The Development and Genetic Variation of *Sophora prostrata* – A New Zealand Divaricating Shrub

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Sophora prostrata **type specimen illustrated by J. Buchanan**

Abstract

This research contributes to our understanding of the development and genetic variation of *Sophora prostrata* Buchanan (Fabaceae). This has provided a basis for further study into the evolution and development of divaricate branching, and relationships within *Sophora*. A comprehensive review of divaricate literature and assessment of published indices has identified the next steps in understanding and defining divaricates. Assembling and reviewing the available literature on *S. prostrata* has highlighted gaps in our knowledge, as well as issues that need to be addressed.

Sophora prostrata is one of approximately 60 species displaying the divaricate habit in New Zealand. Many passionately debated hypotheses have been put forward to explain the evolution of divaricates, including adaptation to climatic factors, browsing by moa, or light intensities. The theories proposed to explain divaricate evolution are reviewed, and the indices of Atkinson (1992) and Kelly (1994) that are used to quantify divarication are assessed, using divaricate and non-divaricate species pairs. Despite Atkinson's focus on branching parameters, and Kelly's focus on aspects of the leaves, the species distinguished as divaricates were correlated in both indices. However, a more robust definition, and therefore a more comprehensive index, would allow the exact number of divaricates within the various potential divaricate subtypes to be established. Clarifying what exactly defines and unifies divaricate subtypes is essential to be able to properly address the evolution of this habit in New Zealand.

The differences in branching and decapitation response were measured and compared between *S. prostrata* and *S. tetraptera* (using three decapitated individuals and three intact of each species), and patterns in axillary bud development along three branches of *S. prostrata* were investigated. Proportions of established branches were found to be the same in both species, contradicting the idea that divaricates have more branches than non-divaricating species. However, in *S. prostrata* short shoots were found to be a significant contributor to the architecture, which allows *S. prostrata* to be able to exploit space that has already been colonised. *Sophora prostrata* also displayed increased growth and node activation in response to decapitation, which could be due to weak apical control resulting in no new leader shoot gaining absolute dominance over the overall plant. Further investigation into the architecture and development of decapitated and intact *S. prostrata* and *S. tetraptera,* along with other divaricate and non-divaricate species pairs, could help in understanding the complexities of apical control and apical dominance in woody species, and contribute to our knowledge of branching in general.

Inter Simple Sequence Repeat (ISSR) markers were used in effort to further test the previously shown distinctness of *S. prostrata,* using a larger sample size across a wider geographic range than had previously been used, and assess the genetic variation across the geographic range of *S. prostrata* (throughout the Canterbury and Marlborough regions). The nine ISSR primers and 29 samples (18 of *S. prostrata*, 5 of *S. tetraptera,* and 6 of *S. microphylla*) were unable to support the distinctness of *S. prostrata*, or identify distinct relationships between the localities. Further research into the population genetics of *S. prostrata* is necessary to clarify any distinct populations, which could then educate ecosourcing. Variation that is shared across taxa could be a result of hybridisation, which has been widely documented in *New Zealand* Sophora species. The high number of fragments shared among all species (55%) is evidence of the close relationship between New Zealand *Sophora* species, which has been previously demonstrated.

Information relevant to *S. prostrata* from the present thesis as well as other published and unpublished sources is presented as a contribution to the New Zealand Biological Flora series. In some areas such as the lower Waimakariri catchment, *S. prostrata* populations are declining due to browse damage, and there is a lack of seedling recruitment due to grazing and competition from grasses. *Sophora prostrata* populations will continue to decline without artificial replacement and other conservation measures.

Increased interest and conservation effort involving *S. prostrata* and other divaricates can ensure that these valuable and iconic plants remain a unique and significant part of New Zealand's native flora. Divaricates are individually and collectively a valuable resource that should be further utilised to answer many important ecological, evolutionary, and developmental questions.

Key words: *Sophora prostrata*; divaricate; branching; apical control; Inter-simple sequence repeats; ISSRs

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Chapter One: Thesis introduction

Sophora prostrata Buchanan (Fabaceae) is one of about 60 divaricating species found in the New Zealand flora. Divaricates are usually defined as small leaved woody shrubs with densely interlacing branches, however the exact definition varies between authors. Divarication is an extraordinary example of convergent evolution, as it has arisen independently at least 18 times within the New Zealand flora, and potentially occurs nowhere else in the world. Species displaying this growth form make up almost 10% of our native woody flora, and there has been much debate surrounding their evolution. The main hypotheses put forward involve adaptation to climatic factors (McGlone and Webb 1981), browsing by large ratite moa (Greenwood and Atkinson 1977), and light intensities (Day 1998, Christian et al. 2006). Some of the difficulties in addressing divaricate evolution are the inconsistencies between authors in what characteristics define a divaricate, and how divarication should be quantified (Kelly 1994).

The divaricate form is often associated with a lack of apical control (Tomlinson 1978, Christian et al. 2006). Apical control is the suppression of growth in lateral meristems by the apical meristem (Bell and Bryan 2008). The lack of apical control in divaricates is thought to cause more outgrowth of lateral branches, which results in many growing points being scattered throughout the canopy. Herbaceous model organisms have been used to study apical control as they are fast growing and relatively architecturally simple (Dun et al. 2006). Increased understanding in this area means that we can now more easily apply this knowledge to the study of non-model organisms. More complex regulatory systems such as those in woody perennials can allow further investigation, as well as opportunities to answer interesting evolutionary and ecological questions. Carswell and Gould (1998) found that in comparison to arborescent species, the timing and number of growth periods observed within divaricating *Sophora* could be indicative of weak apical control. The range of forms within *Sophora*, as well as previous research in this area, makes *S. prostrata,* and its non-divaricating relatives, a good candidate for further research into the architecture and development of divaricates.

Sophora prostrata is endemic to the eastern side of the South Island of New Zealand, and is found in lowland to montane shrubland, river flats, grassland and rocky places (Wilson and Galloway 1993, Godley 2006). There are approximately 50 species of *Sophora* worldwide, and *Sophora prostrata* is one of eight species of *Sophora* in New Zealand. This includes a heteroblastic species with a divaricating juvenile (*S. microphylla* Aiton.) and several arborescent species (e.g. *S. tetraptera* J.S. Muell.). Molecular evidence suggests that New Zealand *Sophora* species are of recent origin

(Hurr et al. 1999, Mitchell and Heenan 2002), and the close relationship between New Zealand *Sophora* species is supported by their frequent hybridisation (Godley 1975, 2006). The taxonomy of New Zealand *Sophora* has had a problematic history, and relationships between species have been difficult to establish (Heenan et al. 2001). There have been numerous hypotheses put forward to explain the origin and evolution of *Sophora* in New Zealand (Pena et al. 2000, Mitchell and Heenan 2002), and for the relationship of *S. prostrata* to the other species (Cockayne 1912, 1928, Godley 1979, 1985). *Sophora prostrata* has been found to be relatively distinct to the other New Zealand *Sophora* using molecular and morphological data (Pena et al. 2000, Heenan et al. 2001, Song 2005), and understanding more about its relationship to the rest of the New Zealand taxa can shed light on the evolution of *Sophora*, which in turn could influence our understanding of divaricate evolution.

1.1 Research objectives

The aim of this thesis research is to enhance the understanding of the branching regulation, divarication, and genetic variation of *S. prostrata*. This will be addressed by the following four objectives:

1. Review the divaricate literature published to date and assess the suitability of the published indices in quantifying divarication.
2. Assess differences in branching, axillary bud development, and apical control between *S. prostrat*a and the non-divaricate *S. tetraptera*.
3. Support the distinctness of *S. prostrata* in relation to other New Zealand *Sophora*, and assess the genetic variation of *S. prostrata* across its geographic range.
4. Assemble and review available literature on *S. prostrata*.

1.2 Thesis outline

Research that addresses each of the objectives above is presented in chapters 2 - 5, which are written as independent papers intended for publication. As a result of this, some repetition of information occurs between the chapters.

Chapter One: Thesis Introduction

This chapter provides a brief introduction to relevant background information, and an outline of the research objectives and thesis content.

Chapter Two: Evolution of the divaricate habit in New Zealand

This chapter aims to review the theories put forward to explain divaricate evolution, and assess the current definitions and indices used to distinguish divaricates. This review is written with the intent of being submitted to an international journal (e.g. Trends in Ecology and Evolution).

Chapter Three: Branching and development of the divaricating shrub *Sophora prostrata*

This chapter assesses differences in branching and decapitation response between *S. prostrata* in *S. tetraptera* in relation to apical control, and investigates patterns in axillary bud features along branches of *S. prostrata*. This chapter forms the basis of a manuscript for submission to an international botanical journal (e.g. American Journal of Botany).

Chapter Four: Assessing broad scale genetic variation in *Sophora prostrata* (Fabaceae) using ISSR markers

This chapter attempts to validate the distinctness of *S. prostrata* as found in previous studies (using morphological and molecular datasets), using a larger sample size and a wider geographic range, as well as assess the genetic variation *of S. prostrata* between different localities. This is intended for submission to submitted to the New Zealand Journal of Botany.

Chapter Five: Biological flora of New Zealand: *Sophora prostrata*, prostrate kōwhai, South Island kōwhai

This chapter assembles and reviews current literature available on *S. prostrata*, with contributions from the current research. It has been prepared in the format of the New Zealand Biological Flora Series (e.g. Bryan et al. 2011, Clarkson et al. 2012) and will be submitted to the New Zealand Journal of Botany for possible publication.

Chapter Six: Synthesis

This chapter provides a summary of the main findings, discussion of their collective implications, and presentation of recommendations for future research.

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Chapter Two: Evolution of the divaricate habit in New Zealand

2.1 Introduction

The history of New Zealand over the last 80 million years has resulted in a unique evolutionary course for New Zealand's flora and fauna. New Zealand's native flora contains approximately 2300 vascular species of which over 80% are endemic (Breitwieser et al. 2012). Distinguishing features of New Zealand's flora include a high proportion of dioecious species, frequent heteroblasty and the presence of distinctive divaricate forms. Divaricate plants are an assemblage of small-leaved woody shrubs with closely interlaced branches, and are one of the most remarkable examples of convergent evolution in plants. The growth form has arisen independently at least 18 times in New Zealand, and divaricate species make up almost 10% of the native woody flora (Greenwood and Atkinson 1977). This growth form is unique to New Zealand, and many passionately debated hypotheses have been put forward to explain its evolution. The two main hypotheses involve adaptation to climatic factors (McGlone and Webb 1981) and browsing by moa (Greenwood and Atkinson 1977), but alternative hypotheses such as light trapping and structural photoprotection have been proposed (Table 2.1) (Day 1998, Christian et al. 2006).

Divaricating species are found in 18 different plant families, including Rubiaceae, Asteraceae, Pittosporaceae and Violaceae (Table 2.2). There are 22 genera with at least one divaricate species, and in 19 of these genera the ancestral non-divaricating form is also present. The genus with the most divaricate species is *Coprosma* (20 species), with *Olearia* (5 species), *Pittosporum* (5 species) and *Melicytus* (5 species) also containing numerous divaricate species. Many of these divaricating species are closely related to non-divaricating species, and will often hybridise (Dawson 1988). There are approximately 55 divaricating species in the New Zealand flora, and an additional 10 which are weakly or possibly divaricate. It is difficult to establish an exact number of divaricate species due to variation in the definition of a divaricate (Wilson and Galloway 1993) (See Section 2.2).

Table 2.1: Summary of the current main hypotheses for divaricate evolution.

Hypotheses	Outline of adaptive benefit	References
Climatic factors	The interlacing branches of the divaricate form creates a microclimate within the plant which shelters internal leaves from wind and frost damage. Heteroblastic change occurs past the range of ground frosts.	Diels 1896; Cockayne 1912; Wardle 1963; Kelly and Ogle 1990; McGlone and Clarkson 1993
Moa browsing	Divaricates resist moa browsing with small, widely spaced leaves concentrated in their centre to reducing area lost by plucking. The strong wide angled branches are difficult to break off and swallow. The transition of heteroblastic species occurs at the height of the tallest moa species.	Carlquist 1974; Greenwood and Atkinson 1977; Atkinson and Greenwood 1989; Bond et al. 2004; Bond and Silander 2007
Light trapping	The small leaves of a divaricate are scattered in three dimensional space by the thin interlacing branches. This means light can be captured more efficiently by distributing light throughout the canopy. Heteroblastic change occurs due to change in light environments from beneath to above the canopy.	Horn 1971; Kelly 1994; Clearwater and Gould 1995;, Day 1998
Structural photoprotection	Self-shading by divaricates dense interlacing branches reduces light intensities enough to avoid photodamage, which inhibits photosynthesis. Photoinhibition is worsened by environmental stresses. Heteroblastic transition occurs above ground frosts which exacerbate photodamage.	Howell et al. 2002; Christian et al. 2006

Divaricates occur in a wide range of environments and latitudes. They are commonly found in open sites, but are also found in forest understory, forest margins and fertile alluvial flats (McGlone and Webb 1981, Wilson and Galloway 1993). They are also particularly frequent on lowland river terraces (Greenwood and Atkinson 1977). The abundance of divaricates increases with increasing latitudes, and reaches greatest density on the eastern side of the South Island (Wilson and Galloway 1993). Divaricates occur significantly less frequently on offshore and outlying islands, and are generally not found on cliffs or as epiphytes (Greenwood and Atkinson 1977). Plants with similar divaricate-like characteristics occur elsewhere in the world, such as in Madagascar, Argentina, and the South West United States (Kelly and Ogle 1990). However these usually have a notably different suite of traits, including the presence of spines. It is interesting to note similarities and differences in climates and histories of herbivores in these areas, as this could help us to tease out the possible selection pressures in New Zealand that have resulted in the unique suite of divaricate traits.

Table 2.2: New Zealand divaricates. Those considered weakly or possibly divaricate are indicated by (?). Based on Wilson and Galloway (1993), with changes from Howell (1999).

Family and Genus	Species
Podocarpaceae	
Prumnopittys	*P. taxifolia* juvenile
Primulaceae	
Myrsine	*M. divaricata*
Araliaceae	
Raukaua	*R. anomalus*
Rutaceae	
Melicope	*M. simplex*
Rhamnaceae	
Discaria	*D. toumatou*
Pennantiaceae	
Pennantia	*P. corymbosa* juvenile
Moraceae	
Streblus	*S. heterophyllus* juvenile
Fabaceae	
Sophora	*S. prostrata*
	S. microphylla juvenile
Argophyllaceae	
Corokia	*C. cotoneaster*
Carpodetus	*C. serratus* juvenile
Malvaceae	
Plagianthus	*P. divaricatus*
	P. regius juvenile
Hoheria	*H. angustifolia* juvenile
	H. populineae var *lanceolata* juvenile
Elaocarpaceae	
Elaeocarpus	*E. hookerianus* juvenile
Aristotelia	*A. fruticosa*
Pittosporaceae	
Pittosporum	*P. anomalum*
	P. divaricatum
	P. obcordatum
	P. ridgidium
	P. turneri juvenile
	(?) *P. virgatum* juvenile
Polygonaceae	
Meuhlenbeckia	*M. astonii*
Myrtaceae	
Lophomyrtus	(?) *L. obcordata*
Neomyrtus	*N. pedunculata*
Labiatae	
Teucridium	*T. parvifolium*

Violaceae	
Melicytus	*M. alpinus*
	M. crassifolius
	M. micranthus
	M. drucei
	M. flexuosus
Asteraceae	
Olearia	*O. bullata*
	O. capillaris
	O. odorata
	O. polita
	O. virgata
	(?) *O. solandri*
	(?) *O. fragrantissima*
	(?) *O. lineata*
Rubiaceae	
Coprosma	*C. acerosa*
	C. areolata
	C. cheesemanii
	C. crassifolia
	C. cuneata
	C. elatirioides
	C. intertexta
	C. obconica
	C. parviflora
	C. pedicellata
	C. propinqua
	C. rhamnoides
	C. rigida
	C. rotundifolia
	C. rubra
	C. rugosa
	C. tenuicaulis
	C. virescens
	C. wallii
	C. decurva
	(?) *C. pseudocuneata*
	(?) *C. foweraker*i
	(?) *C. aff parviflora sp. (t)*
	(?) *C. microcarpa*
	(?) *C. Ciliata*

Some hypotheses in the divaricate debate also claim to account for the high proportions of heteroblastic species in New Zealand (Table 2.1). Heteroblasty is defined by Zotz et al. (2011) pg. 113, as a “rather sudden and substantial change in form of individual metamers or plant habit during ontogeny”. New Zealand has at least 40 heteroblastic species in 17 families (Gamage and Jesson 2007). The only other locations in the world with comparable rates of heteroblasty are New Caledonia and the Mascarene Islands (Bond et al. 2004). The functional significance of heteroblasty is still largely unknown despite considerable research in this area (Zotz et al. 2011). Of the heteroblastic species in New Zealand, 10 species have a juvenile stage with a divaricating habit, which belong to eight different genera (Table 2.2). This high frequency of heteroblasty is another striking example of convergent evolution, which contributes to the debate on what selection pressures may be unique to New Zealand.

Figure 2.1: Map showing the largely submerged continent of Zelandia (shaded). Also showing New Zealand and nearby islands. Taken from Wallis and Trewick (2009).

New Zealand is located in the southern Pacific Ocean on the mostly submerged continent of Zelandia, which separated from Gondwana 80 million years ago (Figure 2.1) (Wallis and Trewick 2009). The climate has changed significantly, with New Zealand being subject to subtropical and tropical climates 60 million years ago, and relatively stable warm to cool temperate climate throughput most of the Oligocene, Miocene and Pliocene (Lee et al. 2001). New Zealand is currently situated on the boundary between the Indo-Australian and Pacific plates, and as a result has been subject to continuous tectonic activity such as earthquakes, mountain formation and volcanism. Tectonic activity has also caused the Earth's crust to stretch and thin, resulting in the gradual submergence of New Zealand (Wallis and Trewick 2009). There has been ongoing debate as to whether New Zealand was completely or partially submerged when it was at its lowest above sea land area 23 million years ago (Pole 1994, Cooper and Cooper 1995, McGlone 2005, Trewick et al. 2007, Wallis and Trewick 2009, Allwood et al. 2010). New Zealand's flora and fauna have been regarded as having evolved in geographical isolation since Zelandia split from Gondwana, however if complete submergence occurred it would mean that the New Zealand flora has arrived primarily via long distance dispersal in the last 22 million years (Landis et al. 2008). The processes of plate collision and crust thickening have then caused land re-emergence to the present level. Two million years ago an Ice Age began, which consisted of fluctuating cool glacial cycles and warmer interglacial periods. The last glacial cycle reached its maximum 22,000 years ago, which resulted in land bridges connecting what are now the three main islands – the North Island (Te Ika-a-Māui), South Island (Te Waipounamu) and Stewart Island (Rakiura) (Wallis and Trewick 2009). New Zealand now consists of these three islands, and numerous outlying islands which span subtropical to subantarctic latitudes. The landscapes of these islands vary from mountainous peaks to gullies and plains. These factors, combined with variation in soil types and rainfall results in a diverse array of available habitats (Breitwieser et al. 2012). Events such as partial submergences, volcanic activity and glaciations have all shaped the face of New Zealand, and contributed to the unique evolutionary course of New Zealand's flora and fauna.

2.2 Definition of a divaricate

Whether New Zealand divaricates are distinct from divaricate-like species elsewhere depends on the various definitions of the term divaricate. The characters that define a divaricate form vary between authors (Table 2.3), the most common of which are interlacing and the presence of small leaves. Cockayne (1912) described divaricates as much branched, often at right angles, with stiff wiry stems that are pressed closely together or interlaced. Greenwood and Atkinson (1977) focused on two criteria for defining a divaricate - firstly, at least 10% of the branches must diverge at least 90 degrees, and secondly, the interlacing of the branches must be three dimensional. Tomlinson (1978) distinguished divaricates by having small leaves, short shoots and interlacing branches, and later Atkinson (1992) suggested a focus on branching angles and the number of branches. Kelly (1994) proposed a formal definition of divarication focusing on interlaced, wide angled branches, small widely spaced leaves, and larger leaves towards the interior of the plant.

Table 2.3: Features included in divaricate definitions from the literature. Y = yes; S = Sometimes, often or not exclusively. The features are shown in order of frequency.

Feature	Cockayne (1912)	Bulmer (1958)	Went (1971)	Greenwood & Atkinson (1977)	Tomlinson (1978)	McGlone & Webb (1981)	Wardle & McGlone (1988)	Dawson (1988)	Atkinson & Greenwood (1989)	Kelly & Ogle (1990)	Wardle (1991)	Wilson (1991)	Atkinson (1992)	McGlone & Clarkson (1993)	Cooper et al (1993)	Wilson & Galloway (1993)	Kelly (1994)	Day (1998)	Carswell & Gould (1998)	McQueen (2000)	Howell et al. (2002)	Bond et al. (2004)	Christian et al. (2006)	Bond & Silander (2007)	Bell (2008)	Wilson & Lee (2012)
Interlaced	Y	Y	Y	Y	Y	Y		Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y		Y	Y
Small leaves		Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y		Y	Y	Y	Y	Y	Y	Y
Wide branch angles	Y			Y	S	Y		Y		Y			Y	Y		S	Y	Y	Y	S	S	Y		Y	S	Y
Long internodes			Y	S			Y		Y		Y		S	Y	Y		Y	Y		S	Y	Y	Y			Y
Much branched	Y	S	Y	S				Y		Y		Y	Y			Y	Y	Y		S	Y					
Fewer leaves at tip				Y		Y			Y	Y			S	Y	Y						S		Y	S		
Smaller leaves at tip				Y					Y				S	Y	Y		Y		Y				S			Y
Reduced apical dominance			Y	Y				Y					Y			Y			S				Y		S	Y
Tough stems		Y		Y					Y				S	Y	Y							S			Y	
Slender stems				S			Y	Y			Y							Y				S	Y	Y		
Branches some >90		Y	Y	Y				Y					Y							S					S	
Stiff wiry stems	S	S		S	S			Y		Y	S														Y	Y
High order branch growth								Y					Y			Y				S				Y		
Many short shoots					Y		S	S		Y	Y					S									S	
Spineless		S		Y	S			S		S	S					Y								S	S	
Lateral flowering					Y					Y															S	
Small buds							Y				Y															
Crooked branchlets												Y				Y										
Recurved branches				S	Y						S															
Zig-zag branching				S	S			S			S									S				S	S	
Fastigate branching					S											S				S					S	
Sympodial branching				S	S																				S	
Entire leaves			S		S					S																
Accessory buds								S																	S	

The divaricate form is often associated with a lack of apical control (Tomlinson 1978, Christian et al. 2006). Apical control results in apical dominance - which is the suppression of growth in lateral meristems by the apical meristem of each shoot (Bell and Bryan 2008). This control is thought to be mediated by hormones such as auxins, cytokinins, and strigolacetones (Hayward et al. 2009). The lack of apical control in divaricates causes outgrowth of lateral branches, which results in many growing points being scattered throughout the canopy. Short-shoot development is also common among many divaricates. This occurs when a branch remains very short, and bears a cluster of leaves (Tomlinson 1978). Wardle and McGlone (1988) suggested that divarication results from an underlying syndrome, where the apical buds are reduced to rudimentary dimensions and consequently exert weak apical dominance. This kind of underlying syndrome could provide a link between the varieties of ways divaricate architecture is achieved.

A. Wide angled branching. E.g. *Coprosma propinqua*

B. Fastigated branching . E.g. *Melicytus alpinus*

C. Sympodial zig-zag branching. E.g. *Elaeocarpus hookerianus*

D. Monopodial zig-zag branching. E.g. *Sophora prostrata*

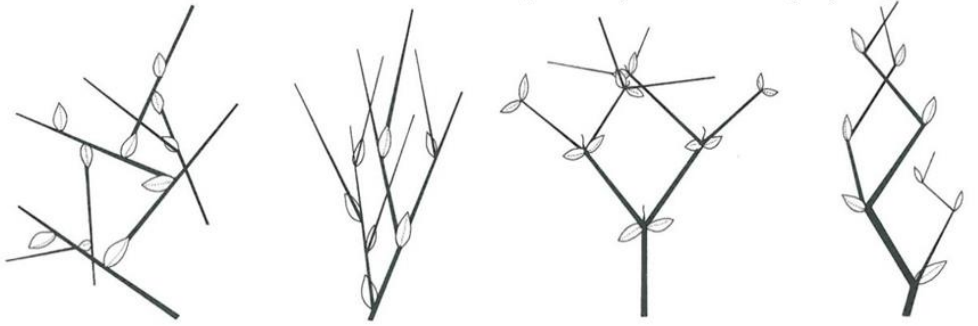

Figure 2.2: Different types of divarication with examples. (Adapted from Bell and Bryan (2008) pg 100, plant photos courtesy of (L-R) Jeremy Rolfe, Steve Atwood, P. B. Pelser and Tony Foster).

As this growth form has arisen independently multiple times within New Zealand, it is achieved in many structurally different ways (Figure 2.2). Divarication can be achieved via wide angled branching (e.g. *Coprosma propinqua.* A. Cunn.), but also branching at a very narrow angle (e.g. *Melicytus alpinus* (Kirk.) Garn.-Jones). Sympodial growth can result in a zig-zag axis (e.g. *Elaeocarpus hookerianus* Raoul), as can monopodial growth where the shoot bends at each node (e.g. *Sophora prostrata* Buchan.)(Bell and Bryan 2008). Tomlinson (1978) also discussed the possible architectural models of Hallé et al. (1978) that divaricates could belong to, and he established that divaricates cannot be categorised by any one model. Halloy (1990) also classified divaricates into five different groups (divaricates *s.s*, antler, lianescent, leaking hose and spout) based on the branching pattern of their stem. None of the attempts to unequivocally define the divaricate form have been widely accepted due to difficulties associated with quantifying this collection of convergent forms (Howell 1999). An exact definition of the divaricate habit is important for establishing whether divaricate plants are unique to New Zealand, as well as exactly which species within New Zealand are truly divaricating. The debate surrounding how the divaricating habit has evolved requires a precise definition, as definitive conclusions cannot be made about the selective advantages of the habit if the features considered are not consistent.

Two mathematical indices to quantify divarication have been proposed by Atkinson (1992) and Kelly (1994). Atkinson's index focuses solely on branching parameters, measuring branching angles and number of branches. Kelly's index measures various aspects of shoot architecture, such as leaf width and internode size as well as branching angle. Kelly (1994) also noted that while the two indices focused on different characteristics, they correlated well and came to the same conclusions about most species that were included in both studies. The equations for calculating the indices are as follows:

Atkinson (1992):

$$Index = Number\ of\ peripheral\ wide\ angled\ branches + \frac{Mean\ branch\ angle}{10} + Number\ of\ branching\ orders + \frac{Branching\ density}{10}$$

Kelly (1994):

$$Index = FILIW + \frac{10}{LWT} + \frac{ANGL}{5}$$

Where *FILIW* is the internode to leaf width ratio($\frac{100/NLT}{LWT}$), *LWT* is the mean leaf width, *NLT* is the number of leaves on the main branch of the sample, and *ANGL* is the mean branch angle.

I applied the indices to four pairs of closely related species comprising one divaricating and one non-divaricating species. The divaricating and a non-divaricating species chosen were respectively *Melicope simplex* A. Cunn. and *M. ternata* J.R.Forst. & G.Forst. (Rutaceae)*; Melicytus micranthus* (Hook.f.) Hook.f. and *M.*

ramiflorus J.R.Forst. & G.Forst. (Violaceae)*; Pittosporum anomalum* Laing & Gourlay and *P. tennuifolium* Sol. ex Gaertn. (Pittosporaceae), and, *Sophora prostrata* and *S. tetraptera* J. F. Mill. (Fabaceae). These species were chosen as they encompass a variety of families and a variety of divaricate forms. Measurements were made on ten juveniles of each species that were of a similar age and size, using methods outlined in Atkinson (1992) and Kelly (1994).

Three of the four species usually considered to be divaricates were distinguished as divaricating according to the criteria of each of the indices – *M. micranthus*, *P. anomalum* and *S. prostrata* (Table 2.4, Figure 2.3 and Figure 2.4)*. Melicope simplex* did not exceed the divaricate threshold (14 for Atkinsons index and 19.2 for Kellys index) in either of the indices, and none of the non-divaricating species had average values above the divaricate threshold in either index. The values obtained were lower than expected based on the values found in Atkinson (1992) and Kelly (1994), which could have been due to the maturity of the plants used. Wilson and Galloway (1993) describe *M. simplex* as a shrub growing up to 5 m tall with slender branchlets which are more or less interlacing, with trifoliate leaves in seedlings and juveniles, and simple leaves on the adults. A possible explanation for the low values obtained from this species is that it could have different divaricate characteristics throughout its development. To assess this, the indices were applied to 10 *Melicope simplex* plants that were approximately 1 – 2m in height at Pukemokemoke Bush Reserve (data not shown). The average value for Atkinson's index was 9.7, and the average value for Kelly's index was 15.4. These values were slightly higher than the juveniles measured, but still not over the thresholds for each index.

Table 2.4: The average, minimum and maximum index values for 10 plants of each species are shown. Divaricates are indicated with a *.The divaricate threshold for Kelly's index is 19.2, and the divaricate threshold for Atkinson's index is 14. Values above the divaricate thresholds are shown in bold.

	Index					
	Kelly			Atkinson		
Species	Average	Min	Max	Average	Min	Max
*Melicope simplex**	13.2	9.5	16.8	8.9	6.1	13.1
Melicope ternata	9.0	7.9	10.7	5.9	4.5	7.4
*Melicytus micranthus**	**23.0**	15.9	**31.9**	**17.6**	10.5	**27.1**
Melicytus ramiflorus	9.1	7.5	12.3	5.5	4.5	6.8
*Pittosporum anomalum**	**25.9**	**21.8**	**30.2**	**19.3**	**15.0**	**28.8**
Pittosporum tennuifolium	13.8	10.9	15.4	10.0	7.3	13.5
*Sophora prostrata**	**24.2**	**20.5**	**31.2**	**18.6**	**15.9**	**26.1**
Sophora tetraptera	14.5	11.6	18.2	9.9	6.5	**14.4**

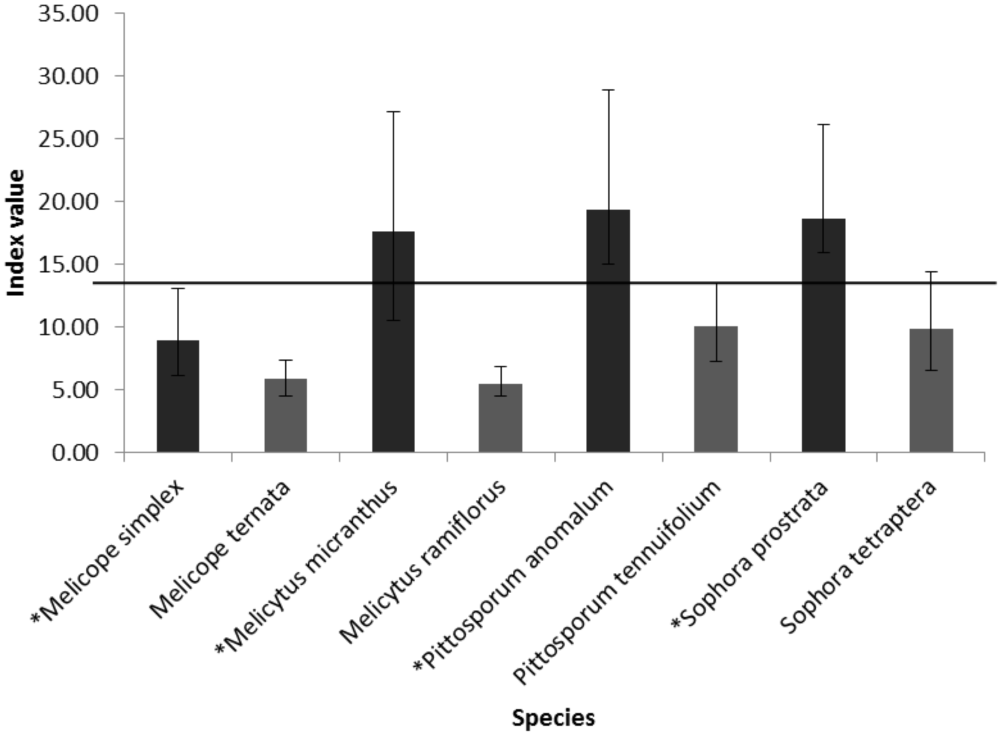

Figure 2.3: Average Atkinson's index values for 10 individuals of each species. The threshold for divarication (14) is shown by the black line, and the range of values is shown by the error bars. Divaricates are indicated with a *, and darker bars.

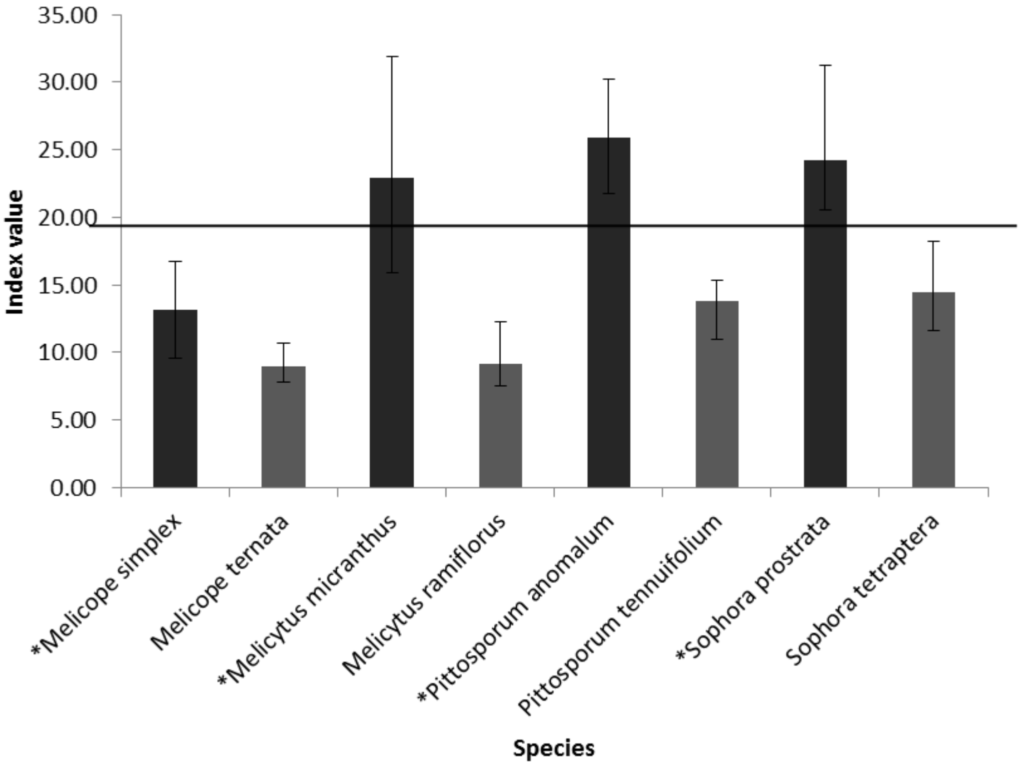

Figure 2.4: Average Kelly's index values for 10 individuals of each species. The threshold for divarication (19.2) is shown by the black line, and the range of values is shown by the error bars. Divaricates are indicated with a *, and darker bars.

Kelly (1994) mentions that his use of mean branching angle may not be the best way to measure interlacing, and that one of the parameters used in Atkinson's index might be more effective. Kelly's index also lacks a measure of branching frequency, which is also a commonly discussed divaricate trait. Kelly also comments that as leaf size and spacing are so frequently associated with the divaricate habit, they cannot be excluded from the calculation of divarication as they are in Atkinson's index. The species that were assessed in both of the initial publications of the indices correlate well, despite the different emphasis of each index. McQueen (2000) used the two indices to compare Patagonian divaricate-like species with New Zealand divaricates. He found no correlation between the indices in Patagonian divaricate-like species, and noted there was correlation between the indices in New Zealand divaricates. This was attributed to the fact that in New Zealand the different aspects the indices focus on are coupled, but in Patagonia they are not. As these two indices show similar trends toward the indices correlating for the species used here, this supports a coupling of characteristics. The fact that both of these indices use different parameters and result in similar conclusions highlights the possibility of combining aspects of these indices into a more complete one. Furthermore, neither of these indices takes into account the variety of other ways that some divaricate habits can be achieved, such as fastigate or recurved branching. Including these subtypes in a divaricate index could help in encompassing the range of divaricate architectures.

The architecture of a plant is derived from the developmental sequences of its shoots. Lateral meristems remain dormant or become shoots or inflorescences. The timing and duration of shoot growth has huge influence on the overall form (Bell 2008). The importance of developmental processes in the overall architecture calls into question the use of only mature dimensions at one point in time to define a growth form. Day and Gould (1997) investigated the development of *Elaeocarpus hookerianus*, which is a heteroblastic species with a divaricating juvenile. They noted that the topology of the form changes over the course of the growing season, and is not stationary. Carswell and Gould (1998) found that morphological parameters did not distinguish divaricate *Sophora* species adequately, and concluded that developmental processes could be better descriptors of divarication than shoot dimensions. The results of these studies highlight the importance of also considering variation over time due to developmental processes when quantifying divarication.

These indices have made a valuable contribution to further define and understand divarication, but there is more work to be done in formulating a more complete definition and therefore a more thorough index. This will allow us to establish the exact number of divaricates within New Zealand, and could clarify whether true divaricates occur elsewhere in the world. Precise agreement as to what defines a divaricate would also alleviate the risk of different conclusions being made regarding the origin of the divaricate form through the use of different species. The architecture of some divaricate species changes over time, and static morphological parameters are not always the best descriptors of divarication. The possibility of an underlying developmental syndrome resulting in the structurally different divaricate forms emphasises the need for more in depth developmental and genetic investigations. It is crucial to understand what characteristics unify divaricate architecture in order to be able to properly address its evolution, and understand divaricate development.

2.3 Climatic hypotheses

Climatic factors were first considered as an explanation for divarication in New Zealand by Diels (1896) and the hypothesis has since been elaborated on by numerous authors (Cockayne 1912, McGlone and Webb 1981, Kelly and Ogle 1990). Diels (1896) suggested that divarication reduced transpiration, and was an adaptation to harsh dry climates. Cockayne (1912) hypothesised that divaricate growth forms were a xerophytic adaptation in response to windy and dry conditions, potentially in past steppe climates of the South Island. Wardle (1963) agreed that Pleistocene climates would have contributed to divaricate evolution, but suggests that divaricates are a response to still existing climates. McGlone and Webb (1981) fully developed the climatic explanation, and they suggested that divaricating plants and heteroblastic juveniles have evolved in response to lowland and montane environments where normal tree growth can be unsuitable due to frost, wind and drought.

The climate hypothesis suggests the divaricating form was a response of New Zealand’s subtropical flora to glacial climates, the lack of refugia during interglacial periods and the variability of New Zealand’s climate (McGlone and Webb 1981). New Zealand was subject to numerous glaciations during the Pleistocene, and as most of New Zealand’s flora originated from sub-tropical and temperate regions, there were very few species that could have occupied the habitats opened up by the changing climate. This resulted in the subtropical flora being forced to adapt to the cooler, drier, more variable climate. During New Zealand’s glaciations, most of the landscape was covered with scrub or grassland, with forested areas being very rare. This would have resulted in large areas in which divaricates could have existed. During the interglacial periods, species adapted to the glacial climates had very few habitats in which they could survive, and would have to be flexible enough to exploit a wide range of habitats. McGlone and Webb (1981) suggest that this lack of refugia has contributed to the evolution of the divaricate habit. Finally, as New Zealand has an aseasonal climate, damaging frosts and high irradiances can occur at any time of the year. Adaptation to this extremely variable and unpredictable climate means that divaricates can function well in any season. This combination of factors is thought to be peculiar to New Zealand, and therefore a possible driver of divaricate evolution.

McGlone and Webb (1981) argue that the densely branched structure shelters internal leaves and creates a microclimate within the plant. Shelter from the wind allows less transpiration, and prevents damage to the inner leaves caused by collisions from wind borne particles, other leaves, or twigs. The small widely spaced nature of divaricate leaves also helps prevent leaf collisions, which can lead to tearing of leaves or abrasion. This type of damage can destroy photosynthetic tissue, and increase transpiration losses. They also suggest that the protective network of branches may act as a frost screen, raising the temperature inside the shrub, preventing frost damage and potential decreases in photosynthesis. The microclimate inside the shrub would allow higher rates of photosynthesis, and the increased humidity reduces water loss via transpiration. The climate hypothesis could also explain heteroblasty, as the transition occurs out of the range of damaging ground frosts. As divaricates reach their highest frequency in the South Island, McGlone and Webb (1981) claim that this distribution correlates with the harshness of the climate. Divaricates are often found in lowland river terraces, which could be explained by the high incidence of frost on such terraces. The lack of divaricates on offshore and outlying island could also be explained by their oceanic climates.

While past climatic conditions cannot be replicated, the effect of current climates on divaricates can be investigated by measuring and comparing physical variables from the inside and the outside of the plant. Kelly and Ogle (1990) measured variables such as leaf temperature, relative humidity and wind speed in *Coprosma propinqua* and *Hoheria angustifolia* Raoul., and found that there was no significant difference in temperature or humidity, but a possible decrease in frost damage inside the plant. They conclude that the physical measurements show too little difference for the divaricate habit to afford much protection against frost damage and drying winds, but they suggest that it is not possible to completely support or refute the climate hypothesis from their data. Kelly and Ogle (1990) suggest that there could be a variable other than temperature or humidity as the key climatic factor. Keey and Lind (1997) investigated airflow patterns around four divaricate shrubs (*Coprosma cheesemanii* W.R.B.Oliv., *Pittosporum obcordatum* Raoul., *Myrsine divaricata* A. Cunn., and *Corokia cotoneaster* Raoul.), and found that densely branched divaricate shrubs produced significantly more calmer zones, which could create a more favourable environment. Darrow et al. (2001) investigated frost resistance in heteroblastic species, most of which had divaricating juveniles. He found that frost resistance in the juvenile forms was not supported by the data, and concluded that the divaricating habit must have evolved from something other than frost avoidance. Darrow et al. (2002) also found that heteroblastic juveniles may even show greater water loss than the adults, which does not support the climatic hypothesis as it predicts less water loss.

2.4 Moa browsing hypotheses

Moa (order Dinornithiformes) were large flightless ratite birds endemic to New Zealand, and browsing by moa has been suggested by numerous authors as having a role in divaricate evolution (Denny 1964, Carlquist 1974, Taylor 1975, Greenwood and Atkinson 1977). Denny (1964) suggested there was a remote possibility that constant grazing by moa could have had an effect on the shape of divaricate shrubs. Carlquist (1974) thought that the tough, tangled branches of divaricates cold act as protective armour, much like thorns in other species. The moa browsing hypothesis was fully explored by Greenwood and Atkinson (1977), who suggested that the divergent and interlacing tough woody stems of divaricates were adaptations in response to browsing by moa, the only large browsing vertebrates in New Zealand until 500 years ago. Since then, the moa browsing hypothesis has been contributed to by several authors (Atkinson and Greenwood 1980, Burrows 1980, Lowry 1980, Bond et al. 2004, Bond and Silander 2007, Wood et al. 2008).

Moa have recently become extinct (500 – 600 years ago), probably due to hunting pressure by Māori. There are currently nine accepted species of moa, from six genera and three families (Worthy and Scofield 2012). Most species were restricted to the south island of New Zealand, though some were found throughout the country (Lee and Gould 2009). They fed on trees and shrubs and were capable of ingesting stems up to 6mm in diameter. They were able to grind up twigs in their large gizzards, and some species were able to shear off twigs (Burrows 1980). Ratite species exist elsewhere, such as emu in Australia, rhea in South America, and ostrich in Africa. In this respect ratite browsers alone cannot considered distinctive to New Zealand, but their presence in the absence of any browsing mammals is unique. It has been hypothesised that moa herbivory is responsible for at least 11 kinds of growth characteristics seen in native New Zealand plants, such as mimicry, spiny tussocks reduced visual apparency (Atkinson and Greenwood 1989). Co-evolution between plant and moa is not unexpected considering their long coexistence (Atkinson and Greenwood 1989).

Greenwood and Atkinson (1977) base their argument on the probable feeding methods of moa, and the consequences of this in the absence of browsing mammals. The features of the divaricating habit that led them to this hypothesis are the small leaves to reduce area lost by plucking, as well as increased leaf biomass and growing points towards the interior of the plant, where they will be protected from browsing. The branches have a high tensile strength, and are thin and difficult to clamp. Divaricating 'zig-zag' branches extend before breaking when pulled, and the wide angled branches are difficult to swallow (Greenwood and Atkinson 1977, Bond et al. 2004). Greenwood and Atkinson (1977) emphasise the probable importance of clamping, pulling and breaking to consume twigs, but mention the possibility of a cutting action. Heteroblastic species with divaricating juveniles usually undergo the transition to adult growth at about 3m - the approximate height of the tallest moa.

Greenwood and Atkinson (1977) also note that in some divaricate species new growth can appear dead, which could deter browsing at vulnerable growth periods. Research into the evolutionary effects of moa browsing has looked at crypsis in heteroblastic species. Fadzly et al. (2009) found a series of ontogenetic colour changes in *Pseudopanax crassifolius* (Sol. ex A.Cunn.) K.Koch*,* a non-divaricating heteroblastic species. They concluded that this could have been defensive strategy against browsing moa. A similar experiment on *Elaeocarpus hookerianus* (a divaricating heteroblastic species), also found that crypsis occurs in juveniles while within reach of browsing (Fadzly and Burns 2010). They found that the variable coloration and morphology of juvenile leaves would make them difficult to locate against leaf litter background. Many other heteroblastic species change colour during ontogeny, which could provide support for moa browsing influencing heteroblastic and divaricate evolution (Fadzly and Burns 2010).

Despite moa being extinct, some understanding can be indirectly gained from extant ratites – such as emu, ostrich and rhea. They generally eat grass, seeds, buds, leaves and occasionally twigs of trees and shrubs. Moa appear to have fed on similar vegetation, specializing on twigs (Burrows 1980). These relatives of moa browse by clamping their beak over a branch or leaf, and pulling their heads back to tear off food (Bond et al. 2004). If moa browsed in the same way, the resistance of divaricates to ratite browsing can be investigated using extant relatives. Bond et al. (2004) used emu and ostrich to observe feeding behaviour and rates on two heteroblastic species with divaricating juveniles (*Penantia corymbosa* J.R.Forst. & G.Forst. and *Plagianthus regis* (Poit.) Hochr.). They compared leaf and branch loss between branch types, and analysed the plant traits most effective in reducing loss to browsing. They conclude that the high tensile strength of the narrow branches reduces breakage, and the widely spaced, small leaves increase the energy and time required to acquire leaf biomass. Their results supported the hypothesis that the divaricate form helps to defend against browsing moa. However, the suitability of extant relatives as an analogue is criticised by some, especially as moa evolved in absence of browsing mammals, which could limit other ratite use as an ecological equivalent.

The population density of moa in the South Island was approximately twice that of the North Island, and the greatest concentration of moa occurred in the eastern South Island (Anderson 2003). The eastern South Island is also where the greatest abundance of divaricates occurs (Wilson and Galloway 1993). Divaricates are often found in lowland river terraces, which Greenwood and Atkinson (1977) suggest is due to the high productivity of such sites supporting many moa. This would mean the plants growing in those areas would be subject to frequent browsing. Divaricates occur significantly less frequently on offshore and outlying islands, on cliffs, and as epiphytes. This could be due to the lack of moa in these areas (Greenwood and Atkinson 1977).

Other parts of the world have divaricate-like species, and comparing these areas with New Zealand can help to understand possible selection pressures on divaricate evolution. Bond and Silander (2007) compared divaricate traits such as the angle of branching, thinness of twigs, and lack of spines in wire plants from Madagascar, which has had a very different climate and phylogenetic history. One of the few features that Madagascar and New Zealand have had in common is the presence of large ratite browsers. Many of the divaricate-like features of the plants are similar, except the wire plants of Madagascar did not have a concentration of leaves in the centre of the shrub, and had a far laxer less cage like form. They suggest that the combination of ratite browsers and New Zealand climate has shaped the distinct form of New Zealand divaricates (Bond and Silander 2007). McQueen (2000) compared New Zealand divaricates with divaricate-like species in Patagonia (e.g. *Escallonia virgata* (Ruiz & Pav.) Pers. (Escalloniaceae), *Berberis microphylla* G. Forst. (Berberidaceae), *Condalia microphylla* Cav. (Rhamnaceae)), which has had similar climatic gradients and latitudes but a different history of herbivores. Patagonia has had and still has browsing mammals, as well as a browsing ratite bird - Darwin’s rhea. He found that the majority of Patagonian species had spines, which may be associated with their evolution in the presence of mammals. McQueen (2000) concluded that divaricates in New Zealand may have evolved due to moa browsing, and are therefore able to grow in harsh conditions.

The discovery of subfossil moa gizzards containing twigs from various divaricating plants has been viewed as evidence for and against the moa browsing hypothesis. Burrows (1980) found that many of the twigs found in the gizzards appeared to have been sheared off, rather than broken as initially suggested by Greenwood and Atkinson (1977). However Burrows (1980) concludes that the moa gizzard contents support the hypothesis that moa have influenced divaricate evolution, but that some details such as method of browsing are not supported. Lowry (1980) suggested that rather than a defence against browsing, the divaricate form enhanced survival in the face of browsing by having many well-spaced growing points. Wood et al. (2008) also confirmed that divaricates were part of the moa diet, using coprolites. This supports the idea of a co-evolution between moa and plants. The discovery of a possible divaricate fossil from the early Miocene (Campbell et al. 2000) could also support the moa browsing hypothesis, as the climatic conditions that are thought to have driven divaricate evolution occurred in the late Pliocene.

2.5 Other theories

In addition to climatic and browsing related theories, various alternative theories have been put forward to explain the divaricate form. Went (1971) suggested that chromosome segments containing divarication inducing factors could pass between species, resulting in the presence of divaricates within many different families. Kelly (1994) proposed the light trapping hypothesis, which suggests the small leaves can capture optimal light by being scattered in three-dimensional space, as it allows the total canopy to use more of the incident radiation. It has been hypothesised that heteroblasty arose due to the change in light intensity from below to above the canopy, meaning the juveniles could more efficiently capture the low intensity light (Clearwater and Gould 1995, Day 1998). Day (1998) further suggested that the divaricate form is a result of heteroblastic species moving to more open conditions, losing their adult state and remaining as divaricate juveniles. Howell et al. (2002) suggested that the divaricate habit has evolved so that self-shading by outer branches can reduce irradiance enough to prevent photodamage. The main alternative hypotheses are discussed below.

Horn (1971) initially discussed the light capture efficiency of monolayer and multilayer leaf distribution, which has led to the light trapping hypothesis (Kelly 1994). This involves the small leaves scattered in a three-dimensional pattern of branches caused by long internodes and wide branch angles. This pattern allows the internal leaves to not be in total shade, and instead be in partial shade. This is thought to be a more efficient use of light, as it is distributed throughout the canopy rather than being absorbed by a single layer, with only approximately 40% being utilised (Kelly 1994). Divarication could be a result of more efficient light trapping in the New Zealand environment, perhaps also providing structural photoprotection.

Clearwater and Gould (1995) initially suggested that the evolution of heteroblasty was in response to differences in light environments. Day (1998) then outlined a series of events leading from the development of heteroblasty to evolution of a permanently divaricate form. First, a juvenile phase develops due to change in light intensity from below to above the canopy. The inherent plasticity in juveniles would provide an advantage in the heterogeneous light environments beneath the canopy. Then, as plants moved to more open conditions, the developmental ability to change to an adult phase was lost due to an acquired ability to mature in the juvenile state. He then proposes that the divaricate form then evolved a denser canopy in response to climate. He suggested that the situation is unique, as New Zealand's vegetation may provide a high proportion of forest margins, and New Zealand forests may allow juvenile plants to survive until a gap forms.

The structural photoprotection hypothesis was proposed by Howell et al. (2002), and involves avoidance of photoinhibition caused by excess irradiance. It is hypothesised that self-shading by outer branches reduces light intensities enough to avoid photodamage. Photoinhibition is caused when excess light is absorbed, and inhibits photosynthesis. This occurs when exposed to high irradiance, and can be intensified by environmental factors such as cold or drought. This hypothesis is suggested as divaricate shrubs are abundant in exposed, frosty habitats that may be exposed to high irradiance after a cold night. Howell et al. (2002) state that the environmental stresses that worsen photoinhibition are likely to be unique to New Zealand. They argue that as the New Zealand climate is oceanic and aseasonal, so cold conditions can occur at any time of year. They also suggest that the combination of clear skies and moderate latitudes result in strong irradiances, even in winter.

Howell et al. (2002) investigated the effect of cold induced photoinhibition on the internal leaves of divaricate plants. They showed that in three divaricate species (*Aristotelia fruticosa* Hook.f., *Corokia cotoneaster* and *Coprosma propinqua*) removal of the outer branches reduced photosynthetic capacity and photochemical efficiency in the exposed leaves. Two of the species (*A. fruticosa*, *C. cotoneaster*) also showed little recovery after three months, and remained photosynthetically impaired. These results supported their structural photoprotection hypothesis. This work was criticised by Lusk (2002), as the photoinhibition observed could be due to the internal leaves being acclimated to shade, rather than being adapted to it. Lusk also argues that the benefits of photoprotection are unlikely to compensate for the high cost of divarication, especially as there are less expensive and drastic ways to avoid photodamage. Christian et al. (2006) quantified net carbon gain and structural costs of the divaricate habit, compared with non-divaricating close relatives. They concluded that while divaricates have traits that may be beneficial in avoiding photoinhibition, the architectural costs are so high that they are unlikely to offset carbon gain arising from photoprotection. This suggests that while avoidance of photoinhibition may occur, it is unlikely to have been the primary selection pressure in divaricate evolution.

2.6 Future research

Continued research into answering the evolutionary question of why the divaricate from has evolved requires a clear definition of what defines a divaricate, and exactly how each type of divaricate achieves its overall architecture. Assessing each divaricate characteristic and its relative contribution to the overall form, which can then educate the formation of a robust index, will provide a foundation for future research. A renewed focus on the architectural and developmental basis of divarication, combined with comparison to divaricate-like traits elsewhere in the world, is essential to be able to properly address the evolution of this form in New Zealand.

Research involving divaricates and their non-divaricating close relatives can further our understanding of how and why divaricates have evolved, and also provide a model system in which to investigate the regulation and development of branching. Typical model organisms (eg *Pisum sativum* L. or *Arabidopsis thaliana* L.) do not necessarily have some of the abnormal and complex traits that can contribute a lot to our understanding. Studies in unusual non-model forms such as divaricates can provide opportunities to address interesting questions relating to branching development and regulation.

An example of such a study could be to investigate the expression of branching genes such as *MORE AXILLARY BRANCHING 4* (MAX4). This gene has been found to be responsible for a signal that that inhibits shoot branching (Sorefan et al. 2003), and could potentially be involved in divarication. This could be investigated by sampling the buds of closely related divaricating and non-divaricating species at key developmental stages and quantifying the levels of MAX4 gene expression present (See Baker et al. 2012). As MAX4 inhibits shoot branching, we could expect to see less MAX4 expression in divaricating species. By comparing the levels of MAX4 gene expression, we can begin to establish potential genes that could be involved in divarication.

2.7 Conclusions

New Zealand’s history has resulted in a unique native flora, and despite decades of research and debate we still do not fully understand why some of these characteristics have evolved. Hypotheses to explain divarication include adaptation in response to harsh climatic conditions, browsing pressure from moa, or high light intensities. Perhaps it was New Zealand’s distinct suite of selection pressures that has contributed to the various aspects of the divaricate forms. We can now begin to ask how this form has evolved, as the genetic basis of these convergent forms is unknown. Whether all divaricates use the same genes and genetic networks to achieve the overall architecture is a question that warrants further investigation. Using the current indices we have so far struggled to successfully quantify divarication at the static morphological level. In order to be able to properly address divaricate evolution we need a precise definition of a divaricate. Understanding divarication requires us to look deeper into the underlying developmental processes that result in the various subtypes of the divaricate habit. Formulating a more robust definition, and therefore a more comprehensive index would allow the exact number of divaricates within the various potential subtypes to be established, and could shed light on whether the divaricating form is indeed unique to New Zealand. Clarifying what exactly defines and unifies divaricate forms can add to our understanding of New Zealand’s history, convergent evolution, and plant development and architecture.

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Chapter Three: Branching and development of the divaricating shrub *Sophora prostrata*

3.1 Abstract

Divaricates are small leaved woody shrubs with tightly interlaced branches, that have arisen 18 separate times within the New Zealand flora. Divaricate shrubs are generally associated with weak apical control, and for that reason divaricating species such as S*ophora prostrata* are ideal models with which to study the complex regulation of apical control and shoot outgrowth. The differences in branching and decapitation response were measured and compared between *S. prostrata* and a non-divaricating close relative *S. tetraptera* (three decapitated individuals and three intact of each species), and potential patterns in axillary bud development along three branches of *S. prostrata* were investigated. Proportions of established branches were found to be the same in both species, however short shoots were found to be a significant contributor to the architecture of *S. prostrata*. *Sophora prostrata* displayed increased growth and node activation in response to decapitation, which could be due to weak apical control resulting in no new leader shoot gaining absolute dominance over the overall plant. Further investigation into the architecture and development of decapitated and intact *S. prostrata* and *S. tetraptera,* along with other divaricate and non-divaricate species pairs, could help in understanding the complexities of apical control and apical dominance in divaricates, and contribute to our knowledge of branching in general.

Keywords: *Sophora prostrata*; *Sophora tetraptera*; divaricate; apical control; short shoots; branching; decapitation

3.2 Introduction

Plant development is regulated by a complex system, integrating information from the genetically controlled species specific branching pattern, the developmental stage of the plant, and the surrounding environmental conditions (Leyser 2003). The resulting architecture is not static, and involves continuous gain and loss of morphological features through the action of meristems (Bell and Bryan 2008). Plant stems are comprised of a series of nodes separated by internodes, with each node consisting of a leaf with one or more axillary bud in its axil (Figure 3.1). Each axillary bud is comprised of the pluripotent cells of the axillary meristem, and leaf primordia which protect the meristem (Beveridge 2006). The location, timing and duration of axillary bud outgrowth affects the overall architecture and fitness of the plants form (Bonser and Aarssen 1996), and each axillary bud can develop into a reproductive or vegetative structure, or remain dormant. Hormonal control of axillary bud outgrowth is an intricate network that coordinates signals from throughout the plant and mediates developmental plasticity.

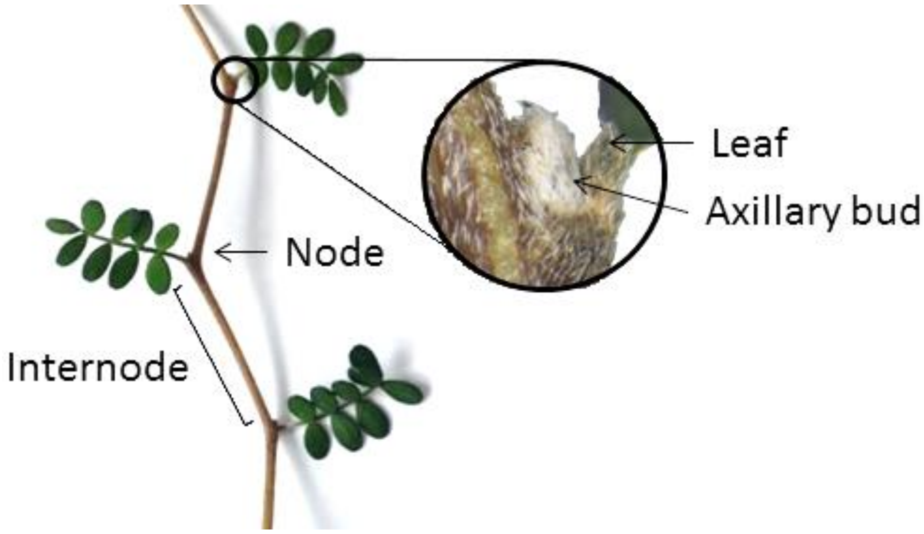

Figure 3.1: The locations of nodes and internodes along the stem of the divaricate *S. prostrata*. Each node consists of a leaf and axillary buds.

Apical dominance is the term used for the control that the shoot tip exerts on lateral bud outgrowth (Cline 1997). Decapitation leads to outgrowth of lateral buds as they are released from apex imposed dormancy. This appears to function as a plant survival mechanism, as the plant is able to compensate for the damage (Cline 1991). Many studies use herbaceous species such as *Pisum sativum* (pea), or *Arabidopsis thaliana* to study apical dominance (Stafstrom 1995, Cline 1997, Dun et al. 2006), however it is significantly more complex in woody plants than it is in herbaceous plants, and may not be the most appropriate term. Brown et al. (1967) introduced the term apical control for use in woody perennials to refer to the overall shape and form of the tree crown as influenced by the top of the tree. Apical dominance in woody species is then applicable to the inhibitory effect of any shoot apex on the lateral buds along its branch.

Axillary bud dormancy has long been manipulated to enhance productivity in agricultural crops (Horvath et al. 2003). Understanding how environmental factors influence branching can help to manage plant growth, and knowledge about the genetic basis of branching plan and control allows for the manipulation of these processes. Using herbaceous model organisms to study apical control is essential as they are fast growing and relatively architecturally simple, however more complex regulatory systems such as those in woody perennials can allow further investigation and insight into natural systems. Model organisms do not necessarily have some of the interesting and complex traits that can add much to our understanding, therefore studies of non-model organisms can provide new opportunities to answer interesting evolutionary and ecological questions (Nawy 2011). Recent advances in the understanding of hormonal signalling and developmental genetic networks have made studies in non-model organisms possible (See Baker et al. 2012). In order to further our knowledge in these areas, research should address abnormalities in the natural world.

One example of such a peculiarity is the branching and development of divaricate shrubs. Divaricate plants are an assemblage of small leaved woody shrubs with closely interlaced branches. They are a remarkable example of convergent evolution as the divaricate growth form has arisen independently at least 18 times, and makes up 10% of the New Zealand native woody flora (Greenwood and Atkinson 1977). The divaricate form has been associated with a lack of apical control resulting in outgrowth of lateral branches (Greenwood and Atkinson 1977, Wilson and Galloway 1993, Christian et al. 2006, Wilson and Lee 2012). The definition of a divaricate varies between authors, but generally includes features such as branch interlacing, long internodes, the presence of small leaves, short shoots, and many branches. As this growth form has arisen independently multiple times within New Zealand, it is achieved in many structurally different ways. The divaricate form can be achieved via wide angled branching (e.g. *Coprosma propinqua*), but also branching at a very narrow angle (e.g. *Melicytus alpinus*). Sympodial growth can result in a zig-zag axis (e.g. *Elaeocarpus hookerianus*), as can monopodial growth where the shoot bends at each node (e.g. *S. prostrata*) (Bell and Bryan 2008)(See Chapter Two).

There has been much debate as to what unique New Zealand selection pressure has shaped the various divaricating forms in so many unrelated taxa. Greenwood and Atkinson (1977) proposed that divarication is a strategy evolved in response to browsing by large ratite moa. They suggest that the small, widely spaced leaves concentrated in their centre could reduce area lost by plucking, and the strong wide angled branches would be difficult to break off and swallow. This view was challenged by McGlone and Webb (1981), when they suggested that divaricating plants evolved in response to frost, wind and drought during past glacial climates. It was thought that the tangled growth form would have offered protection from frost and abrasion. It was later hypothesised by Day (1998) that the divaricate habit was advantageous to light capture, as the small leaves are scattered in three dimensional space by the thin interlacing branches, which would distribute light throughout the canopy. Howell et al. (2002) suggested that self-shading by out branches reduces light intensities enough to avoid photodamage, which inhibits photosynthesis.

Two mathematical indices to quantify divarication have been proposed by Atkinson (1992) and Kelly (1994). Atkinson's index focuses solely on branching parameters, measuring branching angles and number of branches. Kelly's index measures various aspects of shoot architecture, such as leaf width and internode size as well as branching angle. Despite the different focuses and features included in these indices, they correlate well in many species (McQueen 2000) (See also Chapter Two). Day and Gould (1997) investigated the development of divaricating *Elaeocarpus hookerianus*, and noted that the topology of the form changes over the course of the growing season, and is not stationary. They questioned the use of static morphological parameters to define a growth form, and highlighted the importance of developmental processes in the overall architecture. Carswell and Gould (1998) found that morphological parameters did not distinguish *Sophora* species adequately, and concluded that developmental processes could be better descriptors of divarication than shoot dimensions. Grierson (2014: Chapter Two) found that on average, the two indices did distinguish two divaricating and non-divaricating *Sophora* species; however it was not consistent in all individuals. The results of these studies highlight the importance of considering variations over time due to developmental processes when quantifying divarication, and formulating a more robust index.

Sophora prostrata is a prostrate divaricate shrub reaching up to 2m in height with densely interlacing zig-zagging branches, which was first described by Buchanan (1883). It is confined to the eastern side of the South Island of New Zealand, and is found in lowland to montane shrubland, river flats, grassland and rocky places (Wilson and Galloway 1993, Godley 2006). *Sophora prostrata* is one of eight *Sophora* species in New Zealand, and molecular evidence suggests that they are of recent origin due to little to no sequence divergence in normally quite variable regions (Hurr et al. 1999, Mitchell and Heenan 2002). The close relationship between New Zealand *Sophora* species is supported by their frequent hybridisation (Godley 1975, 2006). *Sophora prostrata* is the only *Sophora* that is divaricating at maturity, while *S. microphylla* is a heteroblastic species with a divaricating juvenile and arborescent adult.

Previous research focusing on development and hormonal regulation of divarication has taken advantage of this series of forms within *Sophora*. Carswell et al. (1996) found that divaricating *Sophora* species had greater ratios of active to storage cytokinins, which could impact branch outgrowth. Carswell and Gould (1998) found that the differing growth periods of *S. prostrata*, juvenile *S. microphylla*, and *S. tetraptera* were indicative of the divaricate forms (*S. prostrata* and juvenile *S. microphylla*) having weak apical control. The range of forms within *Sophora*, as well as previous research in this area, makes *S. prostrata* and its non-divaricating relatives a good candidate for further research into the architecture and development of divaricates.

3.3 Aims and objectives

This chapter investigates the development of the divaricate *S. prostrata* in reference to a non-divaricate relative *S. tetraptera*, and attempts to further our understanding of the divaricate form. This work also aims to provide a foundation for further work into the genetic control of branching in *S. prostrata*, through identification and prediction of key developmental stages in which branching gene expression (e.g. MAX4) could be investigated.

The specific objectives of this research were to:

1. Quantify differences in branching between *S. prostrata* and *S.tetraptera*, by recording and comparing the state of each axillary bud along branches of both species.
2. Assess the effect of apical control on branching in *S. prostrata* and *S. tetraptera* by recording the growth and node state change response to decapitation of both species.
3. Investigate patterns in axillary bud features along branches of *S. prostrata* by comparing the internal and external morphology of a series of axillary buds, to identify and predict different developmental stages to educate sampling for future studies.

3.4 Methodology

3.4.1 Study species

This study focused on *S. prostrata* and *S. tetraptera*. *Sophora tetraptera* was chosen to compare with *S. prostrata* (described above), as it is closely related yet has an entirely different form; it is a small tree with spreading branches of up to 12m in height when mature. Ten plants of each species were purchased from Oratia nursery, Auckland in August 2012, and were grown in the Waikato University glasshouse prior to and during the experiments. Examples of a portion of shoot and the mature forms of each species are shown in Figure 3.2.

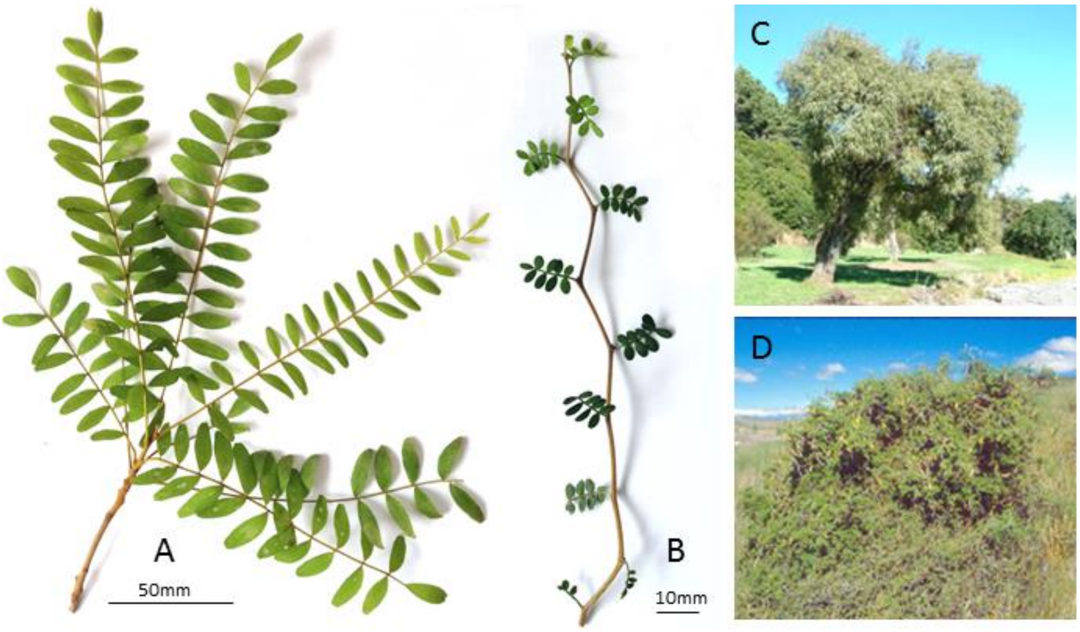

Figure 3.2: Examples of shoots (A and B) and mature forms (C and D) of *S. tetraptera* and *S. prostrata*. A shows a portion of *S. tetraptera* shoot, and B shows a portion of *S. prostrata* shoot. Scale bars are shown to the right of each shoot. C shows mature *S. tetraptera* and D shows mature *S. prostrata.* (Photos C and D courtesy of John Smith-Dodsworth).

3.4.2 Comparison of vegetative development and apical control

The state of the axillary bud and leaf at each node, and any changes in state over time, were compared on six individuals each of *S. prostrata* and *S. tetraptera*. Three individuals were decapitated and three were left intact for each species (Figure 3.3). For each individual, four to six first order branches were chosen from the bottom two thirds of the plant. The state of each node on each of the six branches, and all branches of all orders on that branch were recorded five times over 13 weeks (total of 30,788 observations). The main stem was measured from the base to the tip, and any recent new growth at the apex was not included in the main stem length. The main stem was partitioned into three equal sections, and the top third was removed in three individuals per species, leaving the middle and bottom third for measurements.

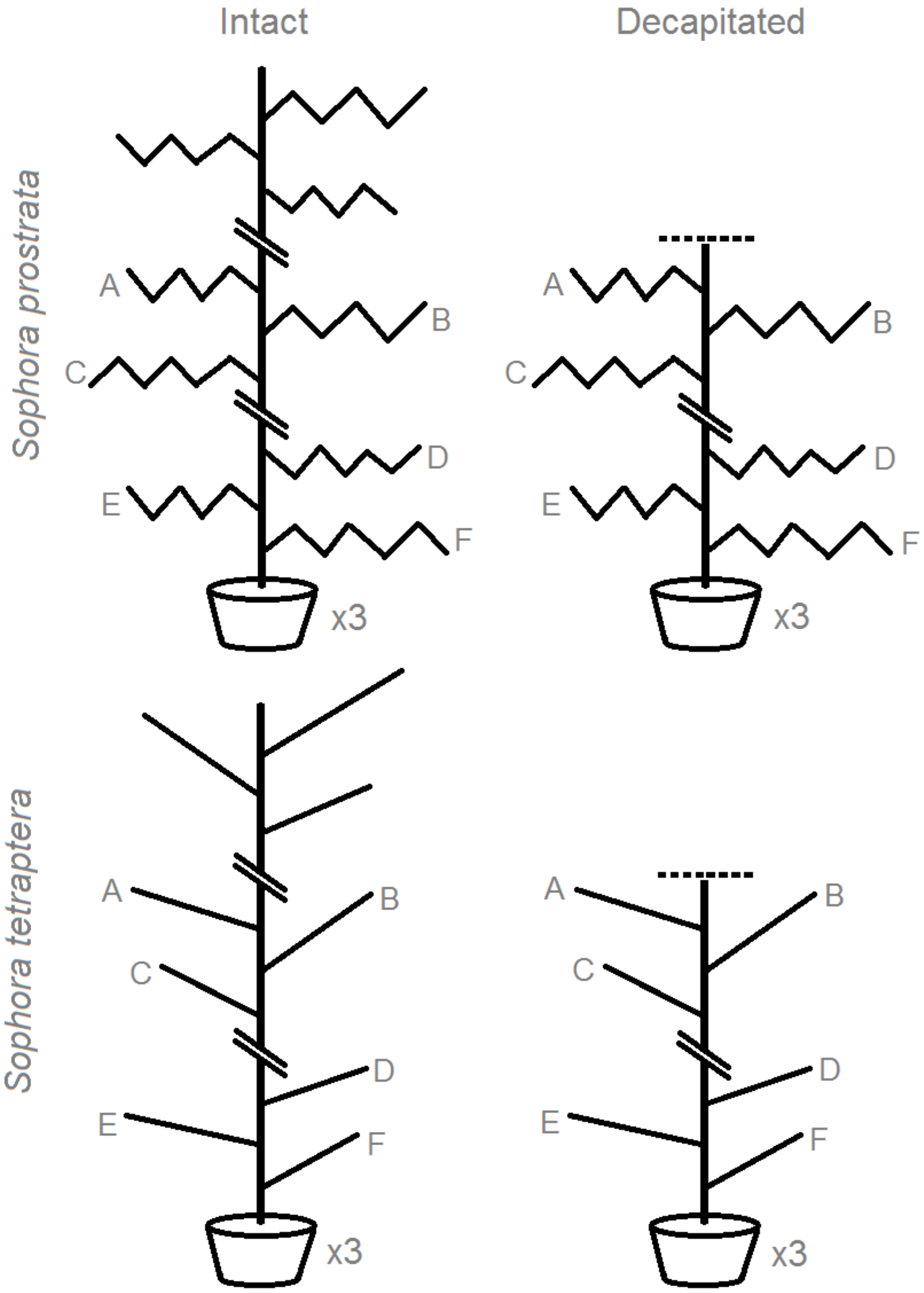

Figure 3.3: Experimental design showing the four classes for comparison of vegetative development between *S. prostrata* and *S. tetraptera*. The stem of each individual was partitioned into thirds, and the individuals from the decapitation treatment then had the top third removed (as shown by the dashed line). Three representative branches from the middle third (labelled A, B and C) of all individuals of both species and treatments were tagged for node state recording, as well as three branches (D, E and F) from the bottom third.

Three branches were chosen from each of the remaining two sections, labelled A, B and C in the middle third and D, E and F in the bottom third. Branches were chosen to be representative of their section. As the *S. tetraptera* plants had fewer branches, it was not always possible to have three branches in each section, or six branches in total. The definitions for the eight possible node states are shown in Table 3.1. This experiment was conducted in late summer and autumn, which is a growth period shared in both of these species (Carswell and Gould 1998).

Table 3.1: The active and dormant states that could be observed in the *Sophora* species included in this study, with definitions of each node state.

Symbol	Node state	Definition
Dormant		
L	Leaf	Leaf, with axillary bud dormant. Classed as a leaf when more than 1mm in length
LS	Leaf scar	Leaf scar, with axillary bud dormant
N	Node	Young unexpanded leaf, less than 1 mm in length. Axillary bud developing.
BR	Transitional branch	Axillary bud appears to have initiated into a branch, but is still developing. It classified as a branch when it develops its first visible nodes or leaves.
Active		
SS	Short shoot	Short shoot, with the number indicating how many leaves are present. Classed as an established branch when internodes are visible – at approx. 1mm in length
BR-	Branch	Branch, with the number indicating its position and order. Eg BRA1 is the first branch off branch A, BRA1.1 is the first branch off BRA1 etc.
BS	Branch scar	Branch or floral structure scar. No axillary bud now present
FL	Floral structure	The peduncle of an inflorescence

A Mann-Whitney U test was performed to assess significant differences between the species. To assess the effect of decapitation, and therefore apical control, a logistic regression was performed in R (version 2.11.1) using only the dormant (leaves, leaf scars and transitional branches) and active (short shoots and established branches) states. A Bayesian survival analysis of the dormant to active transitions is underway but was not complete at the time of thesis submission.

3.4.3 Microscopic observation of axillary buds

3.4.3.1 Scanning electron microscopy

Three complete branches were taken from the middle region of one individual of *S. prostrata.* The branch was divided into four sections – with section one being the most distal (youngest), and section four being the most proximal (oldest). Three axillary buds per section were dissected out from their nodes using hypodermic needles to allow for full visualisation of the bud. The buds were then fixed in gluteraldehyde for at least 48 hours, and then dehydrated through an ethanol series (10 minutes each of 20% EtOH → 50% EtOH → 70% EtOh) and critical point dried. The buds were then fixed to an aluminium stub using double sided carbon tape. Some samples were placed with the abaxial surface facing up, and some with the adaxial surface facing up. Samples were then coated in platinum, and viewed with a FE-SEM (Hitachi S-4700).

3.4.3.2 Light microscopy

Three branches on each of three *S. prostrata* individuals were cut, and the most distal 30 nodes were photographed with a Canon EOS 600D digital camera fitted to an Olympus SZH10 research stereo microscope. Every third node was cut out, with approx. 5mm of branch left either side. The removed nodes were then fixed in FAA for a minimum of 12 hours. Samples were washed in 70% ethanol for 12 hours, dehydrated through a TBA/ethanol series and then infiltrated through paraffin oil to wax (Appendix One). The samples were then embedded into wax using a Thermo Scientific Histostar^{TM} embedding machine onto plastic cassettes, which were then set on the cool tray of the embedding machine. Blocks of embedded samples were then sectioned at a thickness of 9 – 12.5 um using a Leica RM2055 microtome. Ribbons were placed on Polysine^{TM} microscope slides flooded with 2.5ml of 0.2% ethanol on top of a warming table at 37°C. Slides were left overnight for ribbons to flatten and the ethanol to evaporate. Slides were then stained with Safranin O and fast green, using a modified protocol of (Johansen 1940) (Appendix Two). Coverslips were then mounted onto the slides using DPX, and left to dry in a fume hood overnight. Slides were viewed and photographed in on a Leica DMRD microscope fitted with an Olympus DP70 digital camera. Sections were chosen to show the axillary meristem, for comparison, but also the full height of the first leaves and any secondary axillary buds present if possible. Images were taken at 5x –20x magnification. Each axillary bud length was measured from the tip of the meristem to around the midway point between junctions of axillary bud and branch, and axillary bud and leaf. The width of each bud was measured from the centre of the bud. The unexpanded leaves were counted using the serial slides for each bud.

3.5 Results

3.5.1 Comparison of vegetative development and apical control

To look at the differences in node states between *S. prostrata* and *S. tetraptera* species without any effect of decapitation, the state of each node on each branch of each individual at time 0 were used, and the proportions of each state were averaged for each species. The totals of each node state are shown in Appendix Three. Proportions of each node state were used instead of totals to account for differences in node number, which could be due to other factors such as age or prior growing environment. The proportions of each node state (Figure 3.4) show that *S. prostrata* has a larger proportion of total nodes as leaves (shown in blue) than *S. tetraptera* (58% and 31% respectively), and *S. tetraptera* has a larger proportion of leaf scars (shown in blue) than *S. prostrata* (58% and 14% respectively). The proportion of established branches is the same for both species (10%), however *S. prostrata* differs to *S. tetraptera* in the presence of short shoots (12%, shown in green) and transitional branches (6%, shown in orange). When each node is considered as either active (SS or BR-) or dormant (L, LS, BR or N), the proportion of active nodes in *S. prostrata* is over twice the proportion of active nodes in *S. tetraptera* (22% and 10% respectively) (Figure 3.5). Due to the small sample sizes, most of the proportions are statistically insignificant, except for the proportions of short shoots ($p < 0.05$).

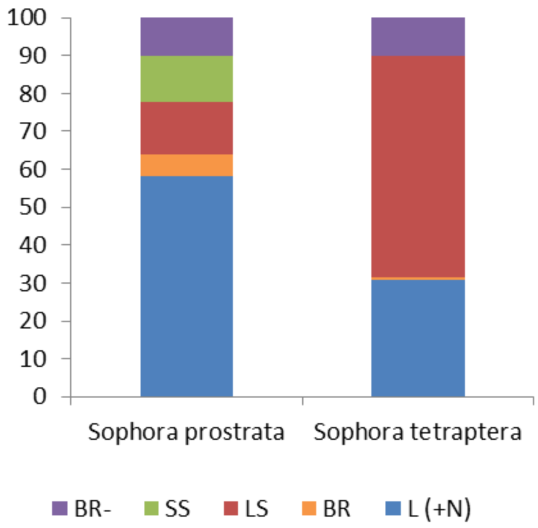

Figure 3.4: Proportions of each node state out of the total nodes nodes at time 0 on all branches of all orders on all individuals of *S. prostrata* and *S. tetraptera.* The N (unexpanded leaf) node state are treated as L (leaves) in this figure.

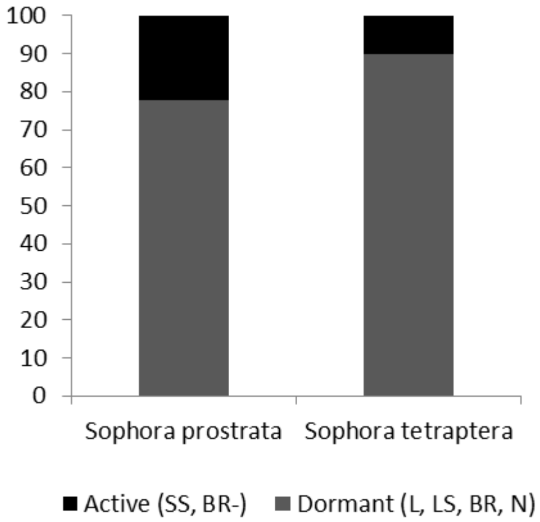

Figure 3.5: Proportions of active (SS, BR-) and dormant (L, LS, BR, N) nodes at time 0 on all branches of all orders on all individuals of *S. prostrata* and *S. tetraptera.*

New nodes are used here as a measure of growth, as they are any new nodes added on any new or existing shoots (this could be as a leaf, leaf scar, short shoot, transitional branch or branch) on all branches of all orders of each individual. Figure 3.6 shows the cumulative percentage increase of new nodes over time for the decapitated and intact individuals of *S. prostrata*. At 13 weeks, the total percentage increase in nodes was greatest for the three decapitated individuals, and lowest for the three intact individuals. However, Soppro03 (decapitated) had an only slightly higher percent increase of nodes (1.1% difference) than Soppro05 (intact). During weeks 2 – 9, Soppro03 (decapitated) had less total percentage increase than Soppro05 and Soppro06 (both intact).

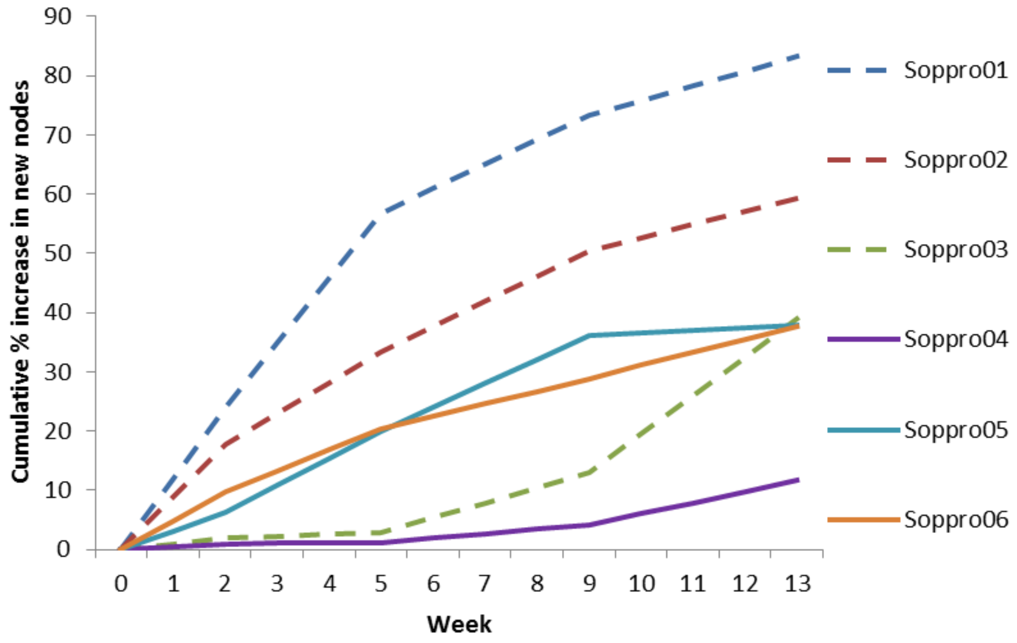

Figure 3.6: Cumulative percentage increase of new nodes over time for six *S. prostrata* individuals. Dashed lines indicate decapitated individuals, solid lines indicate intact individuals.

Figure 3.7 shows the cumulative percentage increase of new nodes added over time for *S. tetraptera* decapitated and intact individuals. At 13 weeks, the number of nodes added was greatest in Soptet04 (decapitated), followed by Soptet08 and Soptet10 (both intact), then the remaining two decapitated individuals (Soptet01 and Soptet06). There were no clear differences in added nodes between the intact and decapitated species.

Figure 3.8 shows the average cumulative percentage increase of new nodes over all branches of the three individuals in each class (*S. prostrata* decapitated, *S. prostrat*a intact, *S. tetraptera* decapitated, and *S. tetraptera* intact). At 13 weeks, decapitated *S.*

prostrata had the highest average cumulative percentage increase of new nodes (60.5%), followed by intact *S. prostrata* (29%), decapitated *S. tetraptera* (24.5%) and then intact *S. tetraptera* (21.4%).

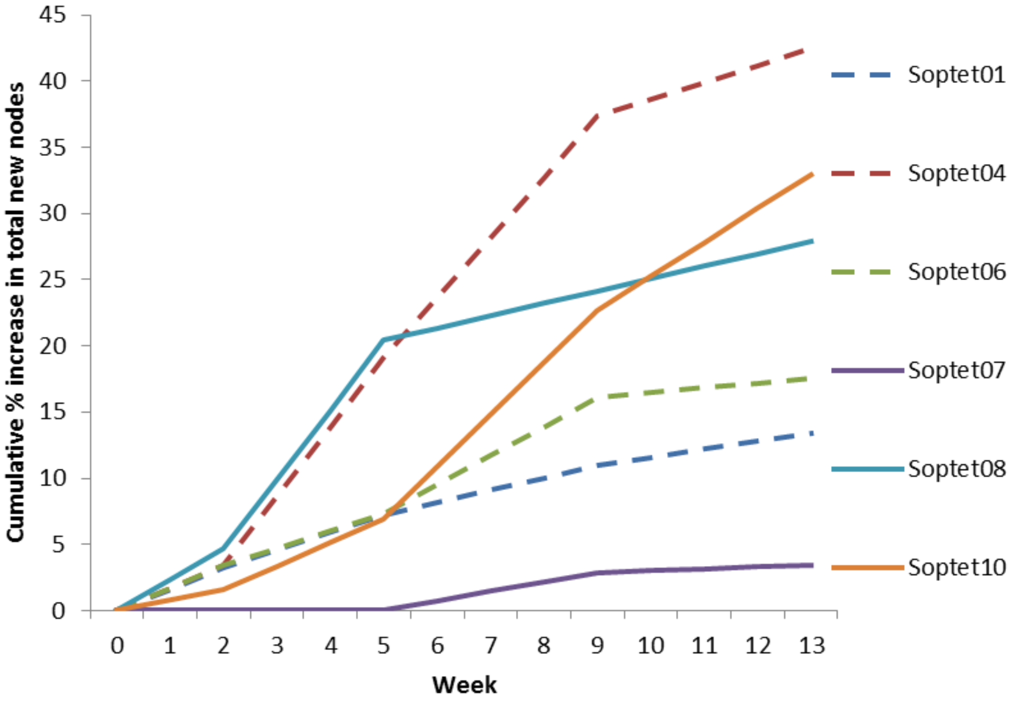

Figure 3.7: Cumulative percentage increase of new nodes over time for six *S. tetraptera* individuals. Dashed lines indicate decapitated individuals, solid lines indicate intact individuals.

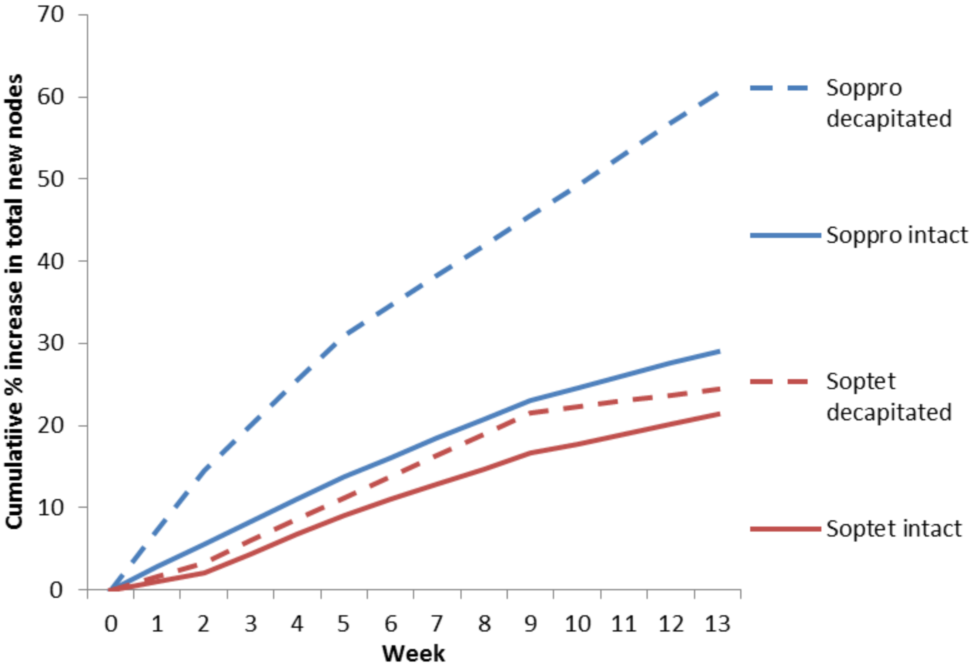

Figure 3.8: Average cumulative number of new nodes added over time for the *S. prostrata* (blue lines) and *S. tetraptera* (red lines). Dashed lines indicate decapitated individuals, solid lines indicate intact individuals.

A logistic regression with a logit link-function was performed in R (version 2.11.1) to assess differences in the probability of transitioning from a dormant to active state in each of the classes. An event was specified as the transition from a dormant to an active state (“DA”), and the transitions from a dormant to a dormant state (“DD”) as a non-event (Table 3.2).

Table 3.2: Output from logistic regression for Dormant-Active events, classifying leaves, leaf scars and transitional branches as dormant and short shoots and mature branches as active.

Coefficient	**Estimate**	**Standard Error**	***p*-value**
Intercept	-5.1625	0.1930	<0.0001
Decapitated	1.7692	0.2047	<0.0001
S. tetraptera	0.4529	0.2816	0.108
Decapitated and *S. tetraptera*	-2.7367	0.3738	<0.0001

The model can be expressed as:

$$\log\left(\frac{p_{\mathrm{DA}}}{1-p_{\mathrm{DA}}}\right) = -4.0447 \text{ for non-decapitated } \textit{S. prostrata}$$

$$\log\left(\frac{p_{\mathrm{DA}}}{1-p_{\mathrm{DA}}}\right) = -3.2563 \text{ for decapitated } \textit{S. prostrata}$$

$$\log\left(\frac{p_{\mathrm{DA}}}{1-p_{\mathrm{DA}}}\right) = -4.4732 \text{ for non-decapitated } \textit{S. tetraptera}$$

$$\log\left(\frac{p_{\mathrm{DA}}}{1-p_{\mathrm{DA}}}\right) = -4.3078 \text{ for decapitated } \textit{S. tetraptera}$$

The probability of transitioning from a dormant to an active state appears highest for decapitated individuals of *S. prostrata*, at around 3.3% of transitions from dormant states (Table 3.3). The next highest probability is for *S. tetraptera* when intact (0.9%); then intact *S. prostrata* (0.6%); and decapitated *S. tetraptera* (0.3%). 95% confidence intervals for the probability of transitioning from a dormant to an active state overlap for the categories of non-decapitated *S. prostrata*, and both decapitated and non-decapitated *S. tetraptera*, which means these estimated probabilities are not significantly different. Decapitated *S. prostrata* has a significantly higher probability of transitioning from a dormant to active state relative to the other classes. The analysis was also performed with the transitional branches considered active, which did not change the significance of the probabilities (data not shown). The logistic regression was performed again with short shoots considered dormant, to assess their significance, which resulted in the 95% confidence intervals overlapping for all classes. There were no significant differences in transition between any of the classes when short shoots were considered dormant.

Table 3.3: The probability of transitions between a dormant and active state between any time interval for each category, with 95% confidence intervals using Bonferroni's correction for multiple comparisons.

Class	p_{DA}	Corrected 95% CI
Non-decapitated *S. prostrata*	0.0057	(0.0030, 0.0084)
Decapitated *S. prostrata*	0.0325	(0.0271, 0.0379)
Non-decapitated *S. tetraptera*	0.0089	(0.0044, 0.0135)
Decapitated *S. tetraptera*	0.0034	(0.0014, 0.0054)

3.5.2 Microscopic observation of organogenesis

All axillary buds observed were vegetative due to the presence of unexpanded leaves. Preliminary SEM results showed that all buds were densely covered in hairs, which prevented observation of bud features (Figure 3.9), so the focus was moved to sectioning for viewing under the light microscope.

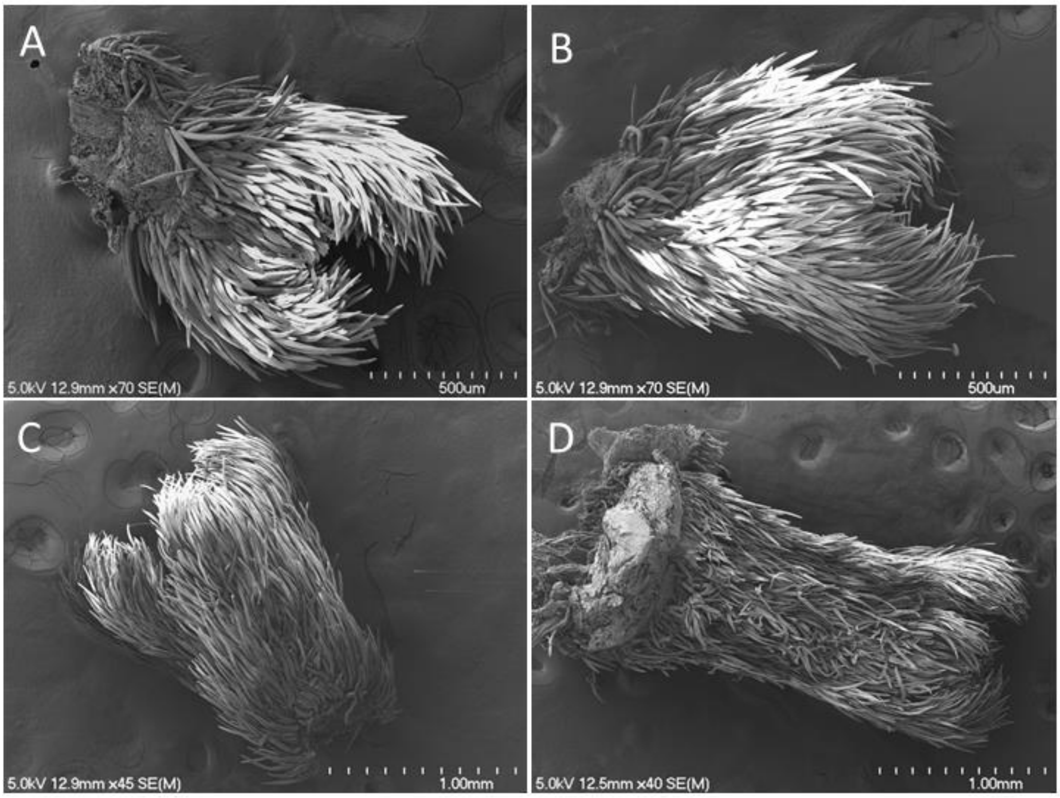

Figure 3.9: Axillary buds of *S. prostrata* removed from leaf axils and viewed under a SEM. A and B show young new growth buds. A shows the adaxial surface, and B shows the abaxial surface. C shows the abaxial surface of an older bud from the middle of the branch. D shows the adaxial surface of a bud from near the base of the branch. All samples show the dense pubescence, and the unexpanded leaves beneath.

Figure 3.10 and Figure 3.11 show the axillary buds along one of the branches (Soppro07C) prior to fixation, and a median longitudinal section of the corresponding bud. Figure 3.12 and Figure 3.13 show the median longitudinal sections of axillary buds along all three branches next to each other. The observed buds consisted of the axillary meristem and 2 – 6 unexpanded leaves. Most axillary buds had secondary axillary buds in their axils (AX2 in figures 3.10 – 3.13). The secondary/accessory buds occasionally had 1 – 2 visible leaf primordia, but often only the meristem was visible. There was no discernable pattern in leaf number in relation to node position along the stem or size. The hairs covering the buds ranged in colour from white - light brown, to dark brown – blackish. There was no correlation between the age or size of the bud and the colour of its hairs. Some of the smallest buds (e.g. node 16 in branch 07C) had large unexpanded leaves, which obscured their size in the external images.

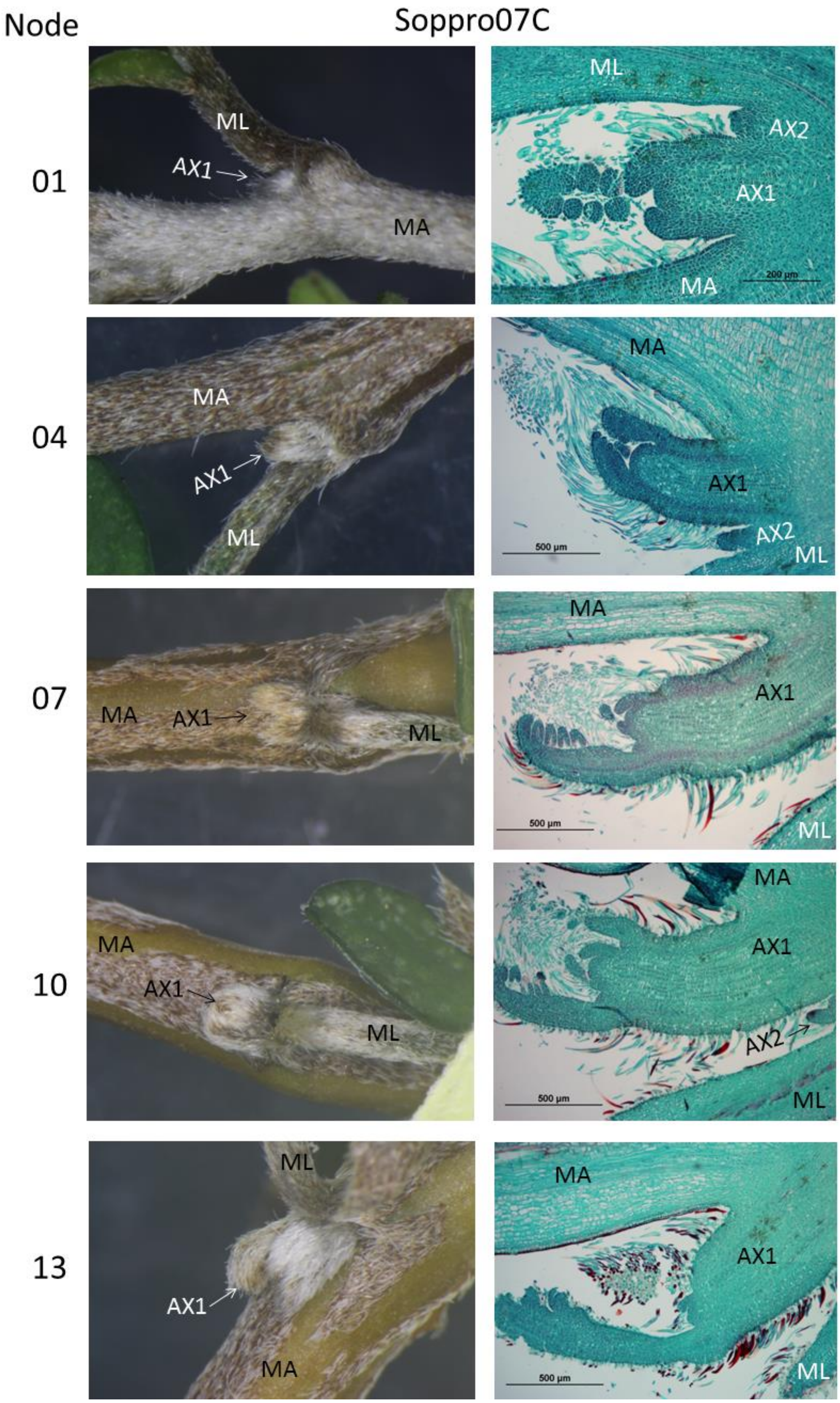

Figure 3.10: Axillary buds from nodes 1 - 13 of *S. prostrata* along the branch Soppro07C. The images on the left show the bud prior to fixation, and the images on the right show a median longitudinal section the bud. MA = Main axis, ML = main leaf, AX1 = the main axillary bud, AX2 = secondary axillary bud.

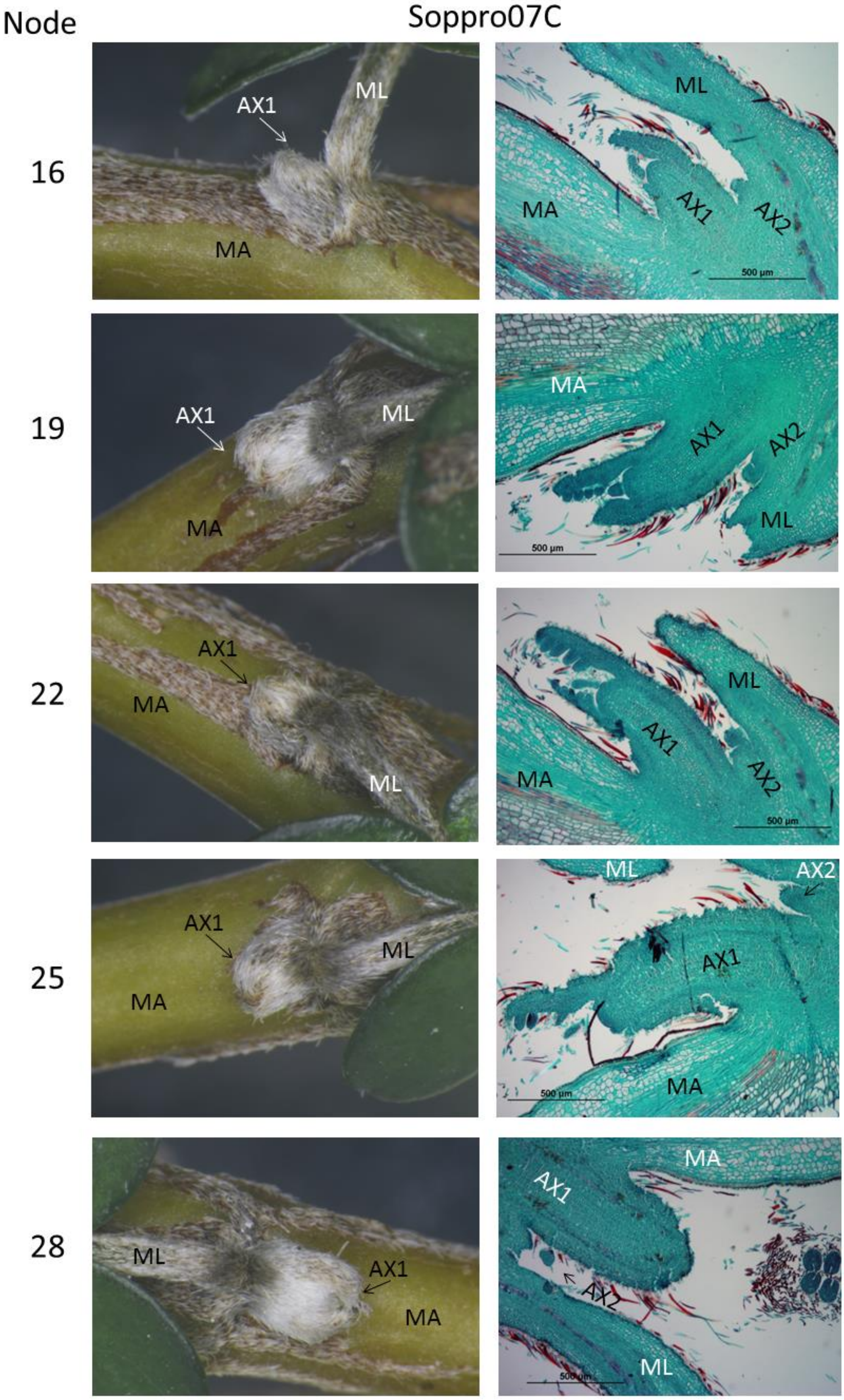

Figure 3.11: Axillary buds from nodes 16 – 28 of *S. prostrata* along the branch Soppro07C. The images on the left show the bud prior to fixation, and the images on the right show a median longitudinal section the bud. MA = Main axis, ML = main leaf, AX1 = the main axillary bud, AX2 = secondary axillary bud.

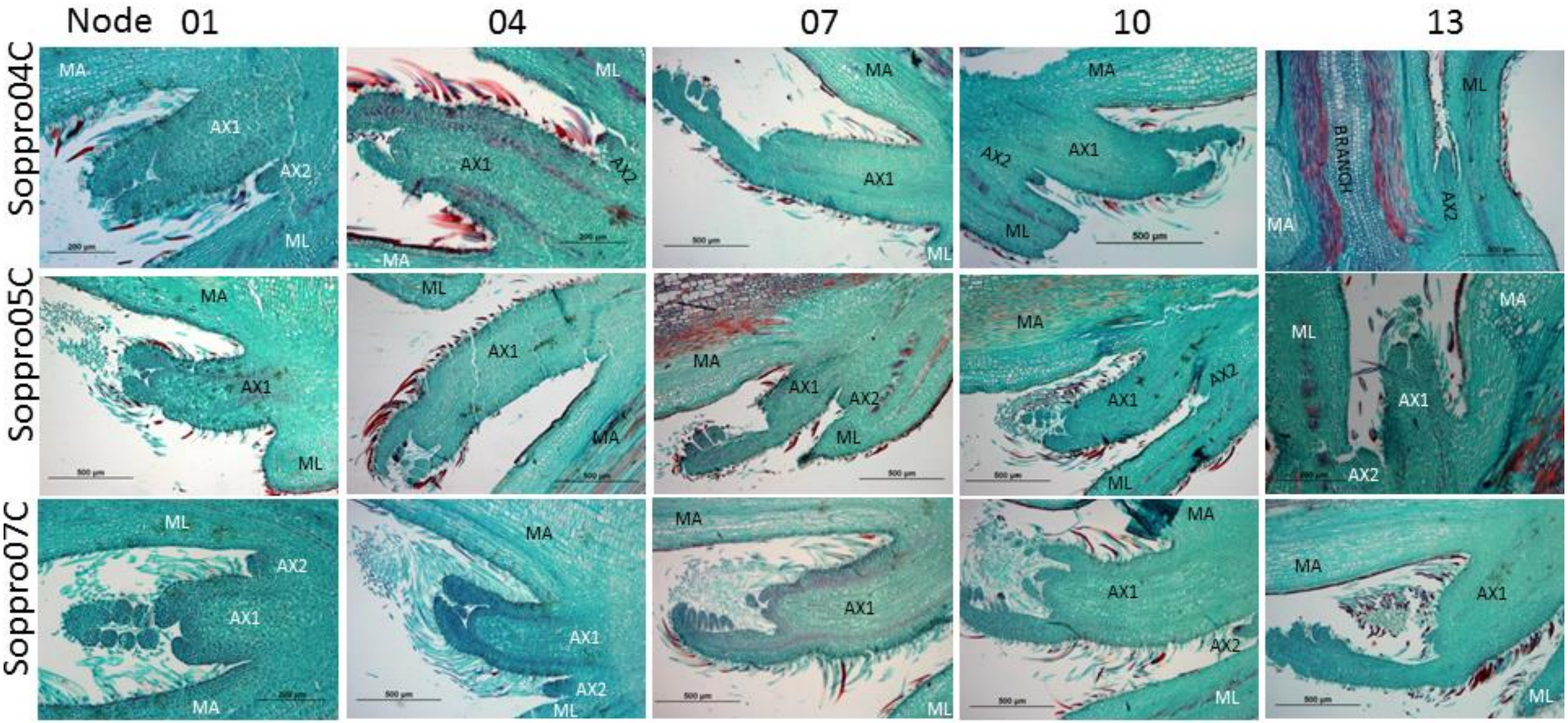

Figure 3.12: Median longitudinal sections of *S. prostrata* axillary buds from node 1- 13 along branches Soppro04C, Sopro05C and Soppro07C. The columns show the same node position across the three individuals. MA = Main axis, ML = main leaf, AX1 = the main axillary bud, AX2 = secondary axillary bud.

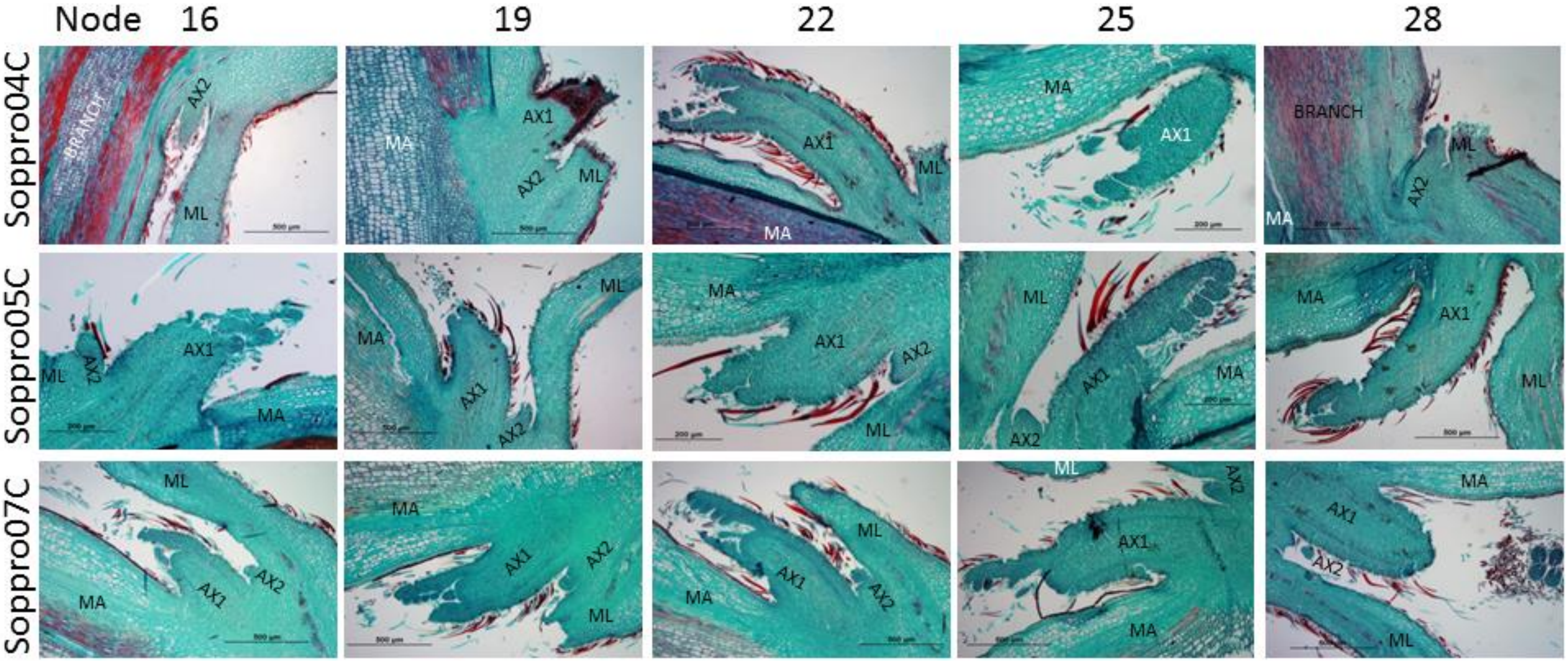

Figure 3.13: Median longitudinal sections of *S. prostrata* axillary buds from node 16 – 28 along branches Soppro04C, Sopro05C and Soppro07C. The columns show the same node position across the three individuals. MA = Main axis, ML = main leaf, AX1 = the main axillary bud, AX2 = secondary axillary bud.

The primary buds ranged in length from 285.7 - 1363.2 µm (Table 3.4). An autocorrelation analysis of the axillary bud length was carried out to determine whether there was any recurring pattern along the branch – from the proximal to the distil node. For all three samples, there was no significant autocorrelation at the 5% level. There did appear to be an almost significant negative correlation between an axillary bud length and the axillary bud length 6 nodes away on one branch (Soppro07C), but this pattern was not evident on either of the other two branches. The buds ranged in width from 214.28 – 556.64 µm (Table 3.4). A linear regression model between length and width show that they are significantly strongly positively correlated ($R^2 = 0.76$, $p < 0.05$).

Table 3.4: Lengths (from the axillary meristem to the base) and widths (from near middle of bud) of *S. prostrata* axillary buds in µm. For nodes 01- 28 on branches 04C, 05C and 07C.

	Branch 04C		Branch 05C		Branch 07C	
Node number	Length (µm)	Width (µm)	Length (µm)	Width (µm)	Length (µm)	Width (µm)
01	314.27	271.42	681.6	431.68	285.7	257.13
04	657.11	328.56	1363.2	471.44	738.4	443.04
07	1022.4	482.8	568	311.413	1363.2	556.64
10	700	350	908.8	340.8	1306.4	533.92
13	Branch	Branch	285.7	199.99	1022.4	511.2
16	Branch	Branch	314.27	257.13	514.26	322.84
19	624.8	340.8	624.8	329.44	681.6	374.88
22	1249.6	403.28	428.55	257.13	738.4	386.24
25	371.41	214.28	399.98	259.99	1079.2	482.8
28	Branch	Branch	1249.6	414.64	852	505.52

3.6 Discussion

Sophora prostrata and *S. tetraptera* both had equal proportions of established branches (10%, Figure 3.4), but *S. prostrata* had a higher proportion of active nodes when short shoots were taken into account (22% compared with 10%, Figure 3.5). Having many branches has been considered a characteristic of divaricates by several authors (Cockayne 1912, Wilson and Galloway 1993, Kelly 1994, Howell et al. 2002), however Tomlinson (1978) assessed the 'branchiness' of several divaricates including *S. prostrata* using bifurcation ratios, and concluded that they were not much branched in that sense. Carswell and Gould (1998) also found that *S. prostrata* did not always branch more frequently than *S. tetraptera* in forest grown material. Neither of these studies take into account the significance of short shoots, which are also occasionally considered as a feature of the divaricate form (Tomlinson 1978, Kelly and Ogle 1990, Bell and Bryan 2008) and have been shown to be a significant component of *S. prostrata* architecture.

There are notable differences in the proportions of many node states in *S. prostrata* and *S. tetraptera*. The higher ratio of leaf scars to leaves in *S. tetraptera* (31 L to 58 LS) is due to it not retaining all of its leaves, while *S. prostrata* keeps a larger proportion of its leaves (58 L to 14 LS) (Figure 3.4). This is due to the larger leaves being borne near the top of the plant in order to harvest maximum light and also because *S. tetraptera* is brevideciduous and loses its leaves in spring at the time of flowering.

Another interesting difference between the species was the presence of transitional branches in *S. prostrata* and not *S. tetraptera*. The proportions used here are from time 0 which was recorded during the few days following decapitation, however when plants are decapitated there is relatively rapid hormonal signalling that releases lateral buds from dormancy into a transitional stage (Morris et al. 2005) (Figure 3.14). Measurable increases in bud size can be detected within a few hours or longer depending on the species (Cline 1997). From this transitional stage, they can either return to dormancy or continue to grow into a branch. Many studies have observed an increase in axillary bud size after decapitation, before dormancy is re-imposed. In decapitated pea seedlings, several axillary buds respond to decapitation by enlarging, and only a few of them continue to form established branches as many then return to dormancy (Morris et al. 2005). Initial growth of axillary buds followed by transition back to dormancy could explain the proportion of what have been classed as 'transitional branches' (BR) in the present study.

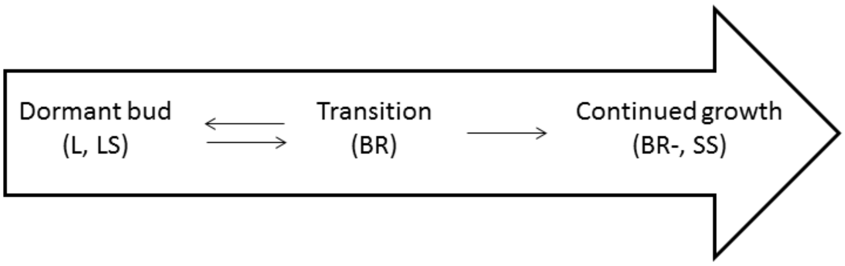

Figure 3.14: Stages in axillary bud outgrowth. A dormant bud can enter a transitional stage (BR), and can then either revert back to dormancy (L, LS), or continue to grow as a short shoot (SS) or branch (BR-).

Aarssen (1995) discussed two ways in which strong apical dominance can be inferred. One of which is a large over compensatory response following decapitation, indicating that the lateral buds had been released from a strong inhibitory influence. Alternatively, strong apical dominance could be inferred through no observable over compensatory growth response, due to another secondary shoot rapidly resuming the role of the dominant shoot, and imposing lateral suppression.

Overcompensation occurs as a response shoot apex damage (Aarssen 1995). Compensatory growth is an important response to damage and biomass removal, as it allows the plant to recover following damage. One of the most widely discussed sources of shoot apex damage is herbivory, but other sources of damage include wind, cold, trampling and heat (Belsky et al. 1993). The greater growth response shown in decapitated *S. prostrata* relative to decapitated *S. tetraptera* (Figures 3.6-3.8) could be due to differences in apical dominance influencing overcompensation. Woody species are generally larger and have a longer life span in comparison to herbaceous species, in which many of these types of studies are carried out. This means that they may not be under the same pressure to compensate quickly in order to complete their life cycle as herbaceous annuals might be (Haukioja and Koricheva 2000). It is possible that it could take several years for the compensatory response to develop (such as Sacchi and Connor (1999)), meaning that short term studies such as this one may underestimate the response. Divaricates are generally viewed as having weaker apical control, and *S. prostrata* specifically has been shown to display weak apical control (Carswell and Gould 1998). In light of previous studies involving divaricates and *Sophora*, the idea that *S. prostrata* is displaying weak apical control through its lack of a new dominant leader seems probable.

Further to this, the increased activation of nodes in decapitated *S. prostrata* could be due to decapitation induced activation of shoots, which were not subsequently supressed due to a lack of a secondary shoot becoming dominant and re-imposing lateral suppression. The non-significant activation response to decapitation displayed by *S. tetraptera* could be due to a secondary shoot resuming the role of lateral suppression. As shown by looking at the proportions of each node state at time 0, *S. prostrata* does not usually have a higher proportion of branches than *S. tetraptera*, but does have a higher proportion of active nodes than *S. tetraptera* due to the presence of short shoots. The logistic regression has shown that a node on a decapitated *S. prostrata* is significantly more likely to become active than a node on an intact *S. prostrata*, or any *S. tetraptera* (Table 3.3). Short shoots and established branches both contributed significantly to the activation of nodes in decapitated *S. prostrata*.

Short shoots have been shown here to be an important contributor to normal development in *S. prostrata*, as well as in the response to decapitation. Long shoots are exploratory and extend out to occupy new space, while short shoots are able to exploit space that has already been colonised (Bell and Bryan 2008). Short shoot development is evident in many divaricate species (Tomlinson 1978), and they allow the plant to increase productivity at little structural cost. Tomlinson (1978) noted that short shoots could be important in compensating for divaricates small leaves.

No significant patterns along the branches were observed in the axillary buds of *S. prostrata* (Figures 3.10 – 3.13, Table 3.4), which is not surprising considering the variability observed in divaricate branching. Carswell and Gould (1998) found that *S. prostrata* was more likely to branch at proximal nodes, but axis development was highly variable between individuals so typical sequence of events could not be established. The lack of clear pattern meant that comparison with the node state data was not possible. The reversibility of dormancy - that the axillary buds can be activated and enlarged, then returned to dormancy - make it difficult to establish which developmental stage each node is at. The axillary buds in the present study appear to reflect the variability of shoot branching in the length of their axillary buds. Due to this variability, the present data is unable to identify different developmental stages that could be used to target sampling to specific gene expression. Further research involving more nodes along more branches could provide a basis for sampling; however preliminary work could be done with pooled axillary bud samples.

Axillary meristems are protected within the bud by the unexpanded leaves, and often trichomes. The axillary meristems of *S. prostrata* are protected by unexpanded leaves and a dense covering of hairs, which are a form of trichomes. Trichomes are outgrowths from the epidermis of a plant, and have a variety of functions depending on their type and location. The dense pubescence found on the buds of *S. prostrata* (Seen in Figures 3.9 – 3.11) is likely to protect the vulnerable bud from extreme temperatures, desiccation, solar radiation and/or insects (Hallahan et al. 2000). *S. prostrata* also had accessory meristems in their axils (Figures 3.10 – 3.13), which is not uncommon (Bell and Bryan 2008). These buds remain undeveloped, but can be activated if the primary bud is damaged. Accessory buds are often associated with harsh environmental conditions, but can also be released from dormancy by herbivory (Bell and Bryan 2008). Some species such as *Pisum sativum* (pea) can have multiple accessory meristems in their axils (Bennett and Leyser 2006).

The analyses reported here are preliminary, and further analysis of this dataset is currently underway. An example of which is a Bayesian analysis (for example, see (King et al. 2006)) using a matrix-based population model for estimating rates of transition between the different node states. Rates of “survival” (remaining as a particular state) and “ageing” (transitioning from one state to another) are estimated using survival analysis which accounts for the heterogeneous lengths between observations, and the censoring of the times of change.

Carswell and Gould (1998) found that *S. prostrata* branched more frequently from the proximal node, while *S. tetraptera* branched from the distil node. This result could be validated from the present dataset. It could also be interesting to establish on what order and when new growth occurred, as Lovell et al. (1991) found that the majority of a seasons growth occurred on shoots initiated in that season in *Muehlenbeckia astonii*. The significance of short shoot outgrowth into long shoots via internode extension could also be assessed, as this type of outgrowth could be useful in response to damage and loss of biomass, as such branches could explore new space while already having expanded leaves. Carswell and Gould (1998) also found that divaricate branch initiation occurred in two discrete growth periods, while non-divaricate branch initiation occurred in one growth period. It would be interesting to assess this in conjunction with decapitation over numerous growing seasons.

Decapitation is widely used to investigate shoot branching, however the regulation of normal shoot branching, and regulation of the response induced by decapitation may be very different (Dun et al. 2006). Decapitation often removes a large portion of biomass, and causes stress on the plant. The responses observed here could be due damage to the primary apex (apical control) or biomass removal, or the specific combination of both. Another issue with the present study is that the intact top third of the non-decapitated individuals was not measured. Lower growth rates in the intact individuals could be due to the majority of their growth occurring on the top third, and therefore not included in the analysis.

The microscopic observation of axillary buds along a branch was conducted using intact individuals. It could be interesting to sample from decapitated individuals as well. As there was a lot of variation between the individuals in axillary bud size, a larger sample size of either more branches or more nodes on a branch could allow us to distinguish a branching pattern. If we are able to identify different developmental stages internally, having the external and internal images of the buds coupled would allow further study into finding external features characteristic of the internal developmental stage.

Sophora microphylla is a heteroblastic species with a divaricating juvenile and an arborescent adult. Including juvenile and adult *S. microphylla* species in a comparison with *S. prostrata* and *S. tetraptera* would encompass the spectrum of growth forms within *Sophora*, and also allow comparison with a divaricate and non-divaricate form within one species.

The present study highlights the complexity of compensatory growth, apical dominance and apical control in woody species. The possibility of transitional branches, a large compensatory growth response and a higher probability of nodes becoming active could demonstrate weak apical control in *S. prostrata*. The lack of an observable pattern in axillary bud features also highlights the developmental variation observed between individuals. This study also demonstrates the significance of short shoots in the structural composition of *S. prostrata*. Studies such as this provide the basis for future work on the evolution, development, and genetics of *S. prostrata* and other divaricates.

Understanding the environmental factors, genes, and hormonal mechanisms that control axillary bud outgrowth and dormancy are essential to be able to manipulate plant forms for use in agriculture, and continued research involving divaricate forms can contribute to our understanding of branching regulation. Divaricate branching could be investigated further by studying the genes involved in branching control, and the expression of these genes throughout the plant at different stages in development. Studies such as this help in gaining a fundamental understanding of the patterns of branching and architecture in divaricate forms. This is necessary to then begin to investigate whether all divaricates utilise the same genes to achieve the divaricate form, and compare the expression of genes implicated in branching regulatory pathways. Divaricates provide a unique opportunity to study the development and genetics of branching, as they provide range of similar forms that have evolved in response to the same selection pressures in slightly different ways. Divaricate research not only addresses interesting ecological questions surrounding convergent evolution, but contributes to the wider understanding of branching and development.

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Chapter Four: Assessing broad scale genetic variation in *Sophora prostrata* (Fabaceae) using ISSR markers

4.1 Abstract

Sophora prostrata Buchan. (Fabaceae) is a divaricate shrub endemic to the eastern side of the South Island of New Zealand. Using morphological and molecular data, previous studies have found *S. prostrata* to be relatively distinct to other New Zealand *Sophora* L. species. The present study seeks to use Inter Simple Sequence Repeat (ISSR) markers to support the distinctness of *S. prostrata* using a larger sample size across a wider geographic range, and assess the genetic variation between different localities across its entire range. ISSRs are highly variable markers that can be useful at the species and population level, where other variable markers are insufficient. This study employs nine ISSR primers which yielded 69 polymorphic bands across three species – *S. prostrata* (18 individuals), *S. tetraptera* (5 individuals) and *S. microphylla* (6 individuals). The present primers and samples were unable to support the distinctness of *S. prostrata*, or identify distinct relationships between the localities. The high number of fragments shared among species is evidence of the close relationship between New Zealand *Sophora*, which has been previously demonstrated. Larger samples sizes and more variable markers may be needed to resolve the relationships of *S. prostrata* at the species and population level.

Keywords: *Sophora prostrata*; *Sophora*; kōwhai; divaricate; Inter-simple sequence repeat; ISSR

4.2 Introduction

4.2.1 Introduction to *Sophora*

The genus *Sophora* contains four sections (*Disamaea*, *Edwardsia*, *Pseudosophora* and *Sophora*), with New Zealand *Sophora* belonging to sect. *Edwardsia.* Section *Edwardsia* consists of 19 species distributed throughout the southern hemisphere (Mitchell and Heenan 2002). The biogeography of this section has been the subject of considerable interest and research, and several hypotheses have been put forward to explain its origin and dispersal. Pena and Cassels (1996) and Pena et al. (2000) hypothesised a South American origin from a North American ancestor, and subsequent dispersal west across the pacific. Pena (2000) tested these hypotheses using chemical, morphological and palynological characters, and their results supported the boreotropic hypothesis. The alternative hypothesis is that sect.

Edwardsia originated in the north or north-west Pacific from a Eurasian ancestor, and was distributed eastward (Hurr et al. 1999, Mitchell and Heenan 2002). Hurr et al. (1999) used the chloroplast DNA region *atp*B-*rbc*L to investigate the origin of sect. *Edwardsia*, and their results supported long distance dispersal from a northwest Pacific origin. An analysis of the nuclear DNA ITS region by (Mitchell and Heenan 2002) also supported a north-west Pacific origin from a Eurasian ancestor in the last 2- 5 million years. It is not known exactly when *Sophora* arrived in New Zealand due to limited fossil evidence, but it does suggest they were not common until the Pleistocene (Polhill 1981, Hurr et al. 1999). The seeds of many *Sophora* species are known to be buoyant and to tolerate saline conditions, so long distance dispersal on ocean currents is the most likely explanation for their present biogeographical distribution (Sykes and Godley 1968, Hurr et al. 1999).

The taxonomy of New Zealand *Sophora* has had a problematic history (Heenan et al. 2001). Early treatments of *Sophora* include *S. microphylla* Aiton. as a variety of *S. tetraptera* J. S. Mill. (Hooker 1853, Cheeseman 1906), and Cheeseman (1925) accepted *S. microphylla*, *S. tetraptera* and *S. prostrata*. Cockayne (1902) distinguished *S. chathamica* Cockayne. from other New Zealand *Sophora* species based on its lack of juvenile stage morphological characteristics. Briggs and Russell (1942) investigated the alkaloids of the seeds of *S. chathamica* and found that they were not chemically dissimilar enough to separate *S. chathamica* from *S. microphylla.* Briggs and Mangan (1948) investigated the seed alkaloids from *S. fulvida* (Allan) Heenan & de Lange, supporting its recognition at the species level. Allan (1961) recognised three species, with an additional two varieties of *S. microphylla* – var. *longicarinata* and var. *fulvida* (Table 4.1). Yakovlev (1967) considered only two species, *S. microphylla* and *S. tetraptera* – with five varieties within *S. microphylla* including *S. microphylla* subsp. *microphylla* var. *prostrata*. Tsoong and Ma (1981) conducted a genus wide revision based on legume structure and other morphological characters, and accepted *S. microphylla, S. tetraptera, S. prostrata* and *S. chathamica*. Heenan (1998) reinstated *S. longicarinata* G.Simpson & J.S.Thomson. based on characters such as its lack of a juvenile growth phase, shrubby slender growth habit, and small dark green leaflets.

Hurr et al. (1999) analysed the intergene region *atp*B - *rbc*L, and found very high levels of sequence similarity between members of *Sophora* sect. *Edwardsia*, and was unable to resolve any taxonomic relationships. The most recent full taxonomic treatment using leaf and growth habit characteristics by Heenan et al. (2001) recognised the current eight species – *S. tetraptera, S. microphylla, S. prostrata, S. molloyi* Heenan & de Lange., *S. longicarinata, S. godleyi* Heenan & de Lange., *S. fulvida* and *S. chathamica* (Table 4.1). Maich (2002) assessed the new species recognised by Heenan et al. (2001) using enzyme electrophoresis and concluded that allozyme data did not support their promotion to species level, and that *S. microphylla* sensu lato was still undergoing adaptive radiation. Mitchell and Heenan (2002) attempted to resolve relationships between New Zealand *Sophora* using nrDNA ITS region, and found no significant sequence differences in *Sophora* sect. *Edwardsia*. Song (2005) also investigated the genetic diversity within New Zealand *Sophora* using ISSR markers, and while considerable genetic differentiation between species was observed, many relationships were unable to be resolved.

Table 4.1: Comparison recent of taxonomic treatments of New Zealand *Sophora*.

Allan (1961)	Yakovlev (1967)	Tsoong and Ma (1981)	Heenan et al. (2001)
S. prostrata	*S. microphylla* subsp. *microphylla* var. *prostrata*	*S. prostrata*	*S. prostrata*
S. tetraptera	*S. tetraptera*	*S. tetraptera*	*S. tetraptera*
S. microphylla var. *microphylla*	*S. microphylla* subsp. *microphylla* var. *microphylla*	*S. microphylla*	*S microphylla*
Synonym of *S. microphylla*	*S. microphylla* subsp. *microphylla* var. *chathamica*	*S. chathamica*	*S. chathamica*
S. *microphylla* var. *fulvida*	*S. microphylla* subsp. *microphylla* var. *fulvida*	-	*S. fulvida*
S. microphylla var. *longicarinata*	*S. microphylla* subsp. *microphylla* var. *longicarinata*	-	*S. longicarinata*
-	-	-	*S. godleyi*
-	-	-	*S. molloyi*

Sophora sect. *Edwardsia* has been shown to be monophyletic using molecular data (Mitchell and Heenan 2002), but the relationships between species have been difficult to establish (Markham and Godley 1972, Hurr et al. 1999, Pena et al. 2000, Mitchell and Heenan 2002). Research has been undertaken to understand *Sophora* relationships using biochemical (Markham and Godley 1972, Pena and Cassels 1996), morphological (Pena and Cassels 1996, Heenan et al. 2001, Heenan et al. 2004), and molecular data (Hurr et al. 1999, Mitchell and Heenan 2002, Heenan et al. 2004), but no phylogenetic hypothesis has been well supported. The difficulties in clarifying relationships with the species suggest recent and rapid speciation and dispersal (Hurr et al. 1999, Mitchell and Heenan 2002). Using chemical, morphological and palynological data, Pena et al. (2000) found *S. prostrata* to be near the base of the *Edwardsia* section. *Sophora prostrata* was the most distant group in a cluster analysis of leaf and growth characters (Heenan et al. 2001), and an analysis using ISSR markers in by Song (2005) found that using ISSR markers *S. prostrata* was the most genetically distant of the New Zealand *Sophora.*

Sophora prostrata is a prostrate to bushy shrub with small pinnately compound leaves and stiff, zigzag branches that are densely interlacing. *Sophora prostrata* is confined to the Eastern side of the South Island of New Zealand, between Blenheim and the Waitaki River (Godley 2006). It can be found lowland to montane shrubland, river flats, grassland and rocky places (Allan 1961, Wilson and Galloway 1993, Godley 2006) from 76 – 760m above sea level (Metcalf 2000). *Sophora prostrata* has probably evolved in New Zealand, as its seeds are not buoyant (Hurr et al. 1999). There have been two theories regarding the relationships between *S. prostrata* and the other species. Cockayne (1912, 1928) suggested that *S. prostrata* is derived from the juvenile form of *S. microphylla*, and Godley (1979, 1985) suggested that *S. microphylla* is derived from a hybridisation between *S. prostrata* and *S. tetraptera*. Pena and Cassels (1996) found that using morphological and chemical characters, *S. prostrata* was closer to *S. tetraptera* than *S. microphylla*. Chloroplast DNA analyses by Hurr et al. (1999) favours Cockaynes hypothesis, as *S. prostrata* and *S. microphylla* consistently group together. More molecular data is needed to resolve the relationships between *S. prostrata* and the rest of the New Zealand *Sophora* species (Heenan et al. 2001, Song 2005). The phylogenetic history of *S. prostrata* could influence the theories behind divaricate evolution, as relationships and divergence times could be tied to different theories (Hurr 1996).

4.2.2 Inter Simple Sequence Repeats (ISSRs)

Inter simple sequence repeat (ISSR) markers involve amplification via the polymerase chain reaction (PCR) of the regions between microsatellites (short simple repeats). Microsatellites are repeated sequences of 2-6 base pairs, and are present in coding and non-coding regions throughout eukaryotic genomes (Kalia et al. 2011). A single primer with the microsatellite sequence and two to four anchoring nucleotides amplifies the DNA between two inverted microsatellites of the same type. As these microsatellites can occur numerous times in the genome, multiple amplified fragments can be generated from one primer. This results in a characteristic pattern of PCR products – a genetic fingerprint (Zietkiewicz et al. 1994). Any absence or mutation in the microsatellites, or changes that alter the distance between the two microsatellites, will result in absence or variation in the length of amplified fragments, and therefore polymorphisms between individuals (Zietkiewicz et al. 1994, Kalia et al. 2011).

Other arbitrarily amplified DNA PCR based markers include random amplification of polymorphic DNAs (RAPDs) and amplified fragment length polymorphisms (AFLPs). RAPD uses short oligonucleotide primers of arbitrary sequence to reproducibly amplify segments of DNA (Williams et al. 1990). The AFLP technique involves selectively amplifying a subset of restriction fragments from digested total genomic DNA by ligation of short oligonucleotides (adapters) to the ends of the digested fragments, and then PCR amplification using primers complementary to the added adapter oligonucleotides (Vos et al. 1995). Both RAPDs and AFLPs have unlimited number of possible loci, but AFLPs are more labour intensive and costly to produce (Nageswara-Rao and Soneji 2008). RAPDs, AFLPs, and ISSRs are dominant markers (Kalia et al. 2011), which means they cannot distinguish homozygotes from heterozygotes. All three of these techniques are relatively easy and cost effective to produce, and do not require sequence data (Kalia et al. 2011). As a result of these factors, these techniques are particularly useful for species where little to no genetic research has been done previously (Nybom 2004).

ISSRs have several advantages over the other arbitrary PCR based marker systems. ISSRs are generally more reliable and reproducible than RAPDs because of the longer primers and higher annealing temperature (Zietkiewicz et al. 1994, Singh et al. 2011). Nagaoka and Ogihara (1997) found that in wheat, ISSR primers produced several times more information than RAPDs. Phong et al. (2011) compared RAPD and ISSR markers in their ability to assess the genetic diversity of an endangered species in Vietnam, *Dalbergia oliveri*, and found that the ISSR markers were more efficient. ISSRs tend to produce higher estimates of within population variation than AFLP and RAPD (Nybom 2004). ISSRs are less labour intensive and technically demanding than AFLPs, and AFLPs have higher operational and developmental costs (Bornet and Branchard 2001).

These arbitrary PCR-based markers can be used across various taxonomic levels, and have been described as the interface between phylogenetics and population biology (Bussell et al. 2005) as they allow assessment of inter and intra specific genetic diversity of natural plant populations, and are useful for population genetics and phylogenetic studies. They can be useful in recently radiated species, such as insular lineages, where the usual fast evolving regions such as the internal transcribed spacer regions of nuclear ribosomal DNA (ITS) or the noncoding regions of chloroplast DNA (cpDNA) are insufficiently variable to resolve relationships at the species level (Bussell et al. 2005, Archibald et al. 2006b).

4.3 Research aims and objectives

Sophora prostrata has been shown to be relatively distinct from other New Zealand *Sophora* using morphological and molecular data; however its relationships to other species are unresolved. ISSRs have been shown to provide higher levels of variation than other commonly used variable regions such as ITS. Assessing the genetic variation of *S. prostrata* samples from across their geographic range in reference to *S. microphylla* and *S. tetraptera* will potentially be able to:

1. Support the distinctness of *S. prostrata* in reference to other New Zealand *Sophora*, utilising a larger sample size and a wider geographic range than has been used previously.
2. Assess the genetic variation between localities of *S. prostrata*.

4.4 Methodology

4.4.1 Sample collection and DNA extraction

The DNA samples used were collected and extracted by Lara Shepherd. For each individual, a herbarium specimen (not yet lodged in herbarium) and young leaf tissue for DNA were collected. The DNA sample was stored in silica gel until it was extracted. DNA was extracted from the leaf samples using a modified CTAB method from Doyle and Doyle (1990). Eighteen *S. prostrata*, five *S. tetraptera* and six *S. microphylla* samples were used in this study. A representative leaf of each of the species is shown in Figure 4.1. *Sophora prostrata* is found only in the eastern South Island, *S. tetraptera* is found only in the eastern North Island, and *S. microphylla* is found throughout both islands. Figure 4.2 and Table 4.2 show the samples and their localities.

Figure 4.1: Examples of leaves of the three species used in this study. The leaves on the left are *S. tetraptera*, the upper right leaves are *S. microphylla*, and the lower right leaves are *S. prostrata*. All of these leaves are taken from individuals grown in the University of Waikato greenhouse.

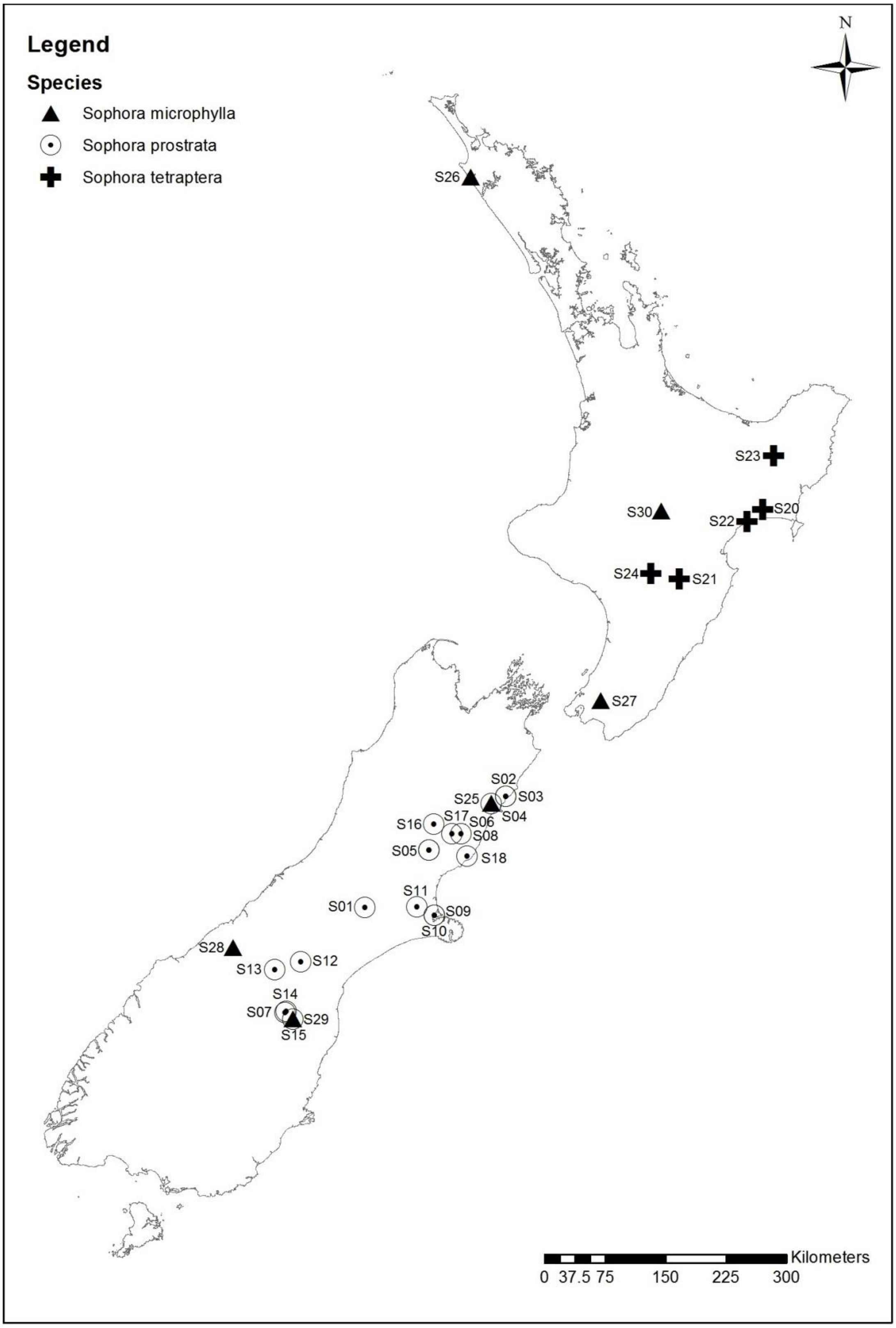

Figure 4.2: Map of New Zealand showing approximate collection locations of *Sophora* samples used in the analyses (See Table 4.2 for details).

Table 4.2: Samples of *S. prostrata*, *S. tetraptera* and *S. microphylla* included in the analyses with their species name and collection location.

Species	Sample	Collection location
Sophora prostrata	S01	Otari ex. Rakaia Gorge
	S02	Blue Duck Scientific Reserve, Marlborough
	S03	Blue Duck Scientific Reserve, Marlborough
	S04	Kōwhai River Valley, Kaikoura
	S05	Waitohi River
	S06	Waiau River
	S07	Deep Stream, Waitaki River Valley
	S08	Waiau River
	S09	The Tors Scenic Reserve, Port Hills
	S10	The Tors Scenic Reserve, Port Hills
	S11	Old West Coast Rd between Christchurch and Darfield
	S12	Burke's Pass near Lake Tekapo
	S13	Lake Pukaki
	S14	Lake Aviemore
	S15	Kurow Hill
	S16	Valley above Waiau River
	S17	Near Waiau township
	S18	Blythe Valley
Sophora tetraptera	S20	Frasertown HB
	S21	Wakarara
	S22	Mohaka
	S23	Gisbourne/Matawai
	S24	Utiku
Sophora microphylla	S25	KōwhaiRiver Valley, Kaikoura
	S26	Whangape
	S27	Rimutaka
	S28	Haast
	S29	Kurow hill
	S30	Waimarino River, Turangi

4.4.2 ISSR amplification

Nine primers used by Song (2005) on *Sophora* and *Clianthus* species were used for initial screening on five samples. The primers trialled were numbers 811, 818, 822, 828, 841, 844, 864, 866 and 868 from UBC primer set no. 9, Biotechnology Laboratory, University of British Columbia. Of those, eight (811, 818, 822, 828, 841, 844, 864 and 866) were used for further analysis (Table 4.3).

Table 4.3: The eight ISSR primers used for the analysis of the 30 *Sophora* species.

Primer	Primer sequence	No. of bands	No. of polymorphic bands
811	$(GA)_8C$	3	2
818	$(CA)_8G$	8	8
822	$(TC)_8A$	11	11
828	$(TG)_8A$	11	10
841	$(GA)_8YC$	13	12
844	$(CT)_8RC$	10	9
864	$(ATG)_6$	11	11
866	$(CTC)_6$	8	7
Total		75	70

PCR was performed in 20µl reactions containing 13.1 µl MQH_20, 0.25µM of each primer, 1.125X MyTaq^{TM} Reaction buffer (Bioline), 0.1 % bovine serum albumin (BSA), 0.05U MyTaq^{TM} Polymerase (Bioline), and 1.0 µl of unquantified DNA (diluted 1:10). A negative control containing no DNA was included in each PCR run to check for contamination. All reactions were run on an Eppendorf mastercycler® pro thermocycler using a program of 5 min at 94°C (initial denaturation); 40 cycles of a 45 s at 94°C(denaturation), 45 s at 50°C (annealing) and 90 s at 72°C (extension), followed by 5min at 72°C (final extension).

4.4.3 ISSR electrophoresis

Separation of PCR products was done on 2% agarose 0.5x TBE gels containing 0.005% volume of RedSafe^{TM} (iNtRON Biotechnology). PCR products were mixed with 5 µl loading buffer, and 10 µl was loaded into each well. A 100bp ladder (Invitrogen^{TM}) was loaded into the first and last lanes to use as a reference for size of bands. Electrophoresis was conducted for 3 to 4 hours at 69 V, and then the gels were viewed under UV light and photographed using an Alphaimager (Alpha Innotech).

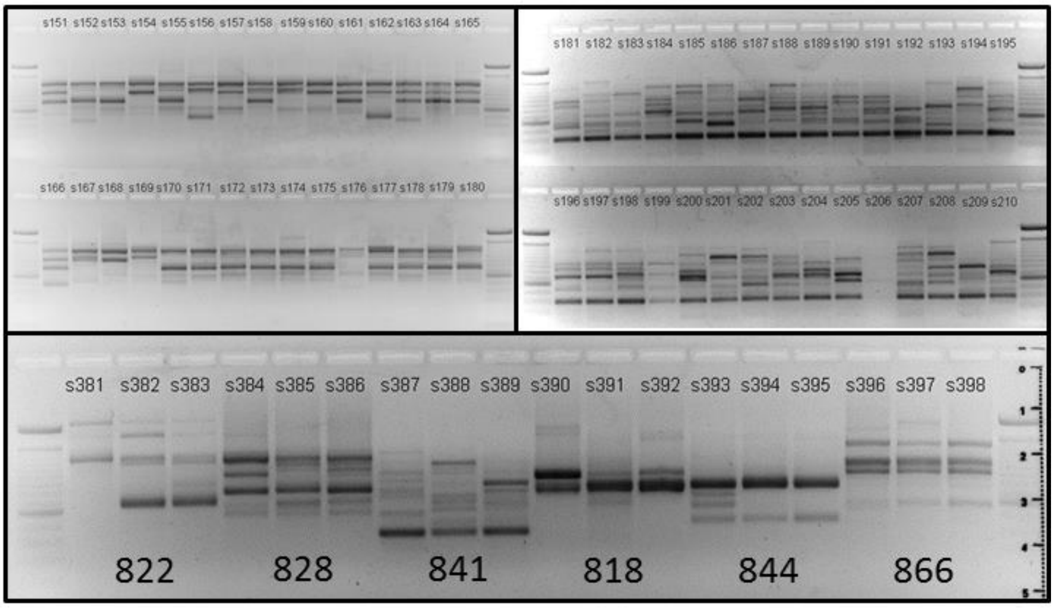

Figure 4.3: Agarose gels showing ISSR products. Top left is primer 828, top right is primer 841. Lane 1, 17, 18 and 34 are the 100kb ladder, lane 2 – 16 and 19 – 22 are *S. prostrata*, lanes 23 – 27 are *S. tetraptera*, and lanes 28 - 33 are *S. microphylla*. The bottom image is a reproducibility test using one sample each of *S. prostrata* (S15), *S. tetraptera* (S22), and *S. microphylla* (S29) respectively, with primers 822, 828, 841, 818, 844 and 866.

4.4.4 ISSR analysis

Each sample resulted in a multi-banded fingerprint (Figure 4.3). Each band was compared to a 100kb ladder to determine its size. Bands of a similar size were then compared between samples and scored as present (1) or absent (0) and a binary data matrix was created. Bands that were of doubtful reproducibility, or were very faint compared to others, were scored as absent. Differences in band intensity were not otherwise considered. Gels were rescored at least twice, and a subset of samples were reamplified and run on a gel next to the original products to ensure reproducibility. The remainder the original product for primers of 841 and 864 was also run on a second gel to assess reproducibility.

The ISSR data matrix was used to generate a distance matrix using Nei-Li genetic distances (Nei and Li 1979) (Appendix Four), which was then used to produce Neighbour Joining (NJ) dendrograms in PAUP* 4.0a129 (Swofford 2002). NJ dendrograms were produced with all species and with *S. prostrata* samples only, and tree topology was assessed by bootstrap analysis (1000 replicates). An AMOVA was not conducted due to the small sample sizes per location.

4.5 Results

Between three and 13 scorable bands were generated for each of the eight primers, resulting in 75 fragments with 69 polymorphic fragments (93.3%) (Table 4.3), which were shown to be reporiducible. Of the 75 fragments, 41 were present in at least one member of all three species (55%), 12 were unique to one species, and four were unique to one individual. The pairwise genetic distance among all samples ranged from 0.00867 (S22 and S25) to 0.04846 (S19 and S03). Within *S. prostrata*, genetic distances range from 0.01166 (S06 and S10) to 0.04846 (S19 and S03) (Appendix Four). Figure 4.4 shows the NJ tree with all species included. No bootstrap support (1000 replicates) was found for any branches. Figure 4.5 shows the NJ tree with *S. prostrata* samples only. Bootstrap values greater than 50% are shown over the branches that received support. The three supported relationships were S08 and S09, S02 and S03, and S05 sister to S02 and 203.

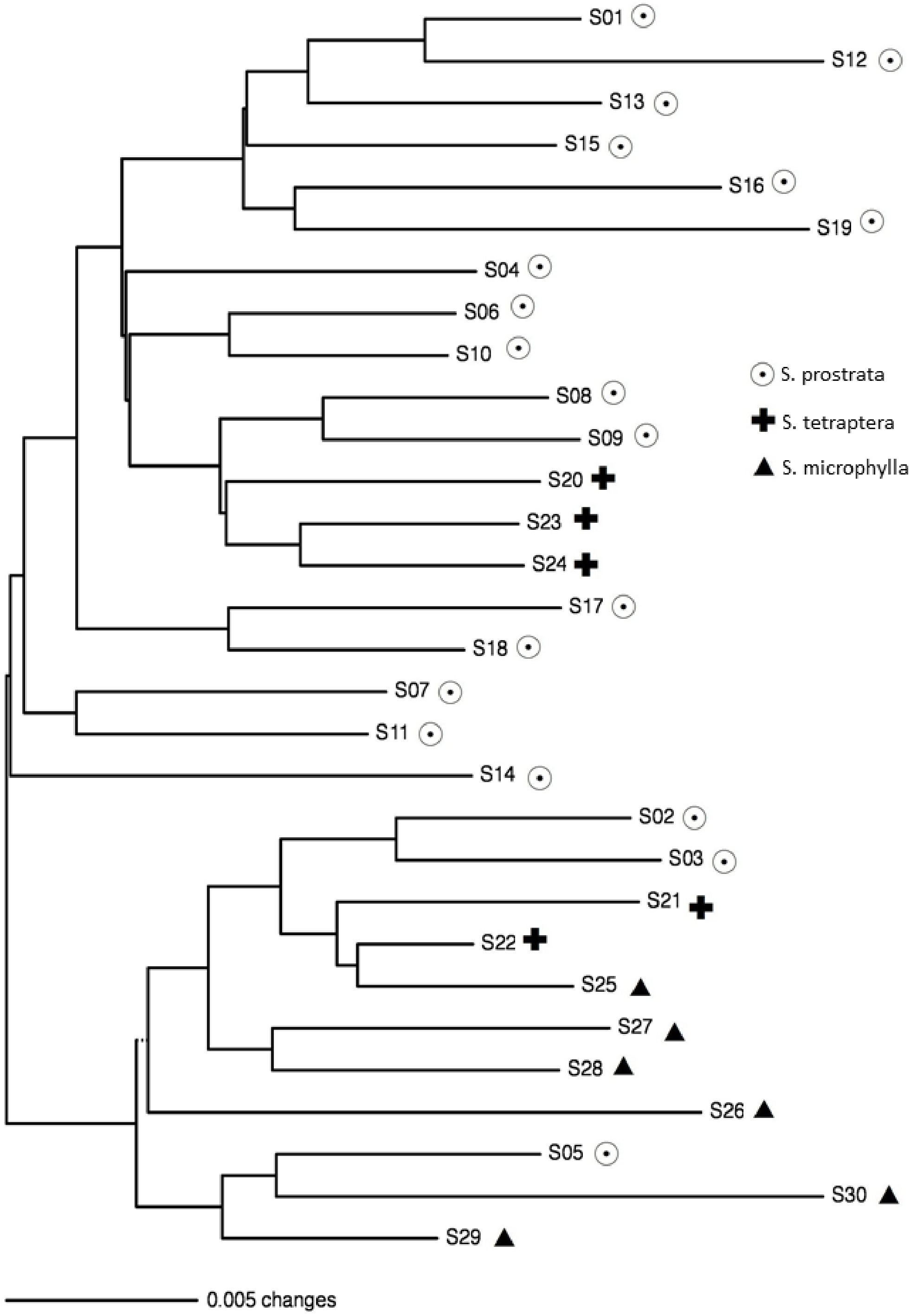

Figure 4.4: NJ dendrogram using Nei's genetic distance between 30 New Zealand *Sophora* samples from three species, based on 75 loci from 8 ISSR primers. *S. prostrata* samples are indicated by the circle, *S. microphylla* by the triangle, and *S. tetraptera* by the cross.

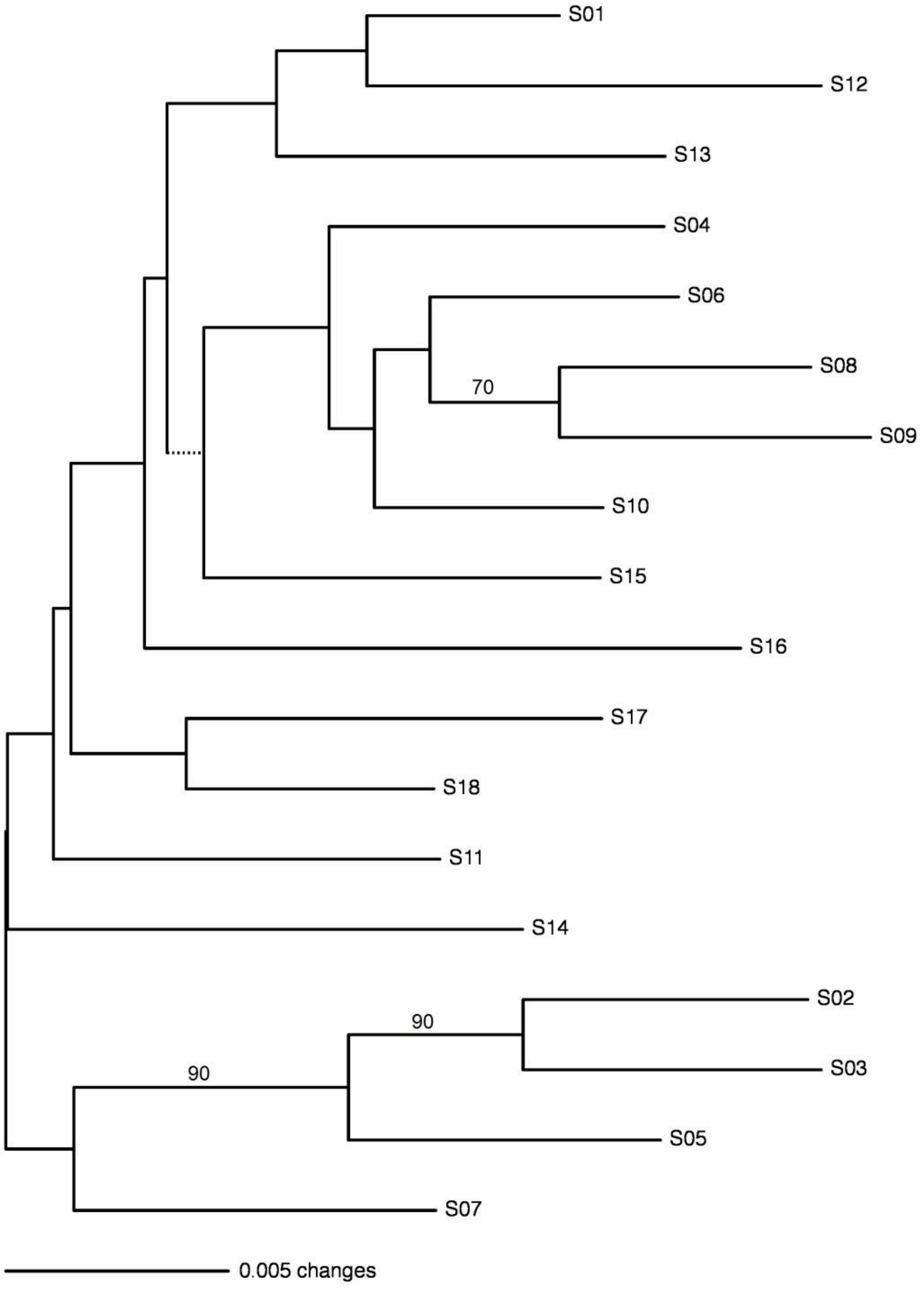

Figure 4.5: NJ dendrogram using Neis genetic distance between 18 individuals of *S. prostrata* based on 75 loci from 8 ISSR primers. Numbers above branches show bootstrap values from 1000 replicates.

4.6 Discussion

Despite the relative morphological distinctness of *S. prostrata* shown in previous studies (Hurr et al. 1999, Mitchell and Heenan 2002) and the hyper-variability of ISSR markers, the present primers and methods were unable to resolve relationships within the New Zealand *Sophora*. The number of fragments (75) (Table 4.3) is within the range of other published studies using ISSRs (Ramp Neale et al. 2008, Nguyen et al. 2013), but many fragments (45) were shared across one or more individuals of each species. There were 12 species specific fragments, but their utility in discriminating between species is limited, as none were found in all individuals of that species. The high number of fragments shared among species is evidence of the close relationship between New Zealand *Sophora*. High genetic similarity has been previously shown for *Sophora* using relatively variable regions (Hurr et al. 1999, Mitchell and Heenan 2002). Variation that is shared across taxa could be a result of hybridisation. Hybridisation between *Sophora* species has been widely documented (Heenan et al. 2001, Godley 2006, Godley et al. in press), and it is suggested that interspecific gene exchange has been common within New Zealand Sophora (Heenan et al. 2001).

ISSRs were used on New Zealand *Sophora* previously by Song (2005) who assessed genetic differences among nine species from *Sophora* sect *Edwardsia*. Song (2005) only included four *S. prostrata* samples, two of which were used in the present study (S05 and S06). The ISSR dataset had relatively high genetic distances (0.05 – 0.49) between species, but was unable to resolve relationships as many species did not cluster together. The four samples of *S. prostrata* formed a distinct cluster, with genetic distances from the other species from 0.33 to 0.83. The samples used in Song (2005) were from the Waitohi river and Waiau river, which are found approximately 30 km apart in Canterbury. The present study found that using the majority of the same ISSR primers (8 out of the 9 primers) with a larger sample size across a wider geographic range, the monophyly of *S. prostrata* is not maintained (Figure 4.4). This could be due to hybridisation and gene flow within New Zealand *Sophora* species, or an insufficient sample size.

ISSRs and other hypervariable markers can be used for species level phylogenetic investigation when the usual variable regions (such as rbcL and ITS) are insufficient to resolve relationships. ISSRs have been successful in other recently specitated lineages, such as *Toplis* (Archibald et al. 2006a) and *Micromeria* (Meimberg et al. 2006) from the Canary Islands. ISSRs have provided strong support for taxonomic relationships within *Jurinea* species native to Turkey, as well as supporting their biogeographic distribution patterns (Dogan et al. 2007). They have had less success in some lineages, such as *Clermontia* in Hawaii (Givnish et al. 2013), and *Amarathus* in the Eastern United states (Nolan et al. 2010). The lack of genetic distinctness within *S. prostrata* and the other New Zealand *Sophora* found with such a hypervariable marker suggest a recent and rapid speciation.

Considerable morphological variation coupled with the lack of genetic distinctness in the present study and others (Hurr et al. 1999, Mitchell and Heenan 2002), supports the idea that morphological and molecular evolution are not always coupled. This decoupling of molecular and morphological evolution occurs quite frequently in oceanic island lineages, as they have often recently and rapidly radiated into a variety of ecological niches within a relatively small area (Baldwin et al. 1998, Archibald et al. 2006a). Ganders et al. (2000) investigated the molecular phylogeny of *Bidens* using nrDNA regions of ITS and 5.8S, and found that the Hawaiian taxa had identical sequences, despite displaying more morphological diversity than any other members of the genus. Gemmill et al. (2002) investigated the insular and morphologically diverse Hawaiian *Pittosporum* using the ITS region, and also found no genetic difference between the Hawaiian species.

The markers used to investigate relationships and the methods used to separate and score the resulting bands have an effect on the number of fragments that can be visualised, and the information they can provide. ISSRs are dominant markers, meaning that there is no distinction between homozygotes and heterozygotes. Co-dominant markers can be more informative than dominant markers, as they allow heterozygotes to be distinguished from homozygotes (Nageswara-Rao and Soneji 2008). Primers can be combined to produce more fragments per amplification, due to amplification of regions flanked by the same primer as well as those flanked by the two different primers (Liu and Wendel 2001). This can lead to some bands disappearing due to competition in PCR (Bussell et al. 2005), but still yields more fragments and is reproducible (Liu and Wendel 2001).The type of gel used can have significant effects on the number of fragments that can be resolved (Liu and Wendel 2001, Bussell et al. 2005). Godwin et al. (1997) showed that the same primer amplification on banana DNA resulted in 26 bands on an agarose gel, 32 on a polyacrylamide gel, and 77 on a radiolabelled sequencing gel. The previous study that used ISSRs on New Zealand *Sophora* used polyacrylamide gels, which could explain some of the differences observed between the present study and that of Song (2005).

The markers and methods used can increase resolution, especially in combination with an increased sample size. A preliminary study by Mort et al. (2003) on Canary islands *Toplis* species used five primers across 80 individuals, which resulted in 48 loci. A follow up study by Archibald et al. (2006a) used six primers across 264 individuals which yielded 1628 loci. The initial study visualised the ISSR fragments on agarose gels, while the follow up study used polyacrylamide gels. These two studies demonstrate that the increase in sample size coupled with the use of polyacrylamide gels can allow even greater resolution in *Toplis*, at the species and population level.

There are three relationships within the *S. prostrata* individuals that were supported (Figure 4.5). The samples S02 and S03 are both from the Blue Duck Scientific reserve in Marlborough, so are potentially from closely related populations. Sample S05 is shown to be sister to S02 and S03 clade. All three samples are found in the upper half of *S. prostrata* distribution, approximately 150 km apart. The other supported relationship is that between S08 from the Waiau River and S09 from The Tors Scenic reserve in the Port Hills, which are approximately 100 km apart. Sister to this group is sample S06 is also from the Waiau River, but the relationship is not supported. The samples used in this study were chosen to encompass a wide geographical range, and did not include many samples from the same locality. As shown by samples 02 and 03 from the Blue Duck Scientific Reserve in Marlborough, higher levels of genetic similarity can be observed between samples within the same locality. Larger sample sizes per locality in combination with more primers and gels that allow for more fragment resolution could allow further investigation into the population genetics of *S. prostrata.*

Microsatellite (simple sequence repeat) markers have recently been developed for the New Zealand members of the genus *Sophora* (Etten et al. 2014). Microsatellites are highly variable markers that can be useful for phylogenetic and population level analyses. When the developed markers were tested on *S. microphylla* and *S. chathamica*, species specific alleles occurred at seven loci. They concluded that the 12 most polymorphic loci could probably be applicable to other closely related species, which makes these markers the potential next step in assessing the genetic variation of *S. prostrata* at the population level, as well as distinguishing it from closely related species.

Despite the distinctness of *S. prostrata* found in previous studies and the hyper-variability of ISSR markers, the present study was unable to support the distinctness of *S. prostrata* or clarify the relationships to other New Zealand *Sophora*. There was very little clear structure in the genetic variation within these samples of *S. prostrata* using the present primers and methods. Future studies could benefit from an increased sample size, as well as higher resolution gels and more primers, or utilising the recently developed *Sophora* microsatellite markers. Understanding the position of

S. prostrata in the phylogeny of New Zealand *Sophora* could shed light on the course of *Sophora* evolution, and also on the evolution of the divaricate form.

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Chapter Five: Biological flora of New Zealand: *Sophora prostrata,* prostrate kōwhai, South Island kōwhai

5.1 Abstract

Information relevant to the morphology, chemistry, systematics, distribution, ecology, usage and conservation of *Sophora prostrata* Buchanan (Fabaceae) from published and unpublished sources is assembled and reviewed here. *Sophora prostrata* is a small-leaved woody shrub with densely interlacing branches, and is one of about 60 species of divaricating shrubs in New Zealand. *Sophora prostrata* and the many other divaricate forms are a product of convergent evolution, and are therefore a valuable tool with which to study evolution and branching development There are eight species of New Zealand kōwhai that belong to *Sophora* sect. *Edwardsia*, which is distributed around the Southern Hemisphere. *Sophora prostrata* is endemic to the Eastern side of the South Island of New Zealand, and is found in lowland to montane shrubland, river flats, grassland and rocky places. Many of the habitats where *S. prostrata* can be found are in decline due to extensive land use changes. *Sophora prostrata* populations are declining in some areas due browse damage from domestic animals, and there is a lack of seedling recruitment due to grazing and competition from grasses. *Sophora prostrata* populations will continue to decline without artificial replacement and other conservation measures.. Increased interest and conservation effort involving *S. prostrata* and other divaricates can ensure that these valuable and iconic plants remain a unique and significant part of New Zealand’s native flora.

Keywords: *Sophora prostrata*; Fabaceae; biological flora; prostrate kōwhai; divaricate; morphology; chemistry; taxonomy; distribution; conservation

5.2 Morphology

Sophora prostrata Buchan. is a prostrate to bushy shrub with densely interlacing branches, but it can reach more than two metres in height, especially in sheltered positions (Allan 1961, Godley 2006). The shrub can consist of numerous main trunks, and the branches are stiff and zig-zagging, with younger branchlets being densely pubescent and becoming glabrous with an orange to yellow brown colour when mature (Allan 1961, Metcalf 2000). The axillary buds of *S. prostrata* are covered in a dense pubescence, and usually have accessory buds in their axils (Grierson 2014: Chapter Three). The pinnately compound leaves are 8 – 20 mm in length, and the rachis is glabrous above and thinly pubescent below (Figure 5.1) (Metcalf 2000). Leaf petioles are pubescent and channelled above. Leaflets are entire, subsessile, 3 – 4 mm long, 1 – 5 pairs with or without a terminal leaflet, and oblong-ovate in shape, with an obtuse base and rounded apex (Buchanan 1883, Metcalf 2000, Heenan et al. 2001). The leaflets can be glabrous on both sides, or with sparse pubescence on the abaxial surface. The abaxial surface is a lighter than the adaxial, with main veins visible (Metcalf 2000). *S. prostrata* is not brevideciduous like some other New Zealand *Sophora* species such as *S. tetraptera* J.S.Mill (Godley 1975).

Figure 5.1: Morphological features of *S. prostrata*. A shows a zig-zag branch. B shows examples of the leaves, the three leaves on the left show the adaxial surface, and the three on the right show the abaxial surface. C shows the seed pods with varying seed numbers. D shows the reddish brown seeds.

Sophora prostrata flowers (Figure 5.2) are solitary, paired, or in threes and are borne on short fulvous tomentose peduncles. The flowers are often presented upside down (resupinate) due to twisting of the pedicel (Allan 1961, Metcalf 2000, Heenan et al. 2001). The calyx is oblique, appressed-hairy, up to 1 cm long, with five short broad triangular teeth. The perianth is usually orange but can be brownish yellow to occasionally bright yellow, composed of five free petals. The standard petal is up to

1.5 cm long, orange, and is broader and slightly to distinctly shorter than the orange wing petals. The two keel petals can be up to 2 cm long, greenish yellow, and are slightly longer than the wing petals (Allan 1961, Metcalf 2000). Within the flower there are 10 separate stamens with longitudinally dehiscent anthers (Allan 1961). The pollen is tricolporate and prolate with a reticulate surface (APSA Members 2007). The ovary is superior and consists of a single carpel and many ovules, and the style is incurved and terminal (Allan 1961). The fruits are legume pods, and are downy when young, 3 – 5 cm long, very narrowly winged, and usually contain 1 – 3, but sometimes up to 5, dark to reddish brown seeds. The seeds are up to 7 mm long and have a hard seed coat (Figure 5.1) (Allan 1961, Webb 1993, Godley 2006).

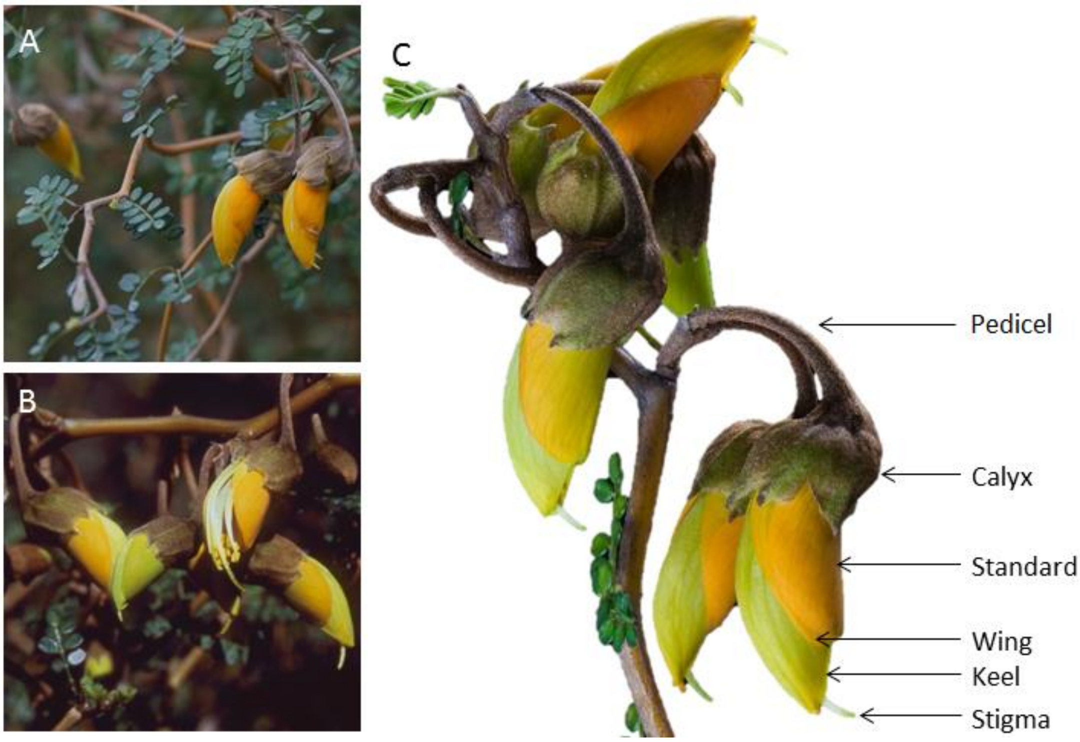

Figure 5.2: The flowers of *S. prostrata*. A shows the orange flowers and leaves. B shows a less tightly folded flower, with the stigma and 10 free stamens visible. C shows the pedicel, calyx, standard petal, one each of the wing and keel petals and the stigma. Also visible is the twisting of the stalk resulting in upside down flowers. Photographs courtesy of A. Jeremy Rolfe, B. Steven Attwood, and C is modified from a photo by Nga Manu Images.

5.3 Divaricate habit

Sophora prostrata is an example of a divaricate growth form. A divaricate is a small leaved shrub with many wide angled interlaced branches and long internodes. This growth form has arisen independently in at least 18 times in the New Zealand flora, and is a remarkable example of convergent evolution. There have been numerous hypotheses put forward to explain the evolution, including adaptation to climatic factors (McGlone and Webb 1981), browsing by moa (Greenwood and Atkinson 1977), and light intensities (Day 1998, Christian et al. 2006)(Grierson 2014: Chapter two). As the divaricate form has arisen multiple times, it can be achieved in many structurally different ways. Divarication can be achieved via wide angled branching (e.g. *Coprosma propinqua*), but also branching at a very narrow angle (e.g. *Melicytus alpinus*). Sympodial growth can result in a zig-zag axis (e.g. *Elaeocarpus hookerianus*), as can monopodial growth where the shoot bends at each node (e.g. *S. prostrata*) (Bell and Bryan 2008).

The divaricate form has been associated with a lack of apical control resulting in outgrowth of lateral branches (Greenwood and Atkinson 1977, Wilson and Galloway 1993, Christian et al. 2006, Wilson and Lee 2012). This lack of apical control makes divaricates an interesting tool in which to study the control of branching, especially in conjunction with their arborescent relatives. Grierson (2014: Chapter Three) investigated apical control in *S. prostrata* in comparison to arborescent *S. tetraptera* by assessing the differences in branching and decapitation response. Short shoots were found to be a significant contributor to the architecture of *S. prostrata*. *Sophora prostrata* also displayed increased growth and node activation in response to decapitation, which could be due to weak apical control resulting in no new leader shoot gaining absolute dominance over the overall plant. Further investigation into the architecture and development of decapitated and intact *S. prostrata* and *S. tetraptera,* along with other divaricate and non-divaricate species pairs, could help in understanding the complexities of apical control and apical dominance in woody species, and contribute to our knowledge of branching in general.

5.4 Anatomy

The wood anatomy of *S. prostrata*, and two close relatives *S. tetraptera* and *S. microphylla,* has been previously described by Patel (1995). The wood for all three species is diffuse porous to semi-ring-porous, and is very hard and conspicuously patterned. The sapwood is cream to very pale yellow, and the heartwood is dark brown. Vessels are usually arranged in a dendritic and oblique pattern. The majority of the features examined were shared by all three species, and only few were of diagnostic value. *Sophora prostrata* has abundant axial and ray chambered parenchyma, where in *S. microphylla* and *S. tetrapter*a they are scarce to occasional. *Sophora tetraptera* and *S. microphylla* also occasionally have their vessels in tangential bands, which does not occur in *S. prostrata*.

Sophora prostrata has the ability to form symbiotic relationships with nitrogen fixing bacteria (Rhizobia). Rhizobia are entrapped the root hairs and then move down to infect the cortical cells which rapidly divide and form nodules.

Carswell (1994) compared the leaf anatomy of fully developed juvenile leaves of *S. prostrata*, *S. microphylla* and *S. tetraptera*. The leaf tissue adjacent to the leaflet midrib consists of an upper cuticle, upper epidermis, hypodermis, mesophyll layer consisting of 2 – 3 palisade layers and 5 – 7 spongy layers, a lower epidermis that has smaller cells than the upper epidermis, and a lower cuticle. The numbers of cell layers present were similar in all three species; however *S. prostrata* had consistently smaller cells than *S. tetraptera*. Idioblasts containing groups of calcium oxalate crystals were present in all three species, but were more concentrated in the adaxial surface of *S. prostrata*.

5.5 Cytology

The chromosome number of *S. prostrata* is 2n = 18 (Dawson 2000). This is also the chromosome number of all other New Zealand *Sophora* (Allan Herbarium 2000, Heenan et al. 2001).

5.6 Chemistry

Leguminous plants are predated by a range of animals and insects, and have evolved a range of secondary compounds in response to this, especially alkaloids (Lewis et al. 2005). Flavonoids have many functions in plants and are involved in floral pigmentation, UV filtration, and symbiotic nitrogen fixation (Dixon and Pasinetti 2010). The genus *Sophora* is rich in alkaloids and flavonoids (Krishna et al. 2012). Flavonoids in the leaves of *Sophora* have been found to distinguish *S. prostrata* from *S. tetraptera*, and flavonoids in the seed coats can differentiate *S. prostrata* and *S. microphylla* (Markham and Godley 1972). Iinuma et al. (1994) and Iinuma et al. (1995) investigated the phenolic constituents in the roots of *S. prostrata,* and found 14 phenolic compounds, including seven new isoflavanones called prostratols A – G. Prostratols A – C are isoflavanones with very rare combinations of side chains, and so are important chemical markers in the chemosystematics in the genus *Sophora* (Boland and Donnelly 1998). Prostratols D and E are pterocarpans, which are derivatives of isoflavanones. Pterocarpans are often found to have antimicrobial properties (Jiménez-González et al. 2008). The seeds contain cytisine – a toxic nicotinic alkaloid (Connor and Fountain 2009). More chemical information on *S. prostrata* can be found in Cambie (1976) and Cambie (1996).

5.7 Taxonomy and relationships

5.7.1 Fabales

The order Fabales is approximately 104 – 89 million years old, and contains four families – Fabaceae (the legumes), Polygalaceae (the milkworts), Quillajaceae and Surinaceae (Stevens 2013). While the order has consistently received strong molecular support, relationships between the four families had been less well understood using molecular datasets (Bello et al. 2009).

5.7.2 Fabaceae

The Fabaceae Lindley, also known by its conserved name Leguminosae Jussieu, is the third largest angiosperm family in the world, with approximately 751 genera (The Legume Phylogeny Working Group 2013). The family name Leguminosae is derived from the leguminous fruits while Fabaceae is derived from Latin *faba* – a bean. The family has been consistently viewed as monophyletic, first with clear morphological characters such as the legume fruits, and then with subsequent molecular analysis (Wojciechowski et al. 2004, Bello et al. 2009, The Legume Phylogeny Working Group 2013). The Fabaceae have had a high rate of diversification over the last 60 million years, with three subfamilies emerging 55 – 50 million years ago – Faboideae or Papilionoideae, Caesalpinioideae, and Mimosoideae (Lewis et al. 2005). The three subfamilies can be distinguished from one another based on morphological features, especially floral characters (Doyle and Luckow 2003). Important agricultural and food plants in this family include *Cicer arietinum* (chick peas), *Arachis hypogaea* (peanut) and *Glycine max* (soybean) (Lewis et al. 2005). Members of Fabaceae in New Zealand include *Sophora* (kōwhai), *Clianthus* Sol. ex Lindl. (kaka beak) and *Carmichalia* R.Br. (native broom).

5.7.3 Sophora

There are about 50 species of *Sophora* found in southeast Europe, Asia, Western South America, Australasia, various Pacific islands and southern Africa (MacKinder et al. 2012). *Sophora* sect. *Edwardsia* (Salisb.) Seem. has a distinct biogeographic distribution, and is comprised of the Australian, Pacific, Mascarene and South American species of *Sophora*. Section *Edwardsia* has been shown to be monophyletic, but there is little genetic difference between species suggesting recent and rapid speciation and dispersal (Hurr et al. 1999, Mitchell and Heenan 2002). Molecular evidence suggests a north-west pacific origin, from a Eurasian ancestor in the last 2 – 5 million years (Hurr et al. 1999, Heenan et al. 2001, Heenan et al. 2004). The seeds of some *Sophora* species are known to be buoyant and to tolerate saline conditions, so transoceanic dispersal is the likely explanation for its distribution (Sykes and Godley 1968, Hurr et al. 1999). It is not known exactly when *Sophora* arrived in New Zealand due to limited fossil evidence, but it does suggest they were not common until the Pleistocene (Hurr et al. 1999).

Two theories have been proposed to explain the relationships between *S. prostrata* and the other species. Cockayne (1912, 1928) suggested that *S. prostrata* is derived from the juvenile form of *S. microphylla* via neoteny, and Godley (1979, 1985) suggested that *S. microphylla* is the result of hybridisation between *S. prostrata* and *S. tetraptera*. Chloroplast DNA analyses by Hurr et al. (1999) favours Cockaynes hypothesis, as *S. prostrata* and *S. microphylla* consistently group together. More molecular data is needed to resolve relationships between New Zealand *Sophora* (Heenan et al. 2001, Song 2005).

Frequent hybridisation and genetic similarity within New Zealand *Sophora* highlight their recent speciation, and make it difficult to resolve relationships (Hurr et al. 1999). However, *S. prostrata* has often been found to be the most distinct relative to other New Zealand *Sophora* species using morphological and molecular data. *Sophora prostrata* was the most distant group in a cluster analysis of leaf and growth characters (Heenan et al. 2001), and an analysis using ISSR markers by Song (2005) found that using Inter Simple Sequence Repeat (ISSR) markers *S. prostrata* was the most genetically distant of the New Zealand *Sophora.* Grierson (2014: Chapter Four) used ISSR markers to support the distinctness of *S. prostrata* using a larger sample size across a wider geographic range, and assessed the genetic variation between different localities. The study was unable to distinguish *S. prostrata* from the other species, or identify many distinct relationships between the localities. The inability of highly variable ISSR markers to resolve relationships between *S. prostrata* and other New Zealand *Sophora* species is further evidence of their close relationship. It was concluded that larger samples sizes and more variable markers such as microsatellites may be needed to resolve the relationships of *S. prostrata* at the species and population level.

5.8 Nomenclature

The taxonomy of New Zealand *Sophora* has had a problematic history (Heenan et al. 2001). Allan (1961) recognised three species, with an additional two varieties of *S. microphylla*. Yakovlev (1967) considered only two species, *S. microphylla* and *S. tetraptera* – with five varieties within *S. microphylla* including *S. microphylla* subsp. *microphylla* var. *prostrata*. This was followed by a genus wide revision by Tsoong and Ma (1981), who accepted *S. microphylla, S. tetraptera, S. prostrata* and *S.*

chathamica. The most recent taxonomic treatment using leaf and growth habit characteristics by Heenan et al. (2001) recognised the current eight species – *S. tetraptera, S. microphylla, S. prostrata, S. molloyi, S. longicarinata, S. godleyi, S. fulvida* and *S. chathamica* (Table 5.1).

Table 5.1: Comparison of taxonomic treatments of *Sophora* in New Zealand.

Allan (1961)	Yakovlev (1967)	Tsoong and Ma (1981)	Heenan et al. (2001)
S. prostrata	*S. microphylla* subsp. *microphylla* var. *prostrata*	*S. prostrata*	*S. prostrata*
S. tetraptera	*S. tetraptera*	*S. tetraptera*	*S. tetraptera*
S. microphylla var. *microphylla*	*S. microphylla* subsp. *microphylla* var. *microphylla*	*S. microphylla*	*S microphylla*
Synonym of *S. microphylla*	*S. microphylla* subsp. *microphylla* var. *chathamica*	*S. chathamica*	*S. chathamica*
S. *microphylla* var. *fulvida*	*S. microphylla* subsp. *microphylla* var. *fulvida*	-	*S. fulvida*
S. microphylla var. *longicarinata*	*S. microphylla* subsp. *microphylla* var. *longicarinata*	-	*S. longicarinata*
-	-	-	*S. godleyi*
-	-	-	*S. molloyi*

The genus *Sophora* L. (1753) was first described in New Zealand by Banks and Solander on Cooks first voyage in 1769-70. The name *Sophora* is derived from Arabic *sufayra* – the name for a leguminous tree (Lewis et al. 2005). The species name *prostrata* is from the word prostrate – meaning growing flat along the ground (New Zealand Plant Conservation Network 2013), derived from the latin *prosternere*, meaning to throw down (Eagle 2007). The Māori name for all species of *Sophora* is kōwhai or kōhai in some areas, which is the Māori word for yellow (Ryan 2012). Common names include prostrate kōwhai, dwarf kōwhai and South Island kōwhai (Allan 1961, Allan Herbarium 2000). Sophora prostrata was first described by Buchanan in 1883 from a type specimen in the Awatere valley, Marlborough (Figure 5.3, specimen: K000759769 at Royal Botanic Gardens Herbarium, Kew, U.K.).

Current synonyms for *S. prostrata* are *S. tetraptera var. prostrata* (Buchanan) Kirk and *Edwardsia prostrata* (Buchanan) W.R.B.Oliv. (Allan Herbarium 2000).

Figure 5.3: *Sophora prostrata* type specimen illustrated by J. Buchanan in Transactions and proceedings of the New Zealand Institute volume 16, plate 36.

5.9 Distribution

Sophora prostrata is confined to the eastern side of the South Island of New Zealand, between Blenheim and the Waitaki River (Godley 2006). It can be found in lowland to montane shrubland, river flats, grassland and rocky places (Allan 1961, Wilson and Galloway 1993, Godley 2006) from 76 – 760m above sea level (Metcalf 2000). *Sophora prostrata* can be found in harsh dry places, and can tolerate wind and drought (Thomas and Spurway 2002). Godley et al. (in press) assessed the distribution of *S. prostrata* and *S. microphylla* in the lower catchment of the Waimakariri River and found that both species grow on a diverse range of soils, and favour those of high soil fertility. Legumes such as *S. prostrata* can also tolerate relatively infertile soils where usable nitrogen is scarce, due to their nitrogen fixing bacteria in nodules on their roots (Winterbourn et al. 2008).

The frost hardiness and lethal temperatures of *Sophora prostrata*, *S. microphylla* and *S. tetraptera* was assessed by Warrington and Stanley (1987). They found that *S. prostrata* was more frost hardy in winter (-6 °C) and had a lower lethal temperature

(-11 °C) than *S. microphylla* and *S. tetraptera* (-4 °C and -6 °C respectively). Darrow et al. (2001) found that divaricating juveniles of *S. microphylla* were more frost resistant than adults. *Sophora prostrata* and *S. microphylla* are both found in the cooler climates of the South Island, and *S. tetraptera* is found in the eastern North Island.

From location data of herbarium specimens, the probable locations in which *S. prostrata* could occur were extrapolated from the Land Environments of New Zealand (LENZ) classifications they occurred in (Figure 5.4). Based on the LENZ data, *S. prostrata* has a high probability of occurring in an area with good soil drainage where the mean annual temperature is between 7 – 13 °C, the minimum temperature in July is between -3.6 – 3.6 °C, there is a vapour pressure deficit of approximately 0.5 kPa, a mean solar radiation of 14.2 MJ/m^2/day, and a ratio of mean annual rainfall to potential evapotranspiration of 1.2 – 3.9.

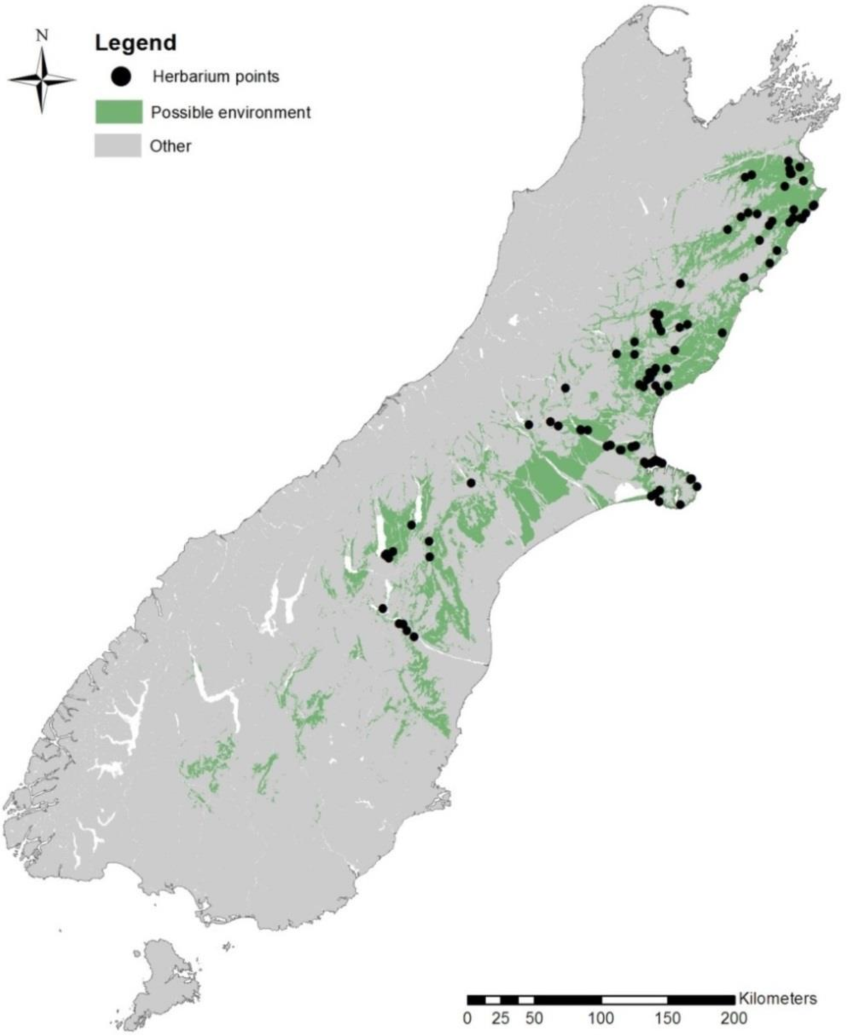

Figure 5.4: South Island of New Zealand showing observed distribution (herbarium points from CHR, MPU and WELT) and predicted possible environmental distribution (shaded green) of *S. prostrata*, based on climate, landform, and soil variables including total annual rainfall, minimum daily temperature, and mean solar radiation.

5.10 Biotic interactions

Most members of the Fabaceae, including *S. prostrata*, have specific symbiotic relationships with nitrogen fixing bacteria (Figure 5.5). New Zealand Sophora species have been shown to be nodulated by a distinct *Mesorhizobium* strain (Weir 2006). Nitrogen fixing legumes contain abundant nitrogen, and can be subject to herbivory by a variety of animals and insects. *Sophora prostrata* is browsed by domestic animals such as sheep and cows, as well as rabbits and hares (Figure 5.6) (Godley et al. in press). This browse pressure can cause a reduction in existing numbers of the species, as well as an inhibition of seedling recruitment. Naturalised grasses can also play a role in preventing the establishment of *Sophora* seedlings through competition (Godley et al. in press).

Figure 5.5: Nitrogen fixing root nodules of *S. microphylla* (Photo courtesy of Wendy John).

Figure 5.6: Many *S. prostrata* individuals that have been heavily grazed in North Canturbury. Image from Bloor (2009).

Kōwhai are hosts to a range of native insects including the kōwhai seed moth (*Stathmopoda aposema*) and the kōwhai moth (*Uresephita polygonalis maorialis*) (Mackay 2004, Godley 2006). Kōwhai seed moth caterpillars eat the young seeds, and the kōwhai moth caterpillar eats the leaflets, and can cause major defoliation (Webb 1993, Godley 2006). *Sophora prostrata* is often parasitized by two mistletoe species - small-flowered mistletoe (*Ileostylus micranthus*) (Allan Herbarium 2000) and pygmy mistletoe – *Korthalsella lindsayi* (Winterbourn et al. 2008).

Sophora prostrata is often found as an emergent and canopy species in dry woodland and shrubland communities, and the presence of *S. prostrata* can indicate succession towards kōwhai forest or scrub (Bloor 2009).

5.11 Reproductive biology

The flowering time of *S. prostrata* varies with environmental conditions, but is usually in early October to late November (Godley and Smith 1977), and can be as early as May on Banks Peninsula or August in north Canterbury (Godley 1978). Kōwhai flowers usually contain abundant nectar and pollen and attract a wide range of pollinators (Godley 1975, 2006), however as *S. prostrata* flowers are smaller, more tightly folded and hidden amongst the branches they are therefore more limited in their pollinators (Godley 1975). They are pollinated by several kinds of small insects including introduced bumble bees and honey bees, and visits by birds have not been recorded (Godley 2006). Cross pollination definitely occurs as hybrids between *S. prostrata* and *S. microphylla* are often observed (Godley 1975, 2006).

The pods of *S. prostrata* are smaller than other kōwhai pods, lack prominent wings, and have usually 2 – 3 dark to reddish brown seeds (Metcalf 2000, Godley 2006). Kōwhai pods are usually partially full, with gaps between the seeds where the ovules have not been fertilised, and unlike other leguminous pods, the kōwhai seed pods do not split open (Godley 2006). The seed has a hard impenetrable coat called a testa, which requires scarification in order for moisture to enter and for the seed to germinate. *Sophora prostrata* seeds can remain dormant for long periods of time (at least 30 years) until scarification and germination (Norton et al. 2002). *Sophora prostrata* seeds have an optimum germination temperature between 10 – 20 °C and do not require light to germinate (Webb 1993, Mackay 2004). The seeds on *S. prostrata* are not buoyant due to the density of the kernel (Sykes and Godley 1968), however seeds may be distributed by floods (Godley et al. in press). The pods are light so can be distributed via gravity or wind (Webb 1993).

5.12 Historic and current uses

Historically, various parts of kōwhai were used medicinally by Maori. There is little mention of *S. prostrata* specifically, probably due to its limited distribution. The medicinal value seems to be the same for the other species so it may apply to *S. prostrata* as well, especially as they closely related (Macdonald et al. 1973).The bark, leaves and roots of kōwhai were used externally and internally (Bell 1890, Riley 1994). An infusion of the bark was used to treat colds and sore throats, internal pains, toothache, various skin rashes, bruises, and to assist in setting fractures (Te Rangi Hiroa 1910, Macdonald et al. 1973). The leaves and bark were boiled an applied as a pack for broken limbs (Kahaki 1941). The juice of the root was taken internally as a cure for gonorrhoea (Bell 1890, Brooker et al. 1987). Dried flowers and seed pods were also used as a yellow dye, and the bark yielded a tan colour (Riley 1994).

Sophora species have had a variety of uses elsewhere in the world (Brooker et al. 1987, Riley 1994).

Sophora prostrata is listed as a suitable plant for plantings in dry woodland, grassland and shrubland (Williams 2005), and should be considered for restoration plantings in some areas such as West Melton reserves (Grove 2014, pers. comm.). *Sophora prostrata* is used for slope stabilisation, sand dune restoration and as a hedge as a windbreak (Mackay 2004). Education surrounding the value of native trees like kōwhai is important in their ongoing conservation, and projects such as Project Gold can help achieve this. Project Gold is an Otago Department of Conservation initiated project, which aims to protect and enhance *S. microphylla* in Otago, and strengthen enthusiasm for dryland forest restoration (Department of Conservation 2014).

A cultivar of *S. prostrata* called 'Little baby', is used throughout the world ornamentally and is often planted in rock gardens (Crowe 1997, Metcalf 2000). *Sophora prostrata* is also frequently used in bonsai (Hughes 2002), and hybrids between *S. prostrata* and *S. microphylla* can be interesting garden plants (Metcalf 2000). The flowers can be used as a yellow dye (Crowe 1999) and the perfumed essences from kōwhai petals have been incorporated into soaps (Cooper and Cambie 1991). Flavonoid compounds found in *S. prostrata* have been included in studies assessing possible anti-cancer effects of natural products (Akihisa et al. 2011, Akihisa et al. 2012).

Due to concern over kōwhai seeds being poisonous, there is occasionally call for kōwhai to be removed from pre-schools and kindergartens (De Lange and Heenan 2006). However, kōwhai are not included in Landcare Research's list of plants not recommended for pre-schools (Sykes 2005).

5.13 Conservation

Sophora prostrata is currently listed as non-threatened using the New Zealand Threat Classification System (de Lange et al. 2013), however as with many other species, natural regeneration will continue to decline as the environment is modified (Godley 2006, Winterbourn et al. 2008). The Canterbury Plains are one of the regions in New Zealand that has experienced the most severe loss of vegetation (Ecroyd and Brockerhoff 2005), and this area is the centre of abundance of many divaricates. Many of the shrubland habitats are in decline, and so many divaricating species including *S. prostrata* are potentially coming under threat (Given 2002). Godley et al. (in press) noted that in the lower Waimakariri catchment the existing mature kōwhai (both *Sophora prostrata* and *S. microphylla*) were in decline, and no seedlings of either kōwhai were observed due to browsing by animals and competition from naturalised grasses. They concluded that without intervention via artificial replacement, kōwhai populations will continue to decline in the face of browsing damage and completion from grasses (Godley et al. in press).

Another threat to New Zealand *Sophora* is hybridisation between different species and mixing with the same species from a different locality. This can be damaging to wild gene pools, and is a risk when revegetation projects occur without ecosourcing seeds, and when non local and foreign species are cultivated in the area (Hughes 2002, De Lange and Heenan 2006, Godley 2006, New Zealand Plant Conservation Network 2013).

5.14 Future research

Identification of the potential threats to *S. prostrata*, such as the browse damage and competition from grasses as discussed above, allows us to address these threats and prevent further decline. Increased interest and conservation efforts surrounding divaricates such as *S. prostrata* can ensure that these iconic plants remain a unique and significant part of New Zealand's native flora (Given 2002). Furthering research by Grierson (2014: Chapter Four) into the population genetics of *S. prostrata* could clarify any distinct populations, and establish where seeds can be collected and grown safely for ecosourcing. Understanding the phylogenetic history of *S. prostrata* could also influence the theories behind divaricate evolution, as relationships and divergence times could be tied to different theories (Hurr 1996). As shown in Grierson (2014: Chapter Three), *S. prostrata* in comparison with other New Zealand *Sophora* can be a useful model to study the development and regulation of shoot branching. Further research into this area can enhance our understanding of *S. prostrata*, divaricates, and potentially the genetics of shoot branching.

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Chapter Six: Synthesis

This research has contributed to our understanding of the development and genetic variation of *Sophora prostrata*, and has provided a basis for further study into divaricate branching and relationships within *Sophora*. Comprehensive review of divaricate literature and assessment of published indices has identified the next steps in understanding and defining divaricates. Assembling and reviewing the available literature on *S. prostrata* has highlighted gaps in our knowledge, as well as issues that need to be addressed. *Sophora prostrata* is part of an interesting series of closely related species displaying a range of growth forms - divarication, heteroblasty and arborescent tree growth. *Sophora prostrata* and its relatives are an appropriate tool to address interesting ecological questions surrounding convergent evolution and to study branching and development.

To be able to properly address divaricate evolution, we need to further work begun by Atkinson (1992) and Kelly (1994) into clarifying divaricate subtypes, and forming robust definitions. Once we have a clearer idea of the characteristics that unify and divide this assortment of forms we can begin to systematically investigate the development and genetics of each type of divarication. Progress cannot be made into understanding divaricate evolution if the species and associated features used are not consistent, and encompassing the range of divaricate types. A renewed focus on the architectural and developmental basis of divarication is essential to be able to properly address the evolution of this form in New Zealand.

Short shoots have been discussed by some authors as a characteristic feature of divaricates, and they are shown here to be a significant contributor to the structure of *S. prostrata*. Short shoots have not been explicitly included in previous indices and analyses (Tomlinson 1978, Atkinson 1992, Kelly 1994), and further investigation into the contribution of short shoots to the structure of all divaricate species could result in their addition to a more robust index.

Divaricates are a remarkable example of convergent evolution, which can provide an ideal framework in which to study evolution, branch development and genetic regulation of dormancy and outgrowth. Previous research into branching regulation involving model organisms has provided the foundation upon which research in non-model organisms can take place. Understanding the complexity of apical control in woody species can allow further manipulation in branching in agricultural cops to increase yield and improve efficiency. The present research is a necessary preliminary step in furthering branching regulation research in divaricates, as it begins to establish ways to carry out divaricate research effectively and to educate sampling for further work. We can now begin to ask how this form has evolved, as the genetic basis of these convergent forms is unknown. Whether all divaricates use the same genes (e.g. MAX4) and genetic networks to achieve the overall architecture is a question that warrants further investigation.

Assessing genetic variation in taxonomically problematic groups like New Zealand *Sophora* is important as it can contribute to our understanding of their evolution. Establishing relationships between *S. prostrata* and other New Zealand *Sophora* can have implications for the theories behind Sophora evolution (Cockayne 1912, 1928, Godley 1979, Godley 1985), and also theories explaining divaricate evolution (Hurr 1996). The distinctness of *S. prostrata* was not supported with the present dataset, however it did confirm the close relationship between *Sophora* species that has been previously demonstrated (Hurr et al. 1999, Mitchell and Heenan 2002). The probable recent origin of *Sophora* species validates that they are an ideal candidate for research into divaricates and their non-divaricating close relatives. The close relationship between *Sophora* species means that primers needed for microsatellite markers or gene expression studies will probably work well across all New Zealand *Sophora*. Further research will need to employ a larger sample size per locality, and potentially the recently developed *Sophora* microsatellites (Etten et al. 2014). The present research could not distinguish any *S. prostrata* populations. Continuing research into the population genetics of *S. prostrata* could clarify any distinct populations, which could then educate ecosourcing.

Combining information about the population genetics, distribution, biotic interactions and conservation of *S. prostrata* provides valuable guidance as to where *S. prostrata* can grow and survive. This could educate where we plant *S. prostrata* in restoration projects, and what measures need to be taken to protect and enhance existing populations. Monitoring of kōwhai populations such as that in the lower Waimakariri catchment (Godley et al. in press) is necessary to establish how and why they are in decline, and be able to take steps to prevent further loss. Many of the shrubland habitats that divaricates like *S. prostrata* are found in are being lost (Given 2002). *Sophora prostrata* is currently not threatened, however the fact that in some sites they are not regenerating means that they may be on their way to becoming threatened (Given 2002). Increased efforts to maintain populations that are not yet classified as under threat is important to prevent further decline, and increased interest and conservation efforts surrounding divaricates can ensure that these iconic plants remain a unique and significant part of the native New Zealand flora (Given 2002).

This research combined investigation of branching regulation and genetic variation of *Sophora* with comprehensive review of literature surrounding *S. prostrata* and the divaricate form. The various aspects of this research have contributed to our understanding of *S. prostrata,* and demonstrated the value of a holistic approach. *Sophora prostrata* and other New Zealand divaricates are a significant part of New Zealand's distinctive flora, and are individually and collectively a valuable resource that should be further utilised to answer many important ecological, evolutionary, and developmental questions.

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Appendices

Appendix One: Dehydration and infiltration protocol

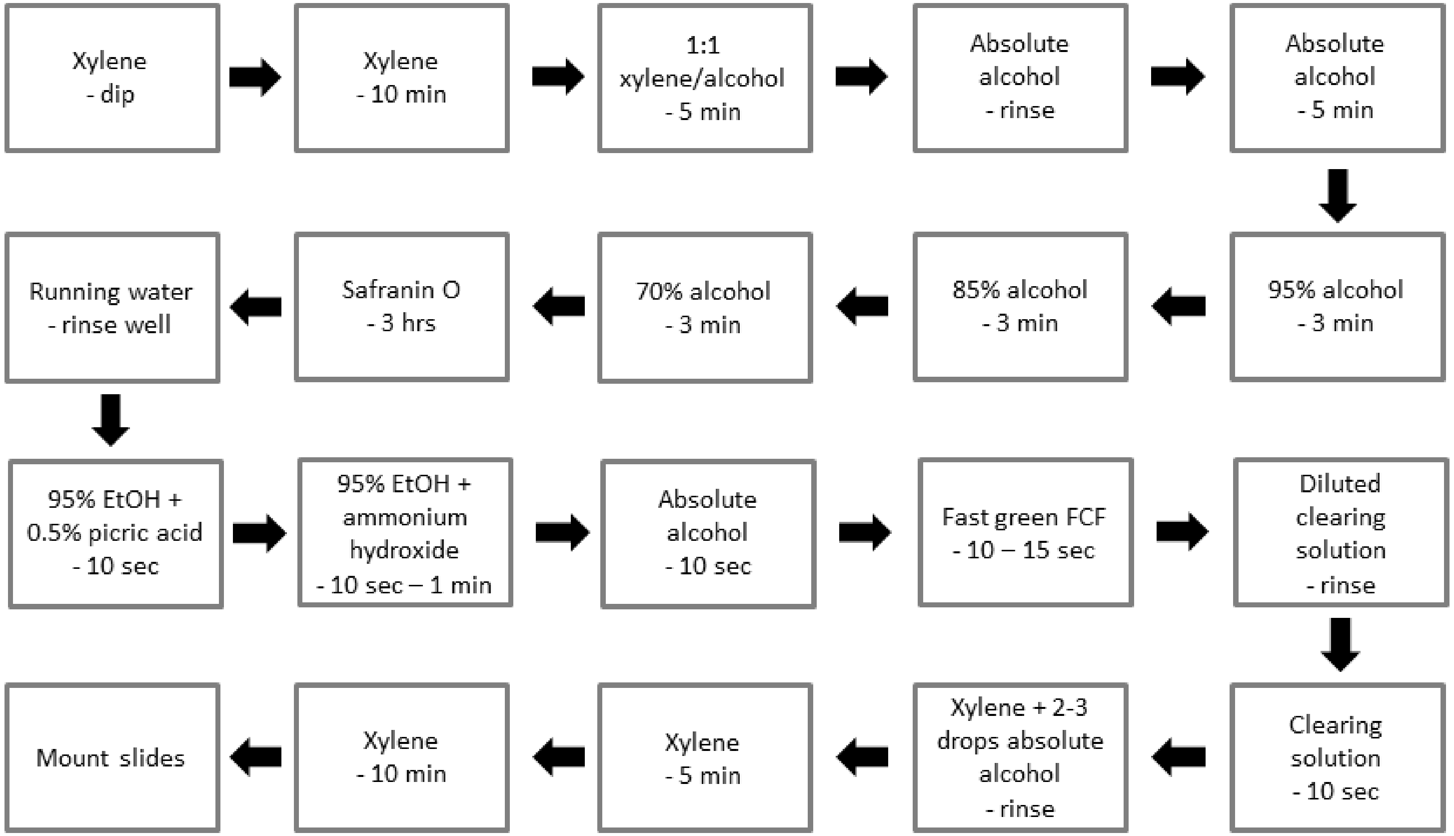
Protocol for dehydration and infiltration for axillary bud histology. Dehydration used a TBA/ethanol series and infiltration was through paraffin oil to wax, in preparation for embedding and microtoming.

	Step 1	Step 2	Step 3	Step 4	Step 5	Step 6	Step 7
Time in solution	Overnight	3 hours	3 hours	3 hours	8 hours (In oven)	8 hours (In oven)	8 hours (In oven)
Total % alcohol	70%	85%	95%	100 %	100% TBA	100% TBA	100% TBA

Step 8	Step 9	Step 10	Step 11	Step 12
1 hour	Until melted (In oven)	10 hours (In oven)	10 hours (In oven)	10 hours (In oven)
Into TBA/paraffin (50:50)	Sample placed onto cooled wax, with enough TBA/paraffin to cover it.	Fresh wax	Fresh wax	Fresh wax

Appendix Two: Staining protocol

Safranin and fast green staining protocol adapted from Johansen (1940).



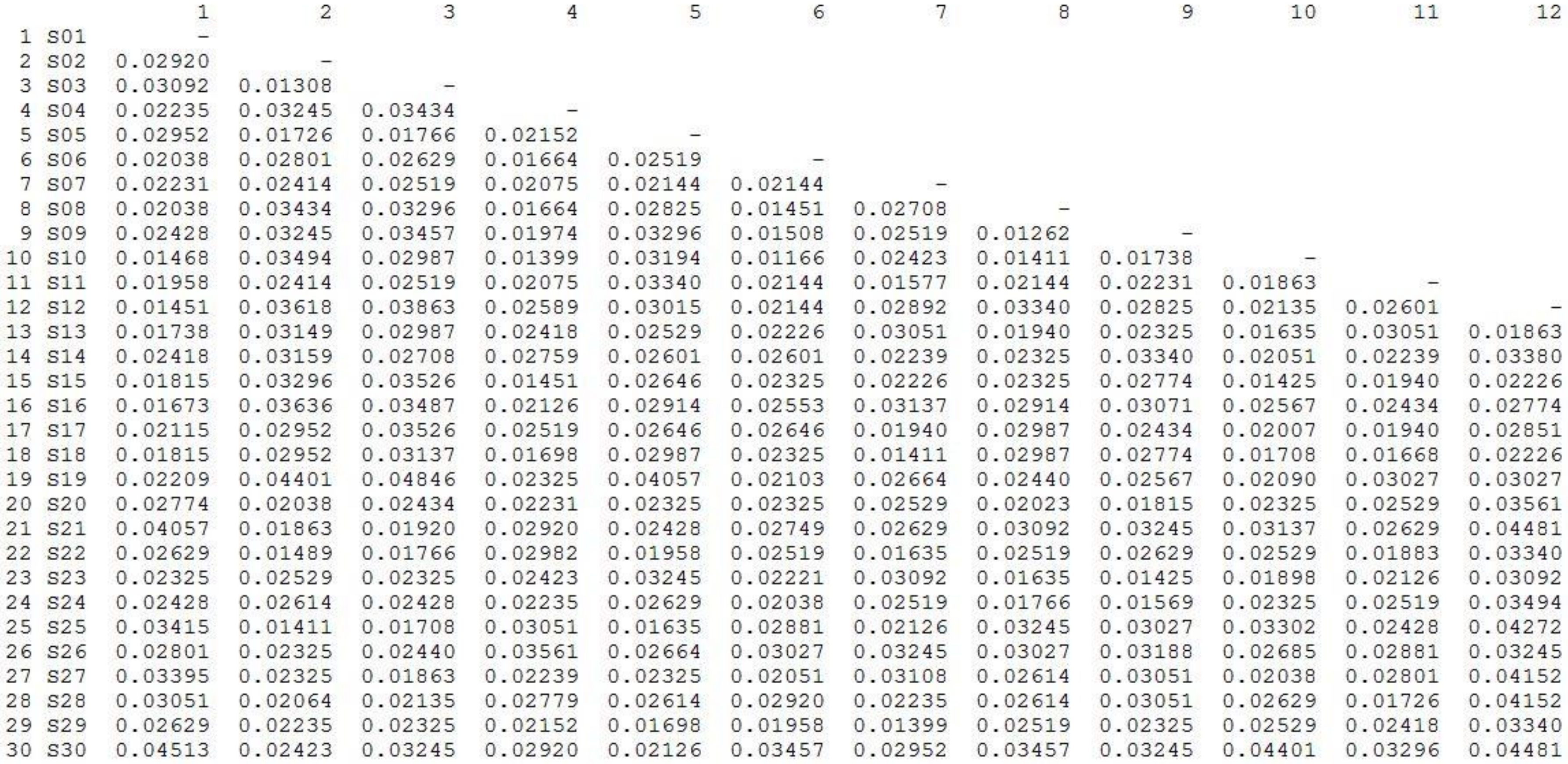
Appendix Three: Node state totals over time

Total number of leaves (L), leaf scars (LS), short shoots (SS), initiated branches (BR), branches with leaves (BR-) and nodes that do not have expanded leaves (N) at each week that the state of nodes were recorded.

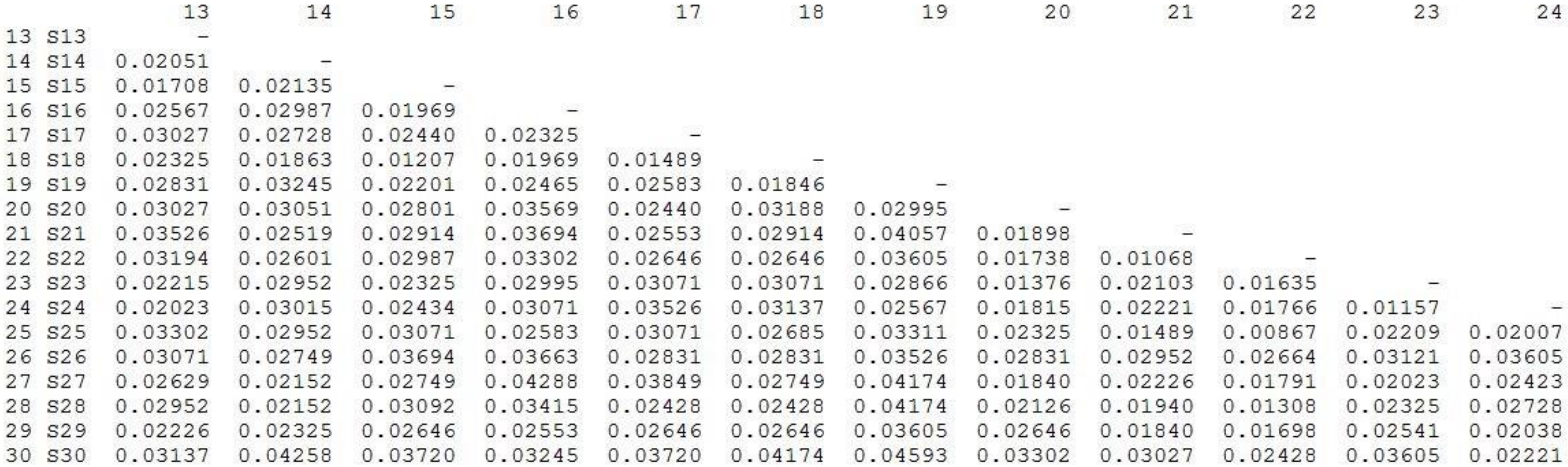
	Decapitated *Sophora prostrata*					Intact *Sophora prostrata*					Decapitated *Sophora tetraptera*					Intact *Sophora tetraptera*				
Week	0	2	5	9	13	0	2	5	9	13	0	2	5	9	13	0	2	5	9	13
	Soppro01					Soppro04					Soptet01					Soptet07				
L	212	309	447	561	643	336	330	326	335	371	66	91	121	146	160	109	109	109	114	115
LS	75	75	76	79	83	135	140	145	154	163	663	662	661	655	653	33	33	33	33	33
SS	89	89	90	90	91	77	77	78	79	79	4	4	4	4	4	0	0	0	0	0
BR	53	59	61	70	74	54	61	61	61	64	3	3	4	10	16	0	0	0	0	0
BR-	43	50	81	82	82	65	65	65	66	69	38	39	40	44	47	31	31	31	31	31
N	3	7	27	29	29	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0
	Soppro02					Soppro05					Soptet04					Soptet08				
L	368	464	575	696	731	329	353	423	494	501	102	102	125	167	176	78	89	109	110	117
LS	161	162	169	173	204	39	39	40	43	48	117	117	120	120	122	72	72	79	81	81
SS	78	94	98	126	155	64	64	63	63	63	0	0	0	0	0	0	0	0	0	0
BR	4	8	8	8	7	37	45	48	71	71	1	9	14	19	25	0	0	0	0	0
BR-	56	57	59	60	61	46	46	48	52	52	14	14	21	25	25	22	23	25	26	27
N	2	2	2	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Soppro03					Soppro06					Soptet06					Soptet10				
L	395	395	400	444	528	118	132	143	154	160	35	43	55	60	52	52	58	77	141	181
LS	59	66	66	70	79	8	8	8	9	9	235	236	236	239	244	286	285	277	269	270
SS	74	74	75	79	154	12	11	12	12	18	0	0	0	0	0	0	0	0	0	0
BR	11	16	16	19	17	10	12	18	22	24	0	0	0	20	28	8	8	9	8	15
BR-	96	96	96	107	128	16	17	18	19	24	26	27	27	27	27	40	40	49	59	60
N	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Appendix Four: Genetic distances between *Sophora* samples

Genetic distances between all *Sophora* samples – 18 *S. prostrata*, 6 *S. tetraptera*, 5 S. *microphylla*.



		1	2	3	4	5	6	7	8	9	10	11	12
1	S01	-											
2	S02	0.02920	-										
3	S03	0.03092	0.01308	-									
4	S04	0.02235	0.03245	0.03434	-								
5	S05	0.02952	0.01726	0.01766	0.02152	-							
6	S06	0.02038	0.02801	0.02629	0.01664	0.02519	-						
7	S07	0.02231	0.02414	0.02519	0.02075	0.02144	0.02144	-					
8	S08	0.02038	0.03434	0.03296	0.01664	0.02825	0.01451	0.02708	-				
9	S09	0.02428	0.03245	0.03457	0.01974	0.03296	0.01508	0.02519	0.01262	-			
10	S10	0.01468	0.03494	0.02987	0.01399	0.03194	0.01166	0.02423	0.01411	0.01738	-		
11	S11	0.01958	0.02414	0.02519	0.02075	0.03340	0.02144	0.01577	0.02144	0.02231	0.01863	-	
12	S12	0.01451	0.03618	0.03863	0.02589	0.03015	0.02144	0.02892	0.03340	0.02825	0.02135	0.02601	-
13	S13	0.01738	0.03149	0.02987	0.02418	0.02529	0.02226	0.03051	0.01940	0.02325	0.01635	0.03051	0.01863
14	S14	0.02418	0.03159	0.02708	0.02759	0.02601	0.02601	0.02239	0.02325	0.03340	0.02051	0.02239	0.03380
15	S15	0.01815	0.03296	0.03526	0.01451	0.02646	0.02325	0.02226	0.02325	0.02774	0.01425	0.01940	0.02226
16	S16	0.01673	0.03636	0.03487	0.02126	0.02914	0.02553	0.03137	0.02914	0.03071	0.02567	0.02434	0.02774
17	S17	0.02115	0.02952	0.03526	0.02519	0.02646	0.02646	0.01940	0.02987	0.02434	0.02007	0.01940	0.02851
18	S18	0.01815	0.02952	0.03137	0.01698	0.02987	0.02325	0.01411	0.02987	0.02774	0.01708	0.01668	0.02226
19	S19	0.02209	0.04401	0.04846	0.02325	0.04057	0.02103	0.02664	0.02440	0.02567	0.02090	0.03027	0.03027
20	S20	0.02774	0.02038	0.02434	0.02231	0.02325	0.02325	0.02529	0.02023	0.01815	0.02325	0.02529	0.03561
21	S21	0.04057	0.01863	0.01920	0.02920	0.02428	0.02749	0.02629	0.03092	0.03245	0.03137	0.02629	0.04481
22	S22	0.02629	0.01489	0.01766	0.02982	0.01958	0.02519	0.01635	0.02519	0.02629	0.02529	0.01883	0.03340
23	S23	0.02325	0.02529	0.02325	0.02423	0.03245	0.02221	0.03092	0.01635	0.01425	0.01898	0.02126	0.03092
24	S24	0.02428	0.02614	0.02428	0.02235	0.02629	0.02038	0.02519	0.01766	0.01569	0.02325	0.02519	0.03494
25	S25	0.03415	0.01411	0.01708	0.03051	0.01635	0.02881	0.02126	0.03245	0.03027	0.03302	0.02428	0.04272
26	S26	0.02801	0.02325	0.02440	0.03561	0.02664	0.03027	0.03245	0.03027	0.03188	0.02685	0.02881	0.03245
27	S27	0.03395	0.02325	0.01863	0.02239	0.02325	0.02051	0.03108	0.02614	0.03051	0.02038	0.02801	0.04152
28	S28	0.03051	0.02064	0.02135	0.02779	0.02614	0.02920	0.02235	0.02614	0.03051	0.02629	0.01726	0.04152
29	S29	0.02629	0.02235	0.02325	0.02152	0.01698	0.01958	0.01399	0.02519	0.02325	0.02529	0.02418	0.03340
30	S30	0.04513	0.02423	0.03245	0.02920	0.02126	0.03457	0.02952	0.03457	0.03245	0.04401	0.03296	0.04481



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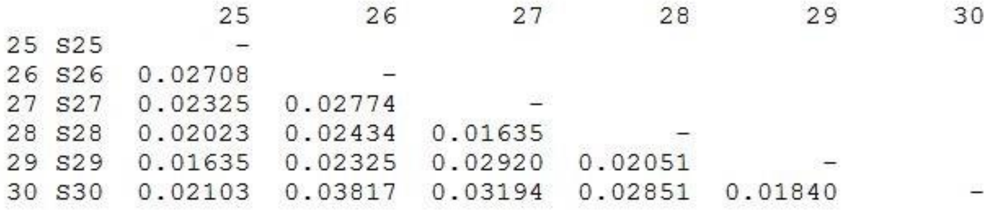
13	S13	-											
14	S14	0.02051	-										
15	S15	0.01708	0.02135	-									
16	S16	0.02567	0.02987	0.01969	-								
17	S17	0.03027	0.02728	0.02440	0.02325	-							
18	S18	0.02325	0.01863	0.01207	0.01969	0.01489	-						
19	S19	0.02831	0.03245	0.02201	0.02465	0.02583	0.01846	-					
20	S20	0.03027	0.03051	0.02801	0.03569	0.02440	0.03188	0.02995	-				
21	S21	0.03526	0.02519	0.02914	0.03694	0.02553	0.02914	0.04057	0.01898	-			
22	S22	0.03194	0.02601	0.02987	0.03302	0.02646	0.02646	0.03605	0.01738	0.01068	-		
23	S23	0.02215	0.02952	0.02325	0.02995	0.03071	0.03071	0.02866	0.01376	0.02103	0.01635	-	
24	S24	0.02023	0.03015	0.02434	0.03071	0.03526	0.03137	0.02567	0.01815	0.02221	0.01766	0.01157	-
25	S25	0.03302	0.02952	0.03071	0.02583	0.03071	0.02685	0.03311	0.02325	0.01489	0.00867	0.02209	0.02007
26	S26	0.03071	0.02749	0.03694	0.03663	0.02831	0.02831	0.03526	0.02831	0.02952	0.02664	0.03121	0.03605
27	S27	0.02629	0.02152	0.02749	0.04288	0.03849	0.02749	0.04174	0.01840	0.02226	0.01791	0.02023	0.02423
28	S28	0.02952	0.02152	0.03092	0.03415	0.02428	0.02428	0.04174	0.02126	0.01940	0.01308	0.02325	0.02728
29	S29	0.02226	0.02325	0.02646	0.02553	0.02646	0.02646	0.03605	0.02646	0.01840	0.01698	0.02541	0.02038
30	S30	0.03137	0.04258	0.03720	0.03245	0.03720	0.04174	0.04593	0.03302	0.03027	0.02428	0.03605	0.02221



		25	26	27	28	29	30
25	S25	-					
26	S26	0.02708	-				
27	S27	0.02325	0.02774	-			
28	S28	0.02023	0.02434	0.01635	-		
29	S29	0.01635	0.02325	0.02920	0.02051	-	
30	S30	0.02103	0.03817	0.03194	0.02851	0.01840	-