulate (Allan), glabrous, weakly rugose (Cooper)	Rugose (Cooper)
Maximum height	10 m (Allan) (Cooper)	9 m (Cooper)	2 m (Allan)	3 m (Allan) (Cooper)
Branchlets	Tomentose when young, glabrate (Cooper)	Divaricating and interlacing branchlets (Allan),	Glabrous (Allan) Branchlets divaricating, opposite or whorled at the nodes, stout often spinose at the tips, tomentulose when young, soon glabrate (Cooper)	Stout, not or hardly divaricate, densely clad when young in ferruginous to pale hairs (Allan)
Habitat	Lowland to montane forest (Allan), higher elevations (Cooper)	Streamsides and forest margins (Allan), forest margins above 1000m sea level (Cooper)	Upper montane forest to forest margins and subalpine scrub (Allan)	Upper montane forest to subalpine scrub (Allan) Along mountain ranges between 650 and 1400m (Cooper)
Distribution	N. S. St. From 38° southwards, occurs west of divide in S. (Allan), From the Waikato and Volcanic Plateau southwards (Cooper)	N. Hauhungaroa range and Waimarino Plateau, 600-1050m (Allan), Central Volcanic Platwau beteen Erua and Waimarino (Cooper)	N. Mountains of volcanic plateau. S. “throughout” (Allan), N. Ruahine Range and Central Volcanic Plateau – Arthur’s Pass (Cooper)	N. along main axis from lat. 38° southwards. S. mountains of N.W. Nelson (Allan) From East Cape to Marlborough (Cooper)
Flowering period	11-12 (Allan)	10-12 (Allan), 11-12 (Cooper)	11 (Allan), 10-11 (Cooper)	10 (Allan), 11-12 (Cooper)
Fruiting period	1-4 (Allan)	1-? (Allan)	1 (Allan)	2 (Allan)