Plant Phylogeography in Southern South America

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Doctor of Philosophy – The University of Edinburgh – 2011

Declaration

I declare that this thesis is composed of work carried out by myself unless otherwise acknowledged and that this thesis is of my own composition. This thesis has not in whole or in part been previously presented for any other degree.

Camila Martinez Araneda

2011

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Preface

This thesis is a phylogeographic investigation into plant species from Patagonia, and aims to infer their past distributions from the study of genealogical lineages. These species have gone through several events such as glacial periods, volcanism and topographical change which are expected to contribute to the divergence of genetic lineages by shaping distributions, isolating populations and therefore changing their genetic structure. So understanding how these processes have affected populations is important to get information on how the biodiversity in the region has been assembled, to identify hotspots of intra-specific diversity and therefore to establish potential conservation priorities. Several multi-species phylogeographic studies have been done in the northern hemisphere, but only few are published for South America and even less for the studied area.

Patagonia is an area of a great interest because is the only area in the southern hemisphere apart from Antarctica that have been covered buy a thick layer of ice within the glacial periods. It has high levels of endemism, due to its natural boundaries and environmental processes, and is a biodiversity hotspot for conservation. Its varied topography (two big mountain ranges with a north-south distribution divided by a low flat area) also makes Patagonia interesting to study, due to the likelihood of this impacting on phylogeographic patterns.

This study encompass seven different Patagonian species of which one is a range restricted conifer and the rest are all angiosperms and include trees, shrubs and herbs with a broad distributions. The reason why I have chosen so many different species is to look for general phylogeographic patterns in species in this region.

The thesis was constructed in five chapters. The first is an introductory chapter that provides background to the study system and concepts. Chapters 2, 3, and 4 are empirical phylogeographic studies. These are written as self-contained chapters with the intention that each will be submitted as a separate paper. This leads to some repetition between chapters, but this is intentional as each will need to 'stand alone' when submitted for publication. Chapter 2 is a general investigation into five different Patagonian plant species: *Discaria chacaye*, *Donatia fascicularis*, *Escallonia virgata*, *Tepualia stipularis* and *Weinmannia trichosperma.* Chapter 3 describes the phylogeographic structure of *Gentianella magellanica* an annual, cold tolerant species with a wide distribution throughout Patagonia. This species was treated separately and in more detail than the previous five species due to its marked phylogeographic structure. Chapter 4 describes the phylogeographic structure of a Chilean endemic conifer *Prumnopitys andina*. This has a small distribution in the Andes and only one known population in the coastal cordillera. It was treated separately due to its restricted distribution and different mode of chloroplast inheritance (paternal). Chapter 5, is a general summary, bring all of the results together and giving a wider explanation of the phylogeographic patterns for all species and an outline of future research areas.

Acknowledgements

Being a PhD as an overseas student is perhaps one of the most difficult and overwhelming experiences I will ever have! However, there is no doubt that what has helped enormously is to be lucky enough to have had unbelievable support from two knowledgeable and very kind supervisors - Peter Hollingsworth from the RBG Edinburgh and Richard Ennos from the University of Edinburgh. Both have been invaluable to my research; guiding me in making the right decisions and helping me to not loose track of what I was doing. I have learned so much from each one of them and I am deeply grateful to them for their friendship and support. Finishing this thesis has been a real test for me, but with their help, support and their continuous belief they have made it possible for me to accomplish this amazing period in my life.

Before I started my PhD, many years ago Martin Gardner from RBG Edinburgh accompanied me on field work in Chile in order to teach me everything that he knew about the native and endemic plants from Chile. This love for the native flora of Chile from a European botanist encouraged me to study more in-depth other aspects of the Chilean flora and led me to become fascinated by the use of molecular genetics. He helped me obtain funding for a short scholarship at RBG Edinburgh to learn molecular methodologies and since then he has always helped me with any difficulties that I have had and taking the time to teach me useful aspects of my research.

A population genetic study and the amount of work it entails can be very overwhelming for research students, but for me this was the aspect of my research that I enjoyed the most. The laboratories at RBG Edinburgh are so well organized and the staff has been so helpful and kind. I am therefore particularly grateful to Michelle Hollingsworth, Alex Clark, Jane Squirrell, Vimi Lomax, Ruth McGregor and Laura Forrest. I thank each and one of them for all their friendship, patience and unbelievable support.

Some of the most important information I needed for my research was distribution data for the different species in order to plan my fieldwork. This information was sourced from several herbaria, including RBG Edinburgh and the Universidad de Concepción. I am grateful to the staff of these herbaria for their help and particularly grateful to Clodomiro Marticorena for giving me electronic data from his database in Concepción. My sampling involved working in many protected areas in central and south Chile and to southern Argentina and I wish to thank the staff of Administracion de Parques Nacionales (APN) in Argentina and Corporacion Nacional Forestal (Conaf) in Chile. I am particularly indebted to the Guardaparques of these parks who went out of their way to help me find my samples – on one occasion even taking me across a remote lake. This selfless help made a huge contribution to my research. I am also grateful to my friends Jorge Andrews and Duncan MacDonald who gave up their holidays to help me find my plants.

On a personal level I had extraordinary help and support from my friends including Kath Evans, and I thank her for the helpful times in the laboratory and great chats at the pub, to Vlasta Jamnicky and her family for making me feel at home in UK and letting me be myself; Estelle Gill for the long walks through Edinburgh. I am also grateful to Colin Beck and Scott Dunbar for letting me stay in their flat and to Sabina Knees for her excellent cooking and help with accommodation. I would also like to thank Pablo Fuentes, Nazre Saleh, Alan Forest, Sumudu Rubasinghe, Rhiannon Crichton, Tobias Marczewski, Daniel Thomas, Markus Ruhsam, Bhaskar Adhikari, Philip Thomas and Claire Morter.

I also had tremendous help and support from my family. A special mention has to go to my mother who is one of the strongest and tireless people I know and the person who encouraged me to carry on during the most difficult times; always finding solutions when they were needed and supporting me throughout. A special thank you has to go to Pancho, my husband and father of my daughter and the most amazing and respectful person who has been so patience and has been my 'pillar' and understood all the difficulties and made huge sacrifices to make my dreams come true. An extra special mention has to go to Emilia, my daughter and the person that

makes my life completely happy. I would also like to thank Paulina my friend and sister-in-law who helped me throughout my thesis in many different and invaluable ways and of course I acknowledge the continuous support from my brother and sister Andres and Pilar and their families. Tia Adriana, Tia Nani and Mane for their friendship and support.

Finally I acknowledge ***The Darwin Trust*** of ***Edinburgh for their*** financial support and for giving me the opportunity to undertake something as important as a PhD.

Abstract

Major perturbations to the earth's climate such as glacial cycles can influence the distribution of plant species. They can also create marked intra-specific genetic structure if a species' range contracts into isolated refugia during periods of sub-optimal climate. Populations in different refugia can diverge, and subsequent colonists from these refugia can show the genetic footprint of their refugial sources. The use of genetic markers to study these phenomena is well established, and numerous detailed phylogeographic studies have been undertaken on species from the Northern hemisphere, particularly in Europe. In the southern hemisphere, however, there is still a shortage of studies. One geographical region of particular interest is Patagonia (southern South America south of 35°S). This is the only region in the southern hemisphere outside of Antarctica to have major ice fields during the Pleistocene glaciations. This region has a varied topography, dominated by the Andes, and the smaller coastal cordillera on the west coast of Chile. Combined, the past effects of glaciations, and the presence of physical barriers in the form of major mountain chains, make the presence of marked phylogeographic structure likely in this region.

I used genetic markers to study phylogeography in seven Patagonian plant species, to assess whether there is marked intra-specific genetic structure and whether this structure can provide insights into the past response of these species to glacial cycles. In particular, I wished to establish whether there is evidence for northern and southern refugia. I also aimed to establish whether there is a major phylogeographic divide between populations in the coastal cordillera and the Andes, and whether the Andes themselves act as a barrier leading to divergent lineages east and west of the Andes.

To undertake this work I sampled a total of 435 individuals from 77 populations of *Discaria chacaye, Donatia fascicularis, Escallonia virgata, Gentianella magellanica, Prumnopitys andina, Tepualia stipularis* and *Weinmannia trichosperma*. These species vary in their life history characteristics (longevity, seed dispersal distances) and levels of cold tolerance. Six of the species are angiosperms, and one *(P. andina)* is a conifer. The species were assayed for variation by sequencing a range of non-coding chloroplast regions.

Despite the wide range of species biologies, some generalities emerged from this study. Private haplotypes (variants restricted to a single population) were far more common in the north of the range than the south, and the presence of private haplotypes is largely concordant with areas to the north of the limit of the ice sheets at the last glacial maxima (42-43°S). This also coincides with the presence of a genetic suture zone previously identified in other species. Levels of population differentiation were high in most species (*Discaria chacaye* Gst = 0.474; *Donatia fascicularis* Gst = 0.580; *Escallonia virgata* Gst = 1.0; *Gentianella magellanica* Gst = 0.780; *Prumnopitys andina* Gst = 0.184*,* *Tepualia stipularis* Gst = 0.727 and *Weinmannia trichosperma* Gst = 0.347), with the main exception being *Prumnopitys* (in which chloroplast DNA is likely to be paternally inherited). There is a general trend for population differentiation to decrease from north-to-south, and populations in the northern Andes and northern coastal cordillera are typically strongly differentiated. Together these results are consistent with the long term survival of these species in northern refugia. In contrast, there is limited evidence for southern refugia, with only two species (*Gentinallea magellanica* and *Escallonia virgata*) showing data which are consistent (but not conclusively so) with the presence of long term *in situ* persistence south of 42-43°S. The results also highlight the conservation importance of isolated populations in the north Andes and the Coastal cordillera, as these populations are reservoirs of unique genetic variants for multiple species. Further south in the species' ranges there was no strong evidence for differentiation between the coastal range and the Andes, and populations from the west coastal shared haplotypes with those east of the Andes suggesting that they have recent common ancestry, and that being either side of the Andean mountain range itself is not a good predicator of populations being genetically divergent.

Chapter 1: Introduction

1.1 Introduction

This thesis is an investigation into the phylogeographic structure of several plant species from southern South America. The primary geographical focus is on the southern most portion of south America, consisting of the parts of Chile and Argentina, south of 35°S, that make up the region of Patagonia (defined here in its broadest sense). This region of southern South America, especially the western area of Patagonia has a diverse flora, including regions designated as global biodiversity hot spots (Arroyo *et al.*, 1999). Key factors which have been considered important in the evolution of this biodiversity include the region's heterogeneous geology and topography, volcanic activity and glacial cycles.

Western Patagonia is the only place in the southern hemisphere outside Antarctica which was covered by extensive ice sheets during glaciations (Figure 1.1) The effect of glacial cycles on plant populations include population isolation, local extinctions, reductions in population size and changes in distribution following survival in restricted refugia and subsequent recolonisation of the landscape. Understanding how plants species have responded to past major environmental changes such as glaciation gives insights into the processes that have given rise to current extant diversity (Hewitt, 2001). Moreover studies of plant population history can help to identify hotspots of intra-specific diversity (Petit *et al.*, 2003). This project aims to investigate the phylogeographic history of a sample of species with different ecological attributes from the southern South American flora in Patagonia. A major aim is to assess whether genetic diversity hot spots exist for these taxa, and to determine whether any diversity hotspots which are identified across these different species are geographically congruent.

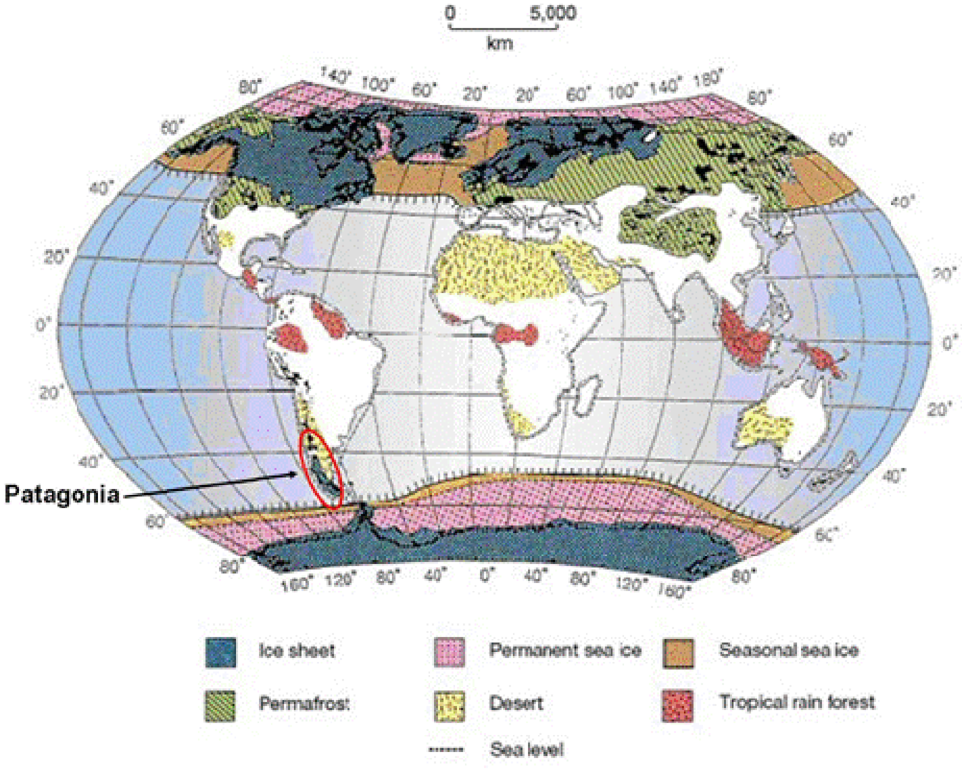


Figure 1.1. Map of the world's ice distribution within the Last Glacial Maximum, showing extensive ice coverage of Patagonia (modify from Hewitt, 2000)

In order to put the study into context it is essential to describe the geographic stage on which these processes have taken place, and to give an account of what has so far been gleaned about vegetation history of the Patagonian flora during and immediately after the last glaciation.

1.2 Study Area

1.2.1 Physical Geography of Patagonia

Patagonia comprises the southern region of temperate South America which goes from 35°S to 56°S (Figure 1.2). It extends over more than 20° in latitude, and covers 2,200 km in an N-S direction. Its northern limit is usually considered as an arbitrary line that runs south-east from the opening of the Chilean Maule River (Cauquenes) to the opening of the Argentinean Colorado River (Buenos Aires). Its south limit is the southern tip of the continent (Cape Horn), and in all it covers an area of about 1,140,000 km^2 (Vuilleumier, 1991).

The area of this study is the west side of Patagonia, which includes Chile and the east Andean slope of Argentina. The area has a very important fauna and flora, as it encompasses biogeographic islands, isolated by natural barriers (Armesto *et al.*, 1995a, b). These barriers are the Atacama Desert in the north, the Drake pass in the south, the Pacific Ocean in the west and the Andean and Coastal Cordilleras on the Argentinean/Chilean border, and in the west of Chile respectively. It contains three Ice fields that are remnants of those occurring on a much larger scale in the Pleistocene glaciations. These ice fields are: (1) Northern Patagonian ice field (46°30’-47°30’S; 73°-74°W); (2) Southern Patagonian Ice field (48°30’-51°S; 73°-74°W) and (3) Darwin cordillera Ice field (54°30’-55°S; 69°-71°W) (Figure 1.2).

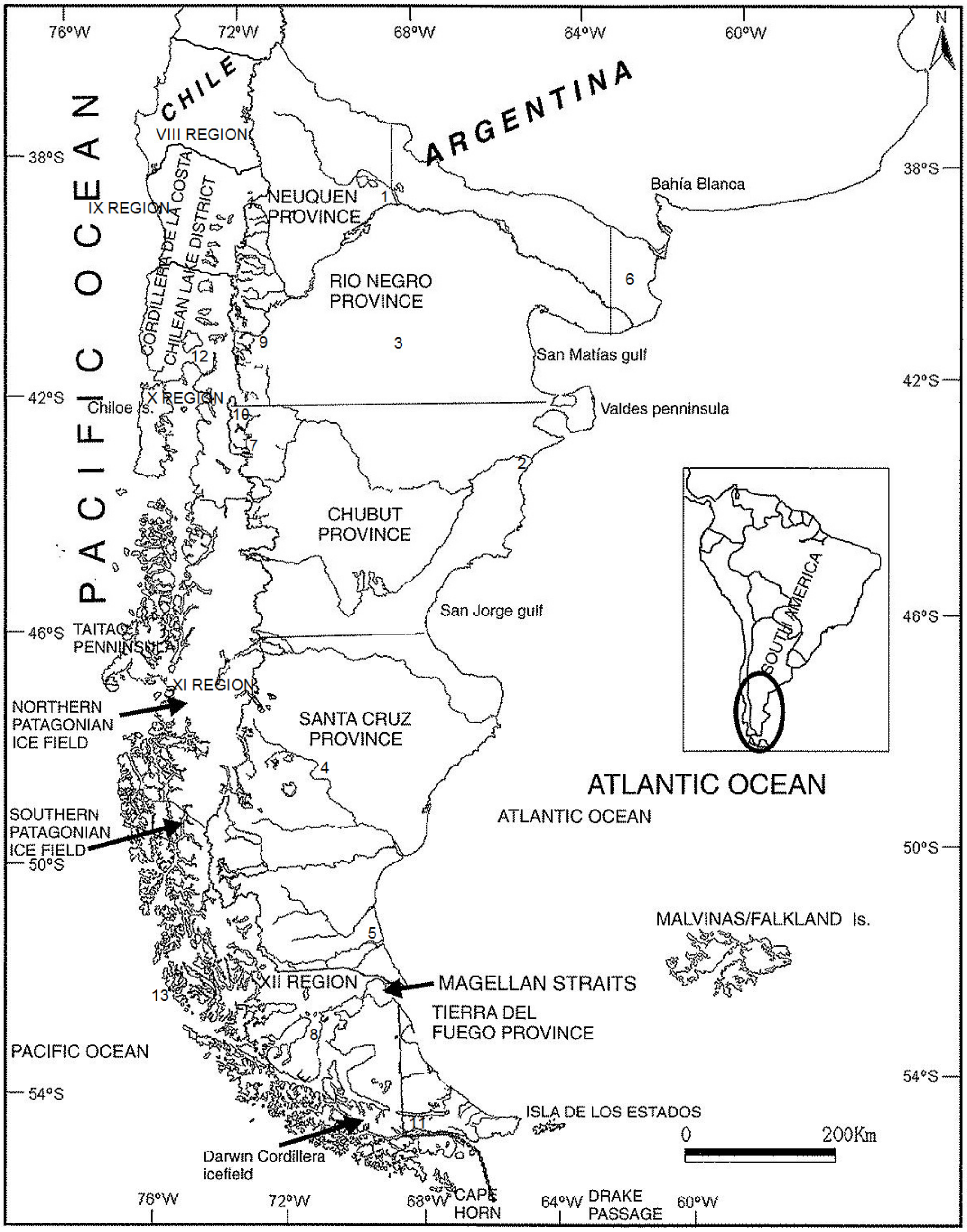


Figure 1.2. Distribution of Patagonia including Ice fields (modify from Rabassa, 2008). Number corresponds to populations used on Figure 1.4

1.2.2 Topography of Patagonia

The western side of Patagonia at the end of the Palaeozoic (230×10^6 yr) was only a marine depression with an accumulation of sediments which started to ascend at the end of the Mesozoic due the subduction of the Nazca plate underneath the South American plate, producing the Pacific Fire Belt which makes this area very seismic and volcanic (Rabassa, 2008). This geological activity, led to the formation of the Andean Cordillera and the Coastal Cordillera (Figure 1.3). There is a longitudinal flat area between both Cordilleras that was filled with volcanic and glacial sediments, called the Central Valley or Central Depression. The coast line of northern Patagonia is very smooth, while its southern coast line has lots of islands, fjords and lakes. The formation of these is related to several glaciations events that have occurred in this area.

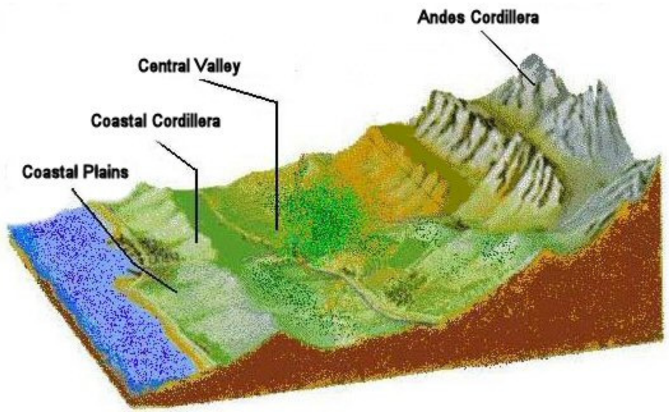


Figure 1.3. Topographic shape of western Patagonia (modify from Inzunza, 2003).

The main topographic features in Patagonia which are relevant for the floral distribution are:

A. Andean Cordillera.

This is a young mountain range located in the west of South America, with a north south distribution, that extends from the Antillas Sea to the Antarctic Peninsula. From the Magellan fault to the Staaten island (54-56°S; 63-72°W) the range curls round in a W-E direction. The Andes are the second highest mountain range in the world, and the highest peak is 6,956 m (Aconcagua). The Andes Cordillera is 7,240 km long and has an average width of 241 km. Its average altitude is 3,660 m and the mountains decrease in elevation from north to south. In its southern distribution due to erosion and glacial processes, the Andes Cordillera sinks in places below the

Pacific Ocean, producing many islands and fjords until its complete disappearance in Cabo de Hornos (Cape Horn). It reappears in the Arco de las Antillas del Sur and later in the Antarctic peninsula, forming the Antarctic Cordillera.

The Andes is characterized by the presence of volcanoes and permanent ice sheets and forms a massive rock barrier between Chile and Argentina. Its uplift started 19 mya and is still ongoing. Blisniuk *et al.* (2006) reported that this mountain range was uplifted by more than 1 km between 14-17x10^6 yr. The Andes create a rain shadow effect on the eastern side of the cordillera, enhancing the aridity on its eastern side and the high precipitation on the western side, which impacts on plant and animal distributions, (Villagran, 1995). The cordillera is crossed by small valleys at high altitude that have a west-east direction, and these areas are where almost all of the evergreen forest grow on the arid Argentinean side of the Andes.

<u>B. Coastal</u> <u>Cordillera</u>. This is an older and smaller mountain range than the Andes Cordillera, which also runs parallel to the Pacific Ocean. It is formed by Precambrian and Palaeozoic sedimentary and metamorphic rocks. Like the Andes it forms a physical barrier, although due to its lower elevation its rain shadow effect is a lot milder. It begins at the northern limit of the border between Chile and Peru and it terminates in the Taitao Peninsula. It is 190 km long with a maximum width of 55 km and a highest peak of 1,440 m. The highest peaks in the Coastal Cordillera (from north to south) are: la Campana (~33°S), Cantillana (~34°S), Nahuelbuta (~38°S), Cordillera Pelada (~40°S) and Piuche (~42°S which is on Chiloe Island). The Coastal Cordillera, like the Andean Cordillera, decreases in altitude from north to south distribution. In its southern distribution, it tapers into the Pacific Ocean, and forms many islands and archipelagos until the coast line of Aysen where it disappears completely below the sea at the end of the Taitao peninsula.

<u>C. Central Valley.</u> This area corresponds to a plain between the Andes and the Coastal Cordillera, which has been formed by the erosive action of the water and ice sheets originating from both Cordilleras. The plain begins at the Chilean-Peruvian border and it continues southwards until strong erosion and the effect of glaciations

have led to the origin of many lakes. Further south, in Puerto Montt, it disappears completely and is replaced by channels and fjords. Its altitude gets lower with the latitude. Therefore in the north it reaches up to 1,400 m and in the south it drops to sea level. The reverse happens with its width, such that it gets wider in the south reaching almost 100 km in Chillan.

1.2.3 Climate of Patagonia

The climate in Patagonia is very variable due to its mountainous topography, geographical position and large latitudinal extent, and includes climates ranging from Mediterranean to polar. The major elements that control the weather along Patagonia are the high pressures produced by the Southern Pacific Subtropical Anti-cyclone (SPSA), the low pressure circumpolar band of migratory systems and the westerly wind belt. A cooling effect on the average climate is caused by the moderating influences of the sea, winds, and the Humboldt Current. This current is formed by the drag of cold water from Antarctica that produces a south-north current along the Patagonian coast line. This current begins at Puerto Montt and is responsible for an increase in the precipitation rates and a decrease in atmospheric temperatures. The Andean Cordillera also influences the climate, forming a barrier to the warm winds that come from the Argentinean steppe and maintaining high humidity in western Patagonia due to maritime influences. Annual temperatures vary from 16°C in the north to 8°C in the south or even down to 4°C in the outermost Magellanic Islands (Coronato *et al.*, 2008). The average rain fall on the eastern side of the Andes is 250 mm (150-1,000 mm) but on the western side it varies between 500 mm and 6,000 mm (Figure 1.4).

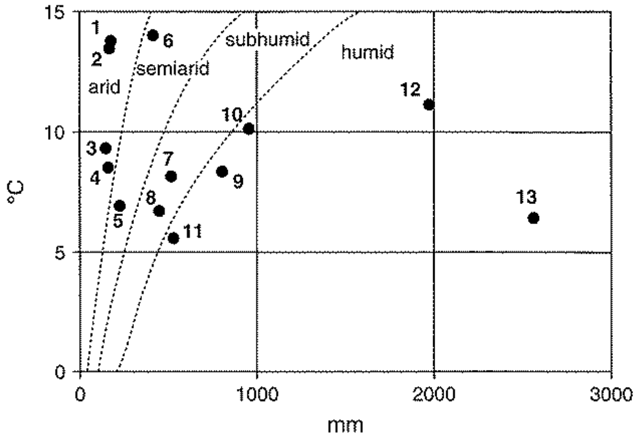


Figure 1.4. Temperature-precipitation of Patagonian climatic stations.
Station 1 Cipolletti; 2 Trelew; 3 Maquinchao; 4 Gobernador Gregores; 5 Rio Gallegos; 6 Patagones; 7 Esquel; 8 Punta Arenas; 9 Bariloche; 10 Lago Puelo; 11 Ushuaia; 12 Puerto Montt; 13 Evangelistas (Senso Coronato *et al.*, 2008). For population distribution see Figure 1.2.

The Patagonian climate was divided in three major climate types for this study (see Figure 1.5). These are summarised below.

Temperate Mediterranean Climate: This is the climate that occurs in the north of Patagonia and varies in relation to the proximity to the sea and latitude, and consists of three types: (a) Warm temperate, with a dry season of 4 to 5 months; with winter rain fall of 1,000 mm, and an average temperature of 12ºC. (b) Warm temperate with one dry season (less that 4-5 months); with a precipitation rate around 1,000 mm, with snow fall in winter. The average annual temperature is 14ºC. (c) Rainy temperate with Mediterranean influence; this climate is distributed in the coastal area, especially in the higher peaks of the Coastal Cordillera. There is an average precipitation of 1,500 mm and an average temperature range in the year of 8ºC.

Rainy Temperate Climate: This climate is characteristic of central western Patagonia (known as the 'Lake District'). There is precipitation throughout the year, and it does not have a distinct dry season. The average rain fall is 2,000 mm and the average temperature is 11ºC, with low temperatures differences between months and also within the day.

Sub polar-Cold Climate: This climate is formed by strong westerly winds and constant fronts of low pressures. Due to differences between the coastal regions, the Andes and the ice sheets surfaces, this climate can be divided into three distinct climate types: (a) Temperate cold from the coast; which is a coastal weather with high levels of winter rain fall, an average of 6,000 mm. It has low differences in temperatures within the year, with oscillations of only 4ºC, and the average temperature is 9ºC. (b) Steppe conditions; this climate is characteristic of the eastern side of Patagonia with precipitations that can vary between 250 to 500 mm. The average high temperature is 10ºC and the average lowest is 2ºC. In winter the only available water comes from the snow. (c) Moorland climate; this climate type occurs in the south of the Magellan Strait and in Tierra del Fuego. There is an annual rainfall of 1,000 mm and the temperatures are very homogeneous with an annual average variation, between the coldest and warmest months, of only 4ºC.

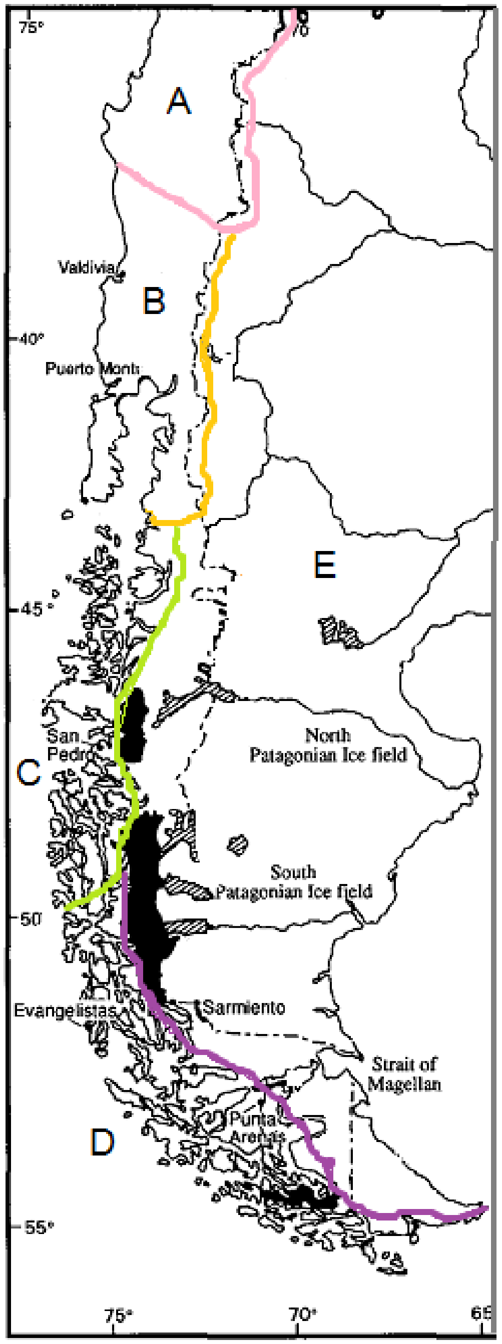


Figure 1.5. Distribution map of Patagonic climate. Lettres and colors correspond to the major climates. A = Temperate Mediterranean; B = Rainy Temperate; C = Temperate cold from the coast; D = Moorland; E = Steppe.

1.3 Historical Climate and Historical Geology

Since the Andes started to ascend, many geological events have happened in Patagonia, involving the action of the active volcanoes and the worldwide glacial periods. The glaciers expanded following global climate change. This was driven by insolation and ice sheet changes from the northern hemisphere due to the Croll-Milankovich cycles.

In southern South America during the early Pleistocene, glaciers started to establish, producing several changes to the fauna, flora and geography. At about 1.2×10^6 yr. the glacial ice sheet was fully developed, lowering the sea levels up to 100-140 m. The Last Pleistocene glaciations in South America had many glaciations of which the most important are as follows:

1) Great Patagonian Glaciation (GPG) named by Rabassa (2008). This is also known as the Initioglacial by Caldenius (1932).

This glaciation was established in the early Pleistocene about 1×10^6 yr, and showed the maximum expansion of the ice outside Andean Patagonia (Figure 1.6).

2) Coldest Pleistocene Glaciation (Rabassa, 2008) or Daniglacial (Caldenius, 1932) This distribution of glaciers was established during the early-middle Pleistocene at about 0.78×10^6 yr.

3) Late Pleistocene Glaciation (LP) named by Rabassa (2008) and also known as the Gotiglacial (Caldenius, 1932).

This was established in the middle pleistocene about 0.128×10^6 yr.

4) Last Glacial Maximum (LGM) named by Rabassa (2008), also known as Finiglacial (Caldenius, 1932) and the Llanquihue glaciation (Clapperton, 1993) due to its biggest extension within the Chilean Lake District.

The LGM was established within the late Pleistocene and its maximum extension was between 25,000 yr and 23,000 yr depending on the latitude. There were five glacial advances at 33,500; 29,600; 26,940; 23,060; 21,000; 15,900 and 14,890 yr. There was a rapid deglaciation starting at 14,000 yr before present (Denton *et al.*, 1999a, b; Moreno, 1997; Villagran, 1995). The overall glacial history and that for the individual regions are outlined below:

- *Overall Picture*

Within the LGM almost all the territory south of 42°30'S experienced glacial advances, as did the central depression. On its western side (Lake District 39° to 42°S) and on the Andean slopes from 36°S southwards (Figure 1.5), the temperatures was 7°C to 8°C lower than at present with twice the modern annual precipitation. Precipitation changed with latitude, due to an intensification and a northern shift of the Westerlies storm track. Thus the southern area (~42°S-55°S) was drier than at present during this period (Manzini *et al.,* 2008; Moreno, 1997).

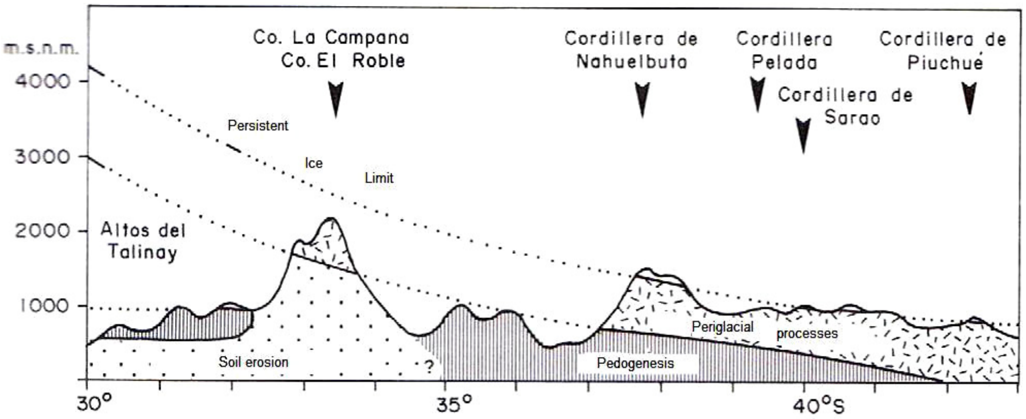


Figure 1.6. Glacial process during LGM (Modify from Armesto *et al.*, 1995a).

In central locations ~34°S (Laguna de Tagua Tagua) the weather was cooler and more humid. There were also many areas lacking morphodynamic (soil evolution). These were areas covered by glaciers and areas located in the plains of the central depression affected by fluvioglacial processes and the tops of high hills subject to solifluxion (displacement of first layers of soil due to deep freezing). The glaciation therefore led to a lack of places for plant species to survive (Figure 1.6).

In Patagonia during the glacial period both the northern and southern Patagonian ice fields together formed a single ice sheet, which was distributed from north of the Chilean Lake District to the south of Tierra del Fuego, covering 1,800 km in total Figure 1.7; Figure 1.8) (Hulton and Sugden, 1994; Caldenius, 1932). This massive ice sheet (up to 1,130 m thick) was cut in two by 12,800 yr at the final stage of the LGM (Last glacial maximum). Figure 1.9 shows a simulation of the southern Patagonia ice sheets by Hulton *et al.* (2002), which suggest shrinkage of the ice sheets from the LGM until the present day. (Note that as this work is focused in the southern Patagonia, the simulation is not so precise in the northern distribution of the ice sheets). Although these studies suggest a wider distribution of the ice sheets, Markgraf *et al.* (1995) has argued that the southern hemisphere glaciation was weaker than that in the northern hemisphere, leaving many areas without ice sheets, due to the proximity of southern Patagonia to the sea.

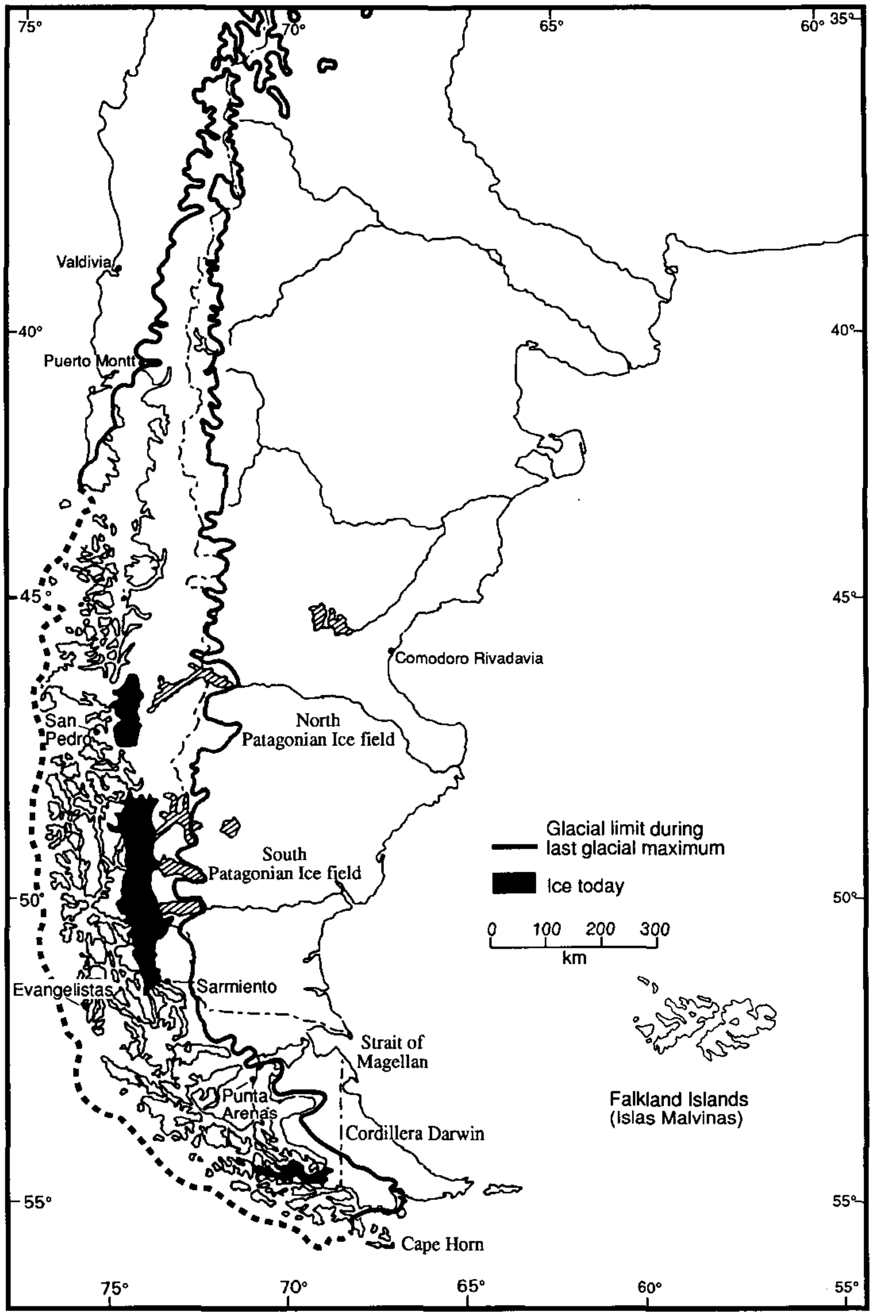


Figure 1.7. Distribution map of South America glaciation during the LGM (Hulton and Sugden, 1994).

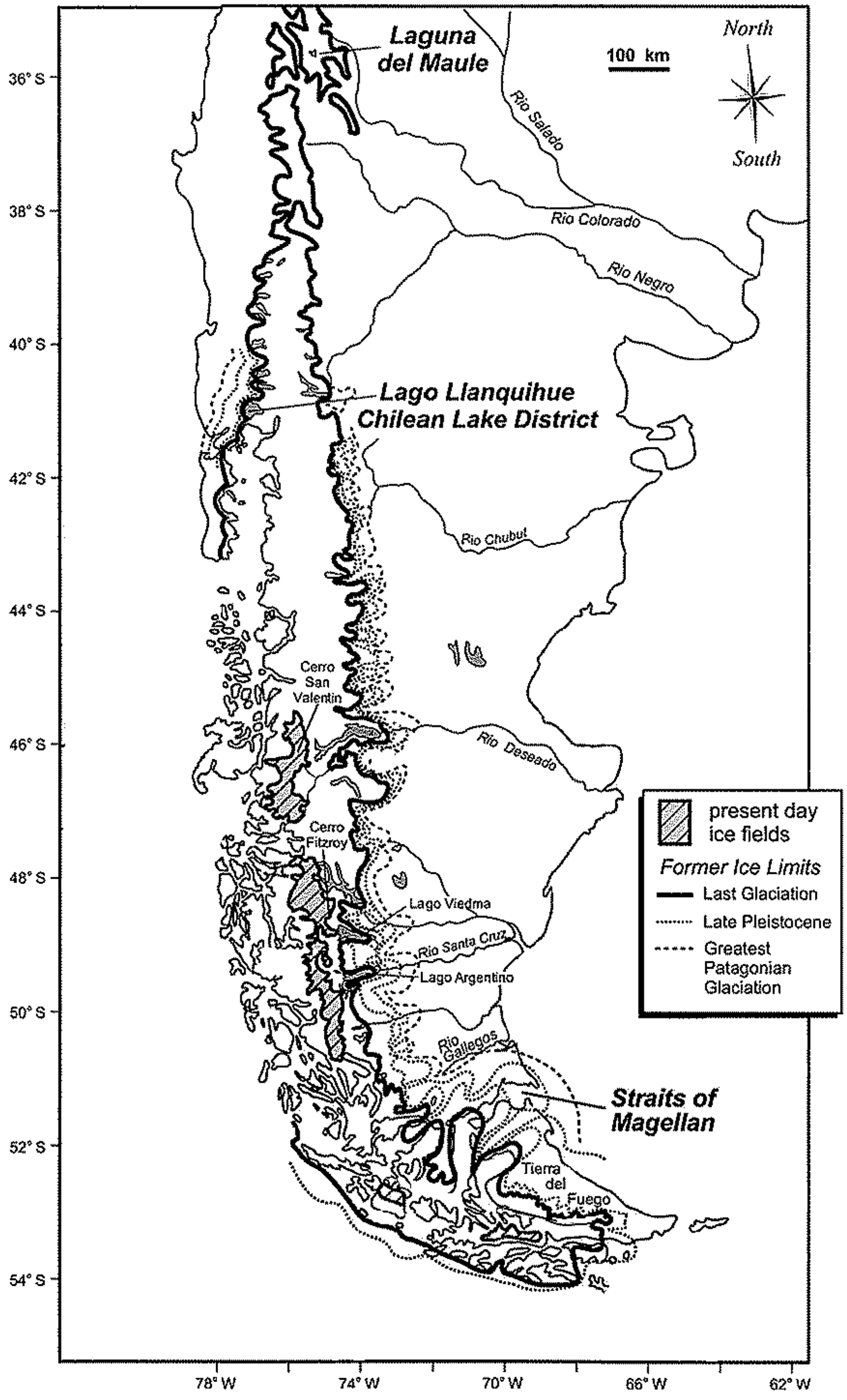


Figure 1.8. LGM and greatest Patagonian glaciation distribution map (Modify from Rabassa, 2008).

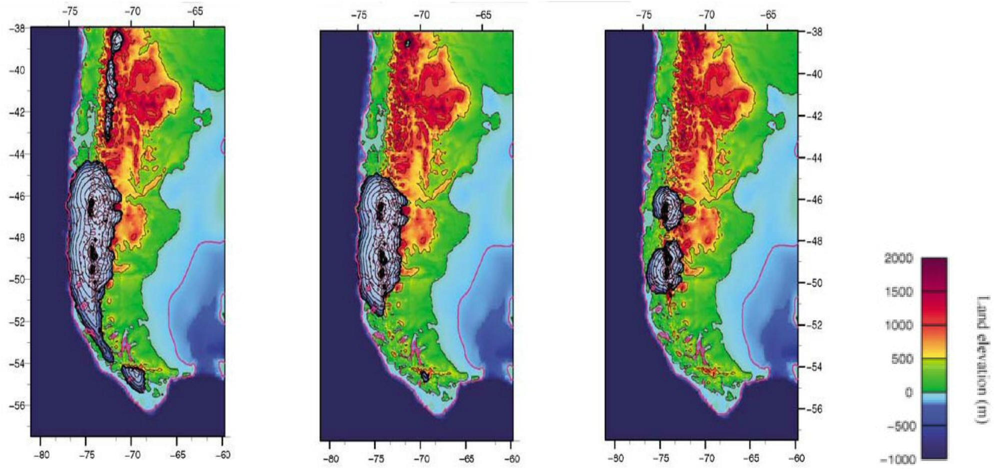


Figure 1.9. Simulation of the Patagonic ice sheets movement during the LGM until present (Hulton *et al*., 2002).

- *Northern Patagonia (Mediterranean region ~34°S)*

Although there is no information about glacier distributions in this area, a few studies of pollen records have suggested the climatic situation during the LGM. A study from coastal Valparaiso showed that the climate was colder and wetter (Groot and Groot, 1966). The same pattern is shown in the central depression at ~34°S, possible due to the northern shift of the westerlies, producing a rise of the precipitation levels (Lamy *et al.*, 1999). On the eastern side of the Andes cordillera there is a different picture. Due to the rain shadow produced by the Andes the climate was drier and colder than on the western slopes (Markgraf *et al.*, 2009).

- *Central Patagonia (Lake District and central south ~37°S-46°S).*

Studies by Denton (1999 a, b), analysed the climate changes during the LGM and the subsequent deglacial period, using moraine chronologies and palynological data from the Lake District. They inferred that the climate conditions were fully glacial between 29,000-14,000 yr, with its maximum expansion between 22,570-22,295 yr. During this period the snow line dropped almost 1,000 m below its current position, together with a lowering of the tree line, suggesting a drop of temperature to 6°C-8ºC. During the LGM period there were several advances of the ice sheets, the most important being at 29,000 yr, 26,000 yr, 22,000 yr and 14,000 yr, and the glacial maxima occurred twice (before 17,000 yr and before 15,000 yr). During the LGM the distribution of the ice within the Lake District was more extensive on the western slopes of the Andes and in the central depression including the southern part of Chiloe island. On the eastern side of the Andes no big glacier surfaces were established. Small glaciers were present only on west-east valleys and in several cirques distributed within the eastern area (Glasser *et al*., 2008).

The post glacial period commenced with warming pulses beginning at 14,000 yr and at 13,000-12,000 yr leading to the retreat of the ice sheets and leaving large regions of bare land. Many pollen studies on this area, also suggest a colder and more humid climate than at present during the LGM (eg; Heusser *et al*., 2006a, b; 2000; 1999; 1996; Markgraf *et al.*, 2009; Villagran and Armesto, 2005; Moreno, 2004; 2000; 1997; Villagran 2001; Moreno *et al*., 1999). However there was always a big climatic difference between the western and the eastern slopes of the Andes. The eastern slopes had an even colder and drier climate than at present (Paez *et al*., 1999).

- *Southern Patagonia (~46°S-55°S).*

Studies by Hubbard *et al*. (2005), Sudgen *et al.* (2005), and McCulloch *et al.* (2005a,b) suggested that around the Straits of Magellan the LGM began some time after 31,000 yr reaching its maximum extension on two or more occasions between 25,000 and 23,000 yr and a smaller extension at 17,000 yr. Within its maximum extensions ice lobes covered almost the entire isla grande de Tierra del Fuego and the Taitao peninsula had one of the biggest LGM glaciers covering the peninsula and many adjacent islands. It has been suggested that the Taitao peninsula glacier was an isolated entity and not an extension of the Northern ice sheet that persist until now (Glasser *et al*., 2008). There was also a glacial advance that only occurred in southern Patagonia, which was coincident with the Antarctic Cold Reversal (ACR) at 15,000-12,000 yr. This could be due to an Antarctic effect on Patagonian glaciation, rather than to northern hemisphere changes, although there is still argument about this (Rabassa, 2008). The climate during the LGM was colder and drier than at present. This could be explained due to the northern shift of the westerlies storm track, leaving the southern most area deprived of rain fall (Paez *et al.*, 1999; Heusser 1995a, b).

Deglaciation was extremely rapid occurring in two steps at 17,000 and at 11,000 yr. In this latter period the ice sheets suddenly produced large amounts of water forming most of the lakes in southern Patagonia, draining into the Pacific Ocean and raising the sea level by 120 m.

1.4 Vegetation

The Patagonian flora is very diverse reflecting the differences in climate, topography, latitude, geology and soils within the region. It has more than 5,000 vascular plants of which 49% are endemic (Villagran and Hinojosa, 1997). This high level of endemism is related to the isolation of the flora by natural boundaries that surround western Patagonia (Armesto *et al.*, 1995a). The amount of endemics and their fragility to anthropogenic disturbances (exotic plantations, crop plantations and wood harvesting), have resulted in the main area of north and central Chile between 20ºS and 47ºS being classified as a hotspot for conservation.

This region has gone through severe changes caused by glaciation and volcanism disrupting its vegetation distributions many times. These geological changes can be seen by the fact that almost all the endemics grow within an area between the Maule valley and Valdivia (35°S-41°S), suggesting the extinction of these species in the south and their survival within this area. Another fact is the discontinuous distribution of species on both cordilleras and the existence of cold tolerant species growing in small populations on top of the coastal cordillera (Villagran *et al.*, 1995).
The main Patagonian vegetation is composed of four different vegetation types (Figure 1.10). These are: Magellanic moorlands; Alpine scrub, *Nothofagus* forest and Patagonian steppes.

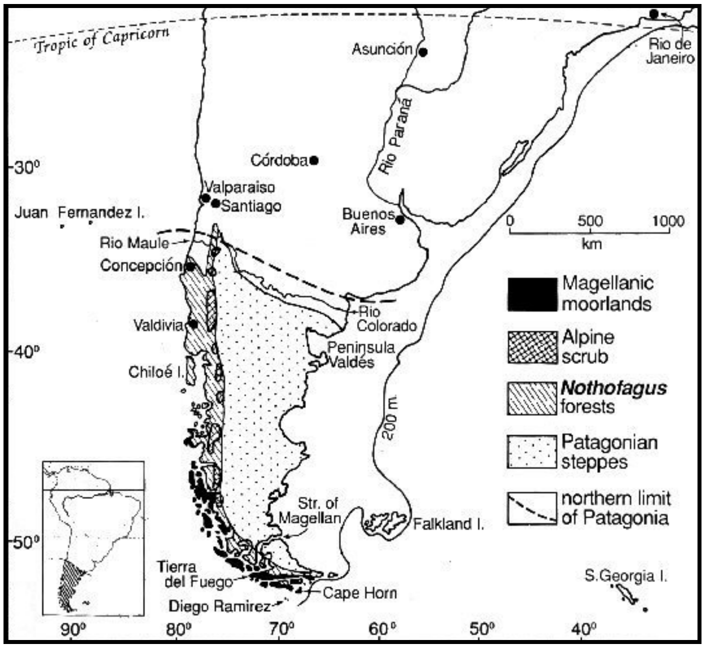


Figure 1.10. Distribution map of Patagonic vegetation (Vuilleumier, 1991).

Magellanic moorlands. These are series of bog communities that are distributed in areas with high rainfall (2,000-2,500 mm). These extend from the Chilean archipelago, in sheltered areas and in the outermost islands (Correa, 1998; Villagran and Hinojosa, 1997; Moore, 1983). These bog communities are mainly represented by prostrate dwarf shrubs, cushion plants, grass-like plants and bryophytes.

Alpine Scrub. This community occurs mostly within the timber line of the Andean Cordillera. It is distributed along all the Patagonian Andes and is associated with high winds, winter snowfall and lack of water in summer (Moore, 1983). This community is made of heath-like plants such as cushions heath and dwarf shrub heath.

Nothofagus forests. This forest type can be divided into two; the deciduous and the evergreen *Nothofagus* forests. Both are distributed along most of the western Patagonia. The deciduous type grows on both flanks of the Andean Cordillera in southern Patagonia from sea level up to 500 m, in sheltered areas where the annual rain fall is 400-800 mm (Donoso, 1998). The dominant species in this type of forest are *Nothofagus pumilio* and *N. antarctica*.

The evergreen forest is distributed in both Cordilleras in northern Patagonia from sea level up to 700 m, in areas with 2,500-6,000 mm of rainfall (Donoso, 1998). This forest type includes a high diversity of species such as *Aextoxicon punctatum*, *Drimys winteri*, *Laurelia philippiana*, *Nothofagus dombeyi*, *Persea lingue* and *Saxegothaea conspicua.*

Patagonian Steppe. This plant community grows along all the latitudinal range of Patagonia in its eastern distribution (Figure 1.7). It contains grassland heath and shrubs and occupies areas with dry and windy climates.

1.4.1 Vegetation History

- Overall Picture

During the Last glacial maximum, south of 43°S and in the Lake District Andes, around two third of the present surface dominated by forest was devastated by glaciers (Villagran and Armesto, 2005; Villagran *et al.*, 1995).

In general, glacial pollen records indicate the presence of Gramineae and Compositae during the glacial periods (
Table 1.1), although in some areas such as Chiloe and the central depression of the Lake District, there are also traces of magellanic moorland. Within the Lake District on the slopes of the coastal cordillera, traces of cold tolerant *Nothofagus* species were present, and further north (~39°S) on the coastal cordillera more thermophilic species were present.

Using these findings Villagran (2001) and Villagran and Armesto (2005) has created a LGM forest distribution model for Chile, which relates the migration of the different species that grow now between 38°S to 48°S to their glacial distributions (Figure 1.8). She suggests that during the LGM the higher peaks of the coastal cordillera were devoid of vegetation due to solifluction processes, and below this area moorland species were growing. Below the moorland species appeared the northpatagonic forest (cold tolerant). In the northern ranges of the coastal cordillera (Nahuelbuta and further north) it is suggested that temperate forest survived on the lower slopes (Figure 1.11; Figure 1.12).

This model suggests that on Chiloe the only species that grew during the LGM were the moorland species while further north, more thermophilic species survived on the coastal cordillera. The central depression, which was covered by glaciers on its eastern side, was apparently only occupied by the cold tolerant moorland species on its western slopes.

As the glaciers retreated it is postulated that there was plant altitudinal displacement. Magellanic moorland shifted to occupy newly available ice-free areas such as the highest mountains of the Coastal Cordillera, leaving these species in small isolated populations surrounded by north patagonic forest. This Patagonia forest grew up to elevations of ~500 m altitude. Below this the Valdivian rain forest was located extending down to sea level, due to its superior competitive ability in warmer climates (Armesto *et al.*, 1995a, b; Villagran *et al.*, 1995).

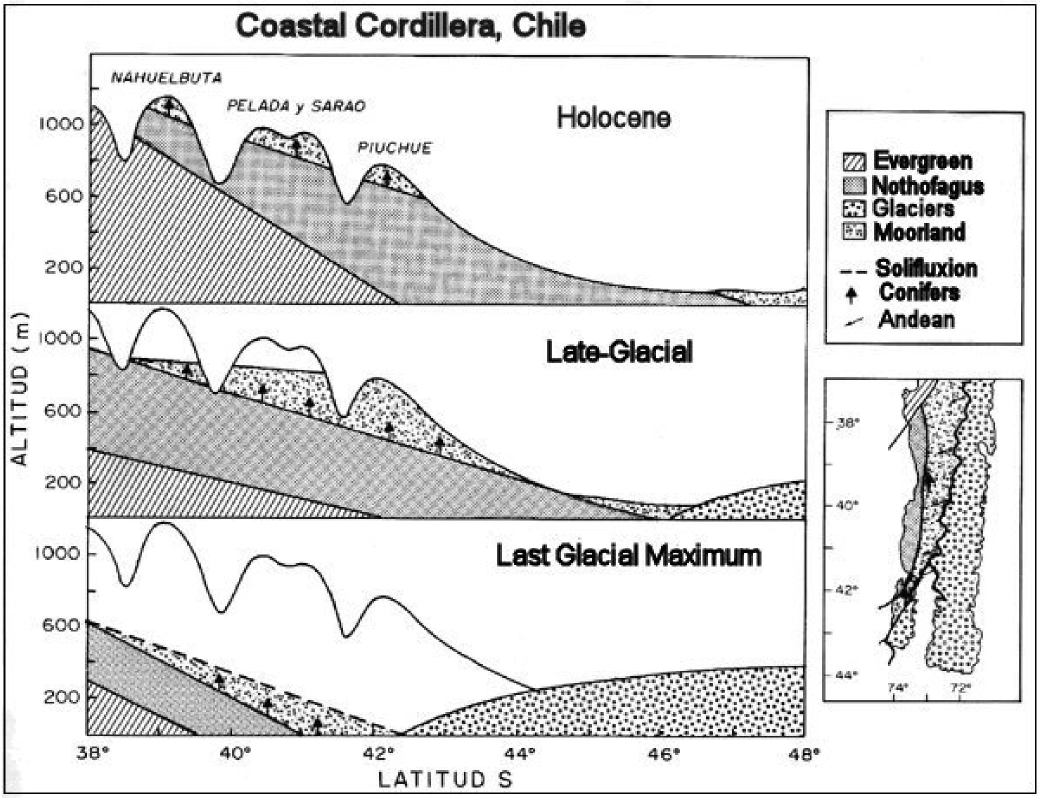


Figure 1.11. Distribution of the vegetation in the Coastal Cordillera during the glacial and post glacial periods (Villagran, 2001).

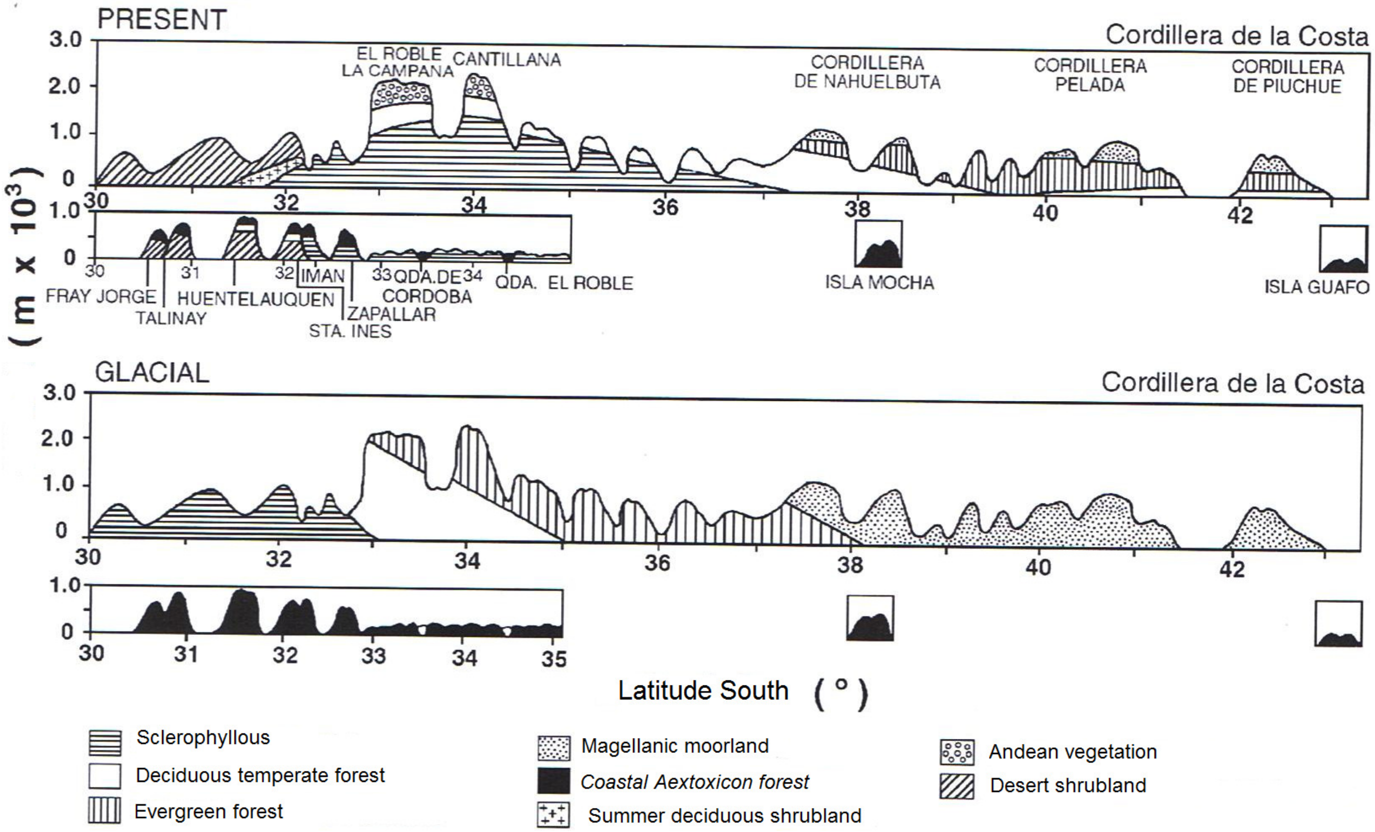


Figure 1.12. Coastal cordillera plant distribution within the LGM and at present (Villagran, 2001).
Large profiles correspond to the coastal cordillera between 30°S to 43°S and shorter profiles correspond to a zoom of the area between 30°S to 35°S plus two islands (Isla Mocha ans Isla Guafo)

Table 1.1. Summary of pollen studies in Patagonia

Core Area	**Area**	**Lat (S)/ Long (W)**	**Reference**	**Vegetation type during LGM (yr)**
Tagua tagua	central depression north	34˚30’-71 10’	Lamy *et al.*, 1999	at ~28000 *Graminea, Nothofagus, Compositae*
Malin vaca lauquen, Neuquen		36˚51’-71 02’	Markgraf *et al.*, 2009	Between 17000 and 14000. *Poacea*, steppe shrubs, *Asteraceae*
Bajada del rahue		eastern ~40	Markgraf *et al.*, 1986	*Gramineae, Compositae, Nothofagus*
Nueva Braunau	central depression	40˚17’-73˚04’	Heusser *et al.*, 2000	subantarctic parkland (*Gramineae*) with Magellanic moorland
Canal de la punilla	central depression	40˚57’-72 54’	Moreno, 1997	*Nothofagus* and Magellanic moorland
Canal de la puntilla, lago llanquihue	central depression	40°57'-72°54'	Moreno *et al.*, 1999	at ~20000 max non arboreal, *Graminea Nothofagus Compositae/ Tubuliflorae*
Pto. octay	central depression	40°58'-72°53'	Heusser *et al.*, 1999	at ~28000 *Graminea* and *Nothofagus*
Fundo llanquihue	central depression	41°13’-73 03’	Heusser *et al.*, 1996	subantarctic parkland with Magelllanic moorland
Llanquihue	central depression	41°16'-72°59'	Heusser *et al.*, 1999	at ~15300 *Graminea, Nothofagus* and *Gunnera*
Alerce	central depression	41°23’-72°52’	Heusser *et al.*, 1996	subantarctic parkland dominated by *Gramineae*
Alerce	central depression	41°23'-72°52’	Heusser *et al.*, 1999	from 16000 to 13000 *Graminea* and *Nothofagus*
Huelmo site	central depression	41°30’73°00’	Moreno and Leon, 2003	*Nothofagus* parkland with magellanic moorland and conifers

Table 1.1. Continued

Core Area	**Area**	**Lat (S)/ Long (W)**	**Reference**	**Vegetation type during LGM (yr)**
Fundo Sta. Helena	central depression	41°30’-73°06’	Heusser *et al.*, 1999	13800 *Nothofagus*, *Graminea* and *Empetrum*
La campana	central depression	41°37’-73°11’	Heusser *et al.*, 1999	at ~18000 *Nothofagus*, *Gramineae* and *Empetrum*
Lago Condorito/ seno reloncavi	central depression	41°45’-73°07’	Moreno, 2004	at ~ 14000 *Nothofagus*, conifers, *Myrtaceae*
El trebol	central south/ Argentina next to Bariloche	41° 71’	Bianchi *et al.*, 1999	absence of pollen during LGM only inorganic sediments, start at 14000
El trebol and mosquitos	Argentina 1 next to Bariloche 2 next to Cholila further south	41°07’-71°49’ 42°50’-71°40’	Whitlock *et al.*, 2006	at 16000 *Poacea*, *Asteraceae*, *Tubuliflorae Nothofagus* and conifers
Fdo. linea pantanosa	central depression	41°05'-73°00'	Heusser *et al.*, 1999	from 19000 to 14000 *Graminea*, *Nothofagus Empetrum, Gunnera*
Frutillar bajo	central depression	41°08'-73°01'	Heusser *et al.*, 1999	at ~26900 *Graminea* and *Nothofagus*
Fdo llanquihue	central depression	41°13'-73°03'	Heusser *et al.*, 1999	from 20600 to 14000 *Graminea*, *Nothofagus Empetrum, Gunnera*
Bella vista	central depression	41°18'-72°58'	Heusser *et al.*, 1999	at ~15600 *Graminea, Nothofagus* and *Gunnera*
Canal Tenglo	central depression	41°28'-72°58'	Heusser *et al.*, 1999	at ~23000 *Graminea, Tubuliflorae and Nothofagus*
Pta. Penas	central depression	41°29'-72°53'	Heusser *et al.*, 1999	at ~16000 *Nothofagus* and *Gramineae*

Table 1.1. Continued

Core Area	**Area**	**Lat (S)/ Long (W)**	**Reference**	**Vegetation type during LGM (yr)**
Taiquemo, Chiloe island	Chiloe island	42°10’- 73°35’	Heusser *et al*., 1999	at ~23000 *Graminea, Tubuliflorae, Nothofagus, Empetrum* and *Lepidothamus*
Teguaco	Chiloe island	42°17’-73°35’	Heusser *et al*., 1999	at ~22300 *Nothofagus, Graminea* and *Tubuliflorae*
Taiquemo, Chiloe island	Central south	42°17’-73°60’	Heusser *et al*., 2006a	21430-23223 *Graminea* in subantarctic park land with moorland species
Dalcahue	Chiloe island	42°20’- 73°39’	Heusser *et al*., 1999	at 21 400 *Graminea* and *Nothofagus*
Dalcahue	Chiloe island	42°21’-73°39’	Heusser *et al*., 1996	subantarctic parkland, dominated by *N. dombeyi, gramineae*, and magellanic mooorland
Taitao peninsula		42°25’-74°24’	Lumley and Switsur, 1993	at ~14000 *Gramineae, Gunnera* and *Ericaceae*
Mayol	Chiloe island	42°38’-73°45’	Heusser *et al*., 1999	at ~ 14900 *Graminea, Nothofagus* and *Empetrum*
Lago Condorito/ seno reloncavi	central depression	42°45’-73°07’	Moreno, 2000	at ~13000 *Nothofagus*, conifers, *Myrtaceae* and *Poacea*e
Laguna Tahui	Chiloe island	42°50’- 73°30’	Abarzua *et al*., 2004	at ~ 16000 *Nothofagus, Lomatia* and *Poaceae*
Laguna Facil	Taiquemo peninsula	44°19’-74°17’	Haberle and Bennet, 2004	at ~15000 *Astelia, Gunnera* and *Empetrum*
Laguna Oprasa	Taiquemo peninsula	44°21’-73°39’	Haberle and Bennet, 2004	at ~16000 *Gunnera, Astelia* and *Empetrum*
Mallin Pollux coyhaique		45°41’-71°50′	Markgraf *et al*., 2009	at ~17000 high aquatic taxa, *Poacea* and *Acaena*

Table 1.1. Continued

Core Area	**Area**	**Lat (S)/ Long (W)**	**Reference**	**Vegetation type during LGM (yr)**
Los toldos	far east	47° 22’-68°58’	Paez *et al*., 1999	at~ 12600 *Ephedra* steppe and grass steppe
Alero Cardenas	east/Sta. cruz	47°18’-70°26'	Mancini, 1998	at~ 7000 steppe, *Graminea, Asteraceae, Tuboliflorae* and *Ephedra*
La Martita cave	east/Sta. cruz	48°24’-69°15’	Mancini, 1998	at ~8000 grass steppe, *Asteraceae* and *Tubuliflorae*
Torres del Paine	Far south	50°59'-72°40'	Heusser, 1995b	at ~11000 *Graminea, Ephedra* and *Acaena*
Gran Campo pen munoz Gamero	Far south	52°48’-72°55’	Fesq-Martin *et al*., 2004	at ~15000 non arboreal pollen *Gunnera, Graminea* and *Compositae*
Puerto del hambre	Far south	53°36’-70°55’	Heusser, 1995b	16000 tundra
Pta Arenas	Far south	53°09'- 70°57'	Heusser, 1995b	at 14000 *Nothofagus, Graminea* and *Empetrum*
Pto del hambre	Far south	53°36'- 70°55'	Heusser, 1995b	at ~16000 *Acaena, Empetrum* and *Graminea*
Bahia Moat	TF.	54°58’-66°44’	Heusser, 1995b	at ~7000 *Graminea, Nothofagus, Botrychium*, and *Filicinae*

- *Northern Patagonia (Mediterranean region ~34°S)*

Although this region was unglaciated, vegetation during the LGM is considered to have been very different to what is known today. A study from coastal Valparaiso showed that during glacials the forest was dominated by cold tolerant species such as *Nothofagus* and *Podocarpus*, and during interglacials a more broad leaved forest predominated (Groot and Groot, 1966). In the central depression (Laguna Tagua Tagua ~34°S), the vegetation that grew during the glacial consisted mainly of species of conifer, Nothofagaceae, Gramineae, Compositae and aquatic taxa (Heusser, 1983). On the east of the Andes the vegetation was dominated by Gramineae and grass steppe, which could be interpreted as a northern extension of the steppe (D'antoni, 1983).

- *Central Patagonia (Temperate region 39°S-46°S, Lake District and Taitao peninsula)*

In this area it has been suggested that many species survived the glacial period in ice free zones (eg. Mathiasen and Premoli, 2010; Tremetsberger *et al.*, 2009; Markgraf *et al.*, 2009; Marchelli and Gallo, 2004). Fossil studies by Villagran (2001) suggest that cold tolerant moorland species were the main vegetation type during the LGM in the Central Valley of the Lake District and on Chiloe Island. At the end of the LGM, these moorland species started to migrate to southern distributions, while some less cold tolerant species from the subantarctic forest such as *Nothofagus* and conifers started to appear. Heusser *et al.* (1999) suggested that less cold tolerant species survived in refugia in small populations at the north of the ice sheet distribution during the LGM period, and when the climate started to warm, these species started to recolonize the Central Valley depending on the temperature tolerance of each species. At the central depression in the lake region and Chiloe island, several studies suggest that between 30,000 and 14,000 yr the vegetation was dominated by Gramineae, compositae and moorland with small amount of *Nothofagus* and conifer thickets, forming a subantarctic parkland and confirming that colder and more humid temperatures prevailed in that area (Whitlock *et al.*, 2006; Heusser *et al.* 2006a, b;

2000; 1999; 1996; Moreno, 2004; 2000; 1997; Villagran and Armesto, 2005; Moreno and Leon, 2003; Heusser, 1999; Moreno *et al.*, 1999; Lumley and Switsur, 1993). Although pollen records suggest the possibility of refugia for cold tolerant species in both the Andean and Coastal Cordillera (Heusser, 1984), there is no clear evidence on which slope of the Andes Cordillera these refugia were established (Villagran, 2001). Eastern Andes pollen records suggest that during the LGM, steppe species were distributed and during the interglacial, dense forests of *Podocarpus*, *Nothofagus* and *Cupressaceae* arrived.

- *Southern Patagonia (~46°S- Tierra del fuego).*

In southern Patagonia there are not as many pollen records as from the Lake District, and they don't go further back than ~17,000 yr. One explanation is that the area was covered by ice and no plants could grow. The other is that there are no good areas to take deep soil cores, or that the area has simply received inadequate study. Samples taken from both sides of the Andes have Gramineae and *Empetrum* as dominant species (Heusser, 1995b). On the eastern side of the Andes there are traces of steppe grasses (Paez *et al.*, 1999), and on the western side there are traces of aquatic taxa (Markgraf *et al.*, 2009). This suggest a west-east precipitation gradient, but the lack of water loving plants characteristic of magellanic moorland, which grows at this latitude at present, suggest a colder but drier climate during the LGM compared with the current climate.

1.5 Phylogeography

The study of phylogeography aims to understand the history of taxa by analysing the geographic distribution of genealogical lineages (Avise, 2000). It deals mostly with inferring historical processes that contribute to the divergence of gene lineages, supported by molecular analysis and fossil pollen records.

Glaciations are one of the main sources of population divergence. Isolation can occur due to the large surfaces of ice sheets, which exist for long time periods, and these can separate populations during glaciations; plant populations can experience local or more widespread extinctions and range shifts occur. On the other hand small ice free areas may represent *refugia* (Bennett *et al*., 1991). These are areas of any size, where species persisted during glaciation. If refugial populations are isolated for a long period of time, they can become genetically divergent. This is facilitated by the often small size of the refugial area, which is helpful for the fixation of existing or new mutations (Ferris *et al*., 1999). It has been suggested that species survive in refugia, due to wetter and warmer microclimate conditions, produced by locations near to mountains slopes or maritime influence, or at lower latitudes.

When the ice retreats a phase of migrations begins. The first step is the fast colonization of new suitable areas, and this can be dominated by long distance dispersants. These rates of migration can be exceptionally high and small leading populations can establish, expand and provide a source for further seed dispersal (Hewitt and Ibrahim, 2001). Large areas may thus be covered by individuals with a narrow genetic base, and drift and founder effects may be important. On the other hand when different migration routes (from different refugia) meet, it produces an admixure of genes leading to high amounts of genetic diversity. These places are called contact zones which can have similar characteristics of refugia (high levels of allele diversity), but are due to the mixture of lineages from different refugia (Petit *et al.,* 2003; 2002b). Where recolonization occurs over vast areas marked effects may be evident. However similar but more subtle processes may occur when species remain in restricted geographical areas but track climate space by altitudinal migrations. Having only small migrations within mountain ranges, the loss of variation and diversity, and differences between the refugia and the colonized areas, is lower.

1.5.1 Molecular Markers

Isolation of populations can be analysed by molecular genetic techniques, and the spatial distribution of genetic diversity can provide phylogeographic inferences. In plants, maternally inherited organelle markers have proved popular in phylogeographic studies. In particular, the chloroplast genome has been widely used.

This is a non-recombinant, predominantly maternally inherited marker in angiosperms and typically shows high levels of population differentiation (Ennos *et al.*, 1999). It can be challenging to find sufficient levels of chloroplast DNA variation to be useful, but once suitable variation has been detected, it can be relatively straightforward to screen large sample sets to assess the distribution of these genetic variants. This can permit inferences of ancestry, past refugia and postglacial colonization. Additional information from the nuclear genome can provide a useful supplement to these data.

1.5.2 European Studies

Many plant species have been analysed from a phylogeographic point of view in Europe. Petit *et al.* (2003; 2002a, b) studied a range of species which suggested that temperate European trees had their refugia during the LGM in the extreme south of Europe, and the most unique populations are in southern and central Italy, Corsica, Iberia and the Balkan peninsula. The same pattern is shown in *Fraxinus excelsior* and *Saxifraga paniculata*, although adding another refugium in the eastern Alps (Reisch, 2008; Puscas *et al.*, 2008; Heuertz *et al.*, 2004). Within Europe, cold intolerant species with lower seed dispersal abilities, showed the clearest phylogeographic patterns whereas more cold tolerant species typically show less structured patterns due to their more extensive distribution during the LGM. Species with mountain distributions showed less founder effects due to the more local migration of the species (altitudinal rather than latitudinal). On the other hand, Petit *et al.* (2003) also suggested that species which showed major post-glacial dispersal had high diversity in areas of central Europe; an area which is an unlikely candidate for refugial sites. The interpretation was that these sites represent admixture of different populations that came from different refugias.

A Phylogeographic study on 82 plant species distributed throughout Europe shows multiple plant refugia with southern and northern distribution, with the biggest concentration of refugia in the western Mediterranean basin (Medail and Diadema, 2009).

1.5.3 North American studies

Phylogeography studies in plants and animals of North America, suggest a marked difference in haplotype distribution between populations from the south and the north. In the east of North America studies of *Liriodendron tulipifera*, suggest two different refugia, one in its southern distribution and one in its northern distribution (Sewell *et al.*, 1996). The same pattern has been analysed in seven different taxa from the coast of western North America (Soltis *et al.*, 1997). However there is a study focused in western North America (Brunsfeld *et al.*, 2000), with a similar geographic scenario to that of the Chilean Lake District. This suggests two different refugia hypothesis; one with two refugias on each mountain range, and a second with a single refugia on the western range which served as source of colonization for the eastern range. The first hypothesis mentioned is supported by molecular data on *Pseudotsuga menziesii* and *Lithocarpus densiflorus* (Gugger *et al.*, 2010; Nettel *et al.*, 2009), where two different haplotypes are shown on each mountain range. A similar pattern has been found for the frog species (*Pseudacris cadaverina*) with a distribution in southern California. However in this area there are two mountain ranges with a north- south distribution, which served as two different glacial refugia (Phillipsen and Metcalf, 2009).

Another different scenario has been found for the monocot coastal plant *Carex macrocephala*, which showed no genetically isolated populations, suggesting that the specie survived as a large metapopulation during the LGM (King *et al.*, 2009).

1.5.4 Patagonia Studies

Beheregaray (2008) indicates a dearth of phylogeographic studies on South America especially from Patagonia. Although several western Patagonian species have been studied using molecular tools, many of the studies have not focused on phylogeography history. While these studies can give helpful data on variation within and between populations, they do not provide extensive phylogeographic signal. In part this is due to the makers being used such as isozymes or RAPD’s which are nuclear markers and therefore are widely dispersed by pollen, and also the distribution of sampling, which does not necessarily sample the complete species distribution and sometimes only samples on one side of the Andes Cordillera. Additionally many studies to date have focused on cold tolerant species (mostly conifers and cold tolerant *Nothofagus* spp.), which limits our general understanding of how more thermophilic species behaved during the LGM.

The cold tolerant species that have been studied, have led to hypotheses of multiple glacial refugia on the coastal cordillera and on both slopes of the Andes. For instance, *Pilgerodendron uviferum* has populations with high diversity in the Coastal Cordillera, Central Valley, Andean Cordillera, and on the eastern slopes of the Andes suggesting it had a wide glacial distribution (Alnutt *et al*., 2003; Premoli *et al*., 2002). It does show a decrease in genetic diversity along a north-south gradient. A similar pattern is observed in the conifers *Araucaria araucana, Pilgerodendron uviferum*, and *Austrocedrus chilensis* (Alnutt *et al*., 2003; Bekessy *et al.,* 2002; Pastorino and Gallo, 2002) and also in two cold tolerant perennail herbs such as *Hypochaeris incana* and *Hypochaeris palustris* (Tremetsberger *et al*., 2009; Muellner *et al*., 2005). This could be interpreted as consistent with a lack of refugia in the southernmost Patagonia. On the other hand, *Fitzroya cupressoides,* another cold tolerant conifer, has its high levels of genetic diversity in its southern distribution, and several refugias were inferred in the eastern slope of the Andes (Allnutt *et al*., 1999) and in the coast and southern west slope of the Andes (Premoli *et al.,* 2000). The cold tolerant and widely distributed proteacea *Embothrium coccineum,* and the conifer *Podocarpus nubigena* have higher levels of genetic diversity in their southern populations, suggesting southern refugia (Quiroga and Premoli, 2010; Souto and Premoli, 2007). The conifer *Austrocedrus chilensis,* has the most genetically diverse populations distributed on the north-eastern slope of the Andean Cordillera, and this was used to suggest refugia in small unglaciated areas between the Andes Cordillera and the Patagonic steppe (Pastorino and Gallo, 2002).

Some cold tolerant species show genetic suture zones between northern and southern populations. The species having this pattern are *Hypochaeris incana*, *Nothofagus* *pumilio*, *Nothofagus antarctica* and *Austrocedrus chilensis.* Many of these taxa have more than one suture zone. The most common suture zones are located at 40°S and at 43°S. On the other hand a few species show a delimited contact zone at around 40°S (e.g *Nothofagus pumilio*, *Nothofagus antarctica*, *Nothofagus obliqua*, *Nothofagus nervosa* - Mathiasen and Premoli, 2010; Pastorino *et al*., 2009; Azpilicueta *et al*., 2009; Marchelli and Gallo, 2001). This is an area where haplotypes from different populations merge in one population.

More thermophilic species should behave differently during the LGM from the cold tolerant species. *Nothofagus nervosa*, a rainforest species, has been studied mainly in its Argentinean distribution, although one study has sampled its entire distribution. From these studies the common pattern are two refugias, one at the coastal cordillera and the other at the Andes-northern distribution of the specie, with no distinction between the slope of the Andes on which the population is distributed (Carrasco *et al*., 2009; Marchelli and Gallo, 2006; 2004; 2001; Marchelli *et al*., 1998). *N. nervosa* shows a suture zone at ~ 40°S. Within the studies that sampled the coastal cordillera, there are indications of a coastal Andes migration during the LGM. This last pattern also occurs with thermophilic species such as *Colliguaja odorifera*, *Aextoxicon punctatum* and *Nothofagus obliqua* (Azpilicueta *et al*., 2009; Nuñez-Avila and Armesto, 2006; Bull-Herenu, 2005) and cold tolerant *Nothofagus pumilio*.

Two Patagonian fishes (*Galaxias platei* and Genus *Percichthy*) share similar patterns suggesting a glacial refugia on the eastern Andes slopes at ~45°S and at western slopes mainly northern populations. Although Percichthy shows a west-east migration after the LGM (Ruzzante *et al*., 2006), in two different studies of *G. platei* there is a mismatch of the migration route taken (west-east v/s east-west) (Zemlak *et al*., 2008; Ruzzante *et al*., 2008). Another aquatic species that shares the western-eastern migration is the water crab *Aegla alacalufi* with higher haplotype diversity at western island populations and a second refugium at ~42°S (Xu *et al*., 2009). A study on the endemic and unique marsupial from Patagonia, *Dromiciops gliroides* refers to two different refugia one on Chiloe Island and the second within populations north of 39°S. This has a haplotype suture zone around 39°S. Rodents show very different patterns within each species. For instance, there are indications that *Abrothrix olivaceus* had 3 different refugia in a north south direction, the northern population having a higher level of gene diversity suggesting a north south migration. On the other hand, *Oligoryzomys longicaudatus* apparently persisted in 3 refugias within its southern distribution, in coastal populations and on both side of the Andes (Rodriguez-Serrano *et al*., 2006; Palma *et al*., 2005).

What is evident from studies done to date is (a) a range of different hypotheses on glacial refugia and migration routes (b) some conflict and ambiguity between studies, in part due to the dearth of studies using cpDNA markers to study plant phylogeography in the region, and (c) a shortage of studies on cold-intolerant species which might provide more clear cut phylogeographic patterns and (d) no studies using annual plant species within Patagonia (Table 1.2).

Table 1.2. Summary of Patagonian plant phylogeographic studies

Author	**Year**	**Studied species**	**Molecular method**	**Entire distribution**	**Cold tolerant**	**Glacial refugia**	**Migration routes**
Nuñez-Avila and Armesto	2006	*Aextoxicon punctatum*	RAPD	Y	N	North of 40°S	North-coast
Bekessy *et al.*	2002	*Araucaria araucana*	RAPD	Y	Y	Coast; North Andes; South Andes	
Marchelli *et al.*	2009	*Araucaria araucana*	cp DNA, mt DNA	N	Y	East Andes 40°S	East-west
Pastorino and Gallo	2002	*Austrocedrus chilensis*	isozyme	N	Y	North of 40°S	
Bull-Herenu	2005	*Colliguaja odorifera*	RAPD	N	N	Coast	West-east
Souto and Premoli	2007	*Embothrium coccineum*	allozyme	Y	Y	Coast; West and East Andes north of 43°; South of 44°S	South-north
Allnutt *et al.*	1999	*Fitzroya cupressoides*	RAPD	Y	Y	Coast; east	West-east
Tremetsberger *et al.*	2009	*Hypochaeris incana*	AFLP, cpDNA	Y	Y	North of 43°S; Between 44°S and 50°S; South of 51°S	North-south
Muellner *et al.*	2005	*Hypochaeris palustris*	AFLP'S	N	Y	Coast; North East Andes	North-south
Pastorino *et al.*	2009	*Nothofagus antarctica*	Cp DNA, isozyme	N	Y	North of 40°S; South of 43°S	
Marchelli and Gallo	2004	*Nothofagus. nervosa*	isozyme	N	N	North of 40°S	North-south
Marchelli *et al.*	1998	*Nothofagus. nervosa*	RFLP	N	N	North of 40°S	
Carrasco *et al.*	2009	*Nothofagus nervosa*	RAPD	N	N	Coastal; North 36°S	West-east

Table 1.2. Continued

Author	**Year**	**Studied species**	**Molecular method**	**Entire distribution**	**Cold tolerant**	**Glacial refugia**	**Migration routes**
Marchelli and Gallo	2001	*Nothofagus nervosa*	Isozyme	N	N	At 40°S	
Marchelli and Gallo	2006	*Nothofagus nervosa*	RFLP	Y	N	Coastal; south east of 40°S	West-east
Mathiasen and Premoli	2010	*Nothofagus pumilio*	Cp DNA, isozymes	Y	Y	North of 41°S; south of 43°S	North-south
Azpilicueta *et al.*	2009	*Nothofagus obliqua*	RFLP	Y	N	Coast; North west Andes 34°S; Eastern Andes 40°S	
Allnutt *et al.*	2003	*Pilgerodendron uviferum*	RAPD	Y	Y	North; Eastern Andes	North-south
Premoli *et al.*	2002	*Pilgerodendron uviferum*	Isozyme	Y	Y	North	North-south
Quiroga and Premoli	2010	*Podocarpus nuvigena*	Isozyme	Y	Y	South of 43°S	South-north
Allnut *et al.*	2001	*Podocarpus salignus*	RAPD	Y	Y	North of 37°S	

It is important to resolve these outstanding issues. The study of Patagonian phylogeography is very important because it is the only Southern hemisphere area outside of Antarctica that was strongly covered by ice sheets within the glacial period. Also its unique flora includes many endemic species, which have likely been shaped by the effect of climatic changes during glacial cycles. Finally this area has had little anthropogenic disturbances so that natural phylogeographic patterns may be easier to find compared to those in Europe where human movement and planting has been more common.

In addition to uncovering patterns of biodiversity to understand evolutionary history, there is also the potential for providing information of conservation value. Chile and Argentina are large and geographically variable countries. It is possible that past environmental change has led to hotspots of genetic diversity for multiple species. Such information could be useful to contribute towards recognising important areas of biodiversity.

1.6 Project Aims

The aims of this study are:

1) To find centres of genetic diversity, which can be related to refugia or contact zones, using molecular tools.
2) To establish whether phylogeographic patterns coincide for multiple plant species, with different attributes.
3) To establish whether the evidence supports the existence of unique refugia or multiple refugia.
4) To produce the first scheme for the migration of Patagonian plant species after the LGM.

To resolve these questions a group of species has been selected with broad geographical distributions and different thermal tolerances. These species will be analyzed using organelle markers (chloroplast markers) to infer their phylogeographic history.

1.7 Project Planning

1.7.1 Species selection

In selecting species for this study, I aimed to include a range of different species attributes. I focused on species which were endemic to Patagonia, had low seed dispersal (to maximize the likelihood of detecting phylogeographic structure), and had different inferred levels of cold tolerance and different life history traits (herbs, shrubs, trees). I prioritized species with a wide current distribution throughout Patagonia, preferably occurring in both cordilleras and on both slopes of the Andes and whose distributions encompassed putative refugias and also areas covered by the ice sheet during the LGM. Using this approach, a list of 10 species was developed using information from different herbaria, botanical books, monographs; and specialist advice from botanists who worked in the studied area and my personal knowledge of Patagonian plants. The selected species were: *Caldcluvia paniculata*, *Discaria chacaye*, *Donatia fascicularis*, *Drimys winteri*, *Escallonia virgata*, *Gentianella magellanica*, *Phacelia secunda*, *Pumnopitys andina*, *Tepualia stipularis* and *Weinmannia trichosperma*. However, during initial field work I discovered that *Phacelia secunda* grows mainly in roads and places that have been disturbed by man and thus natural phylogeographic structure was unlikely to be found. Secondly, I discovered that the records for *Caldcluvia paniculata* in the north were erroneous and thus the species has a more limited distribution than expected. I also did not find any useable genetic markers for inferring phylogeographic structure for this species. The same problem (lack of variable markers) also occurred for *Drimys winteri.* Thus these three species were excluded from the final study which focused on *Discaria chacaye*, *Donatia fascicularis*, *Escallonia virgata*, *Gentianella magellanica*, *Pumnopitys andina*, *Tepualia stipularis* and *Weinmannia trichosperma*.

1.7.2 Field work plans and choice of sampling sites

In designing my field work I aimed for good coverage across species' ranges, and covering previously glaciated, and putative refugial regions, and encompassing the topographical variation in Patagonia (coastal and Andean Cordilleras) and a broad latitudinal range. In the first instance at least four populations from 10 species were sampled, choosing populations from the northern, southern, western and eastern edges of their range. To obtain information about population distributions I consulted records from different herbaria as well as books and internet sites. Following this initial pilot study more intensive field work was undertaken on the selected study species. This involved a considerable time input, as many of the sample sites are in remote locations. At each site I made a rough approximation of population size, and sampled 20 individuals per species. Individuals were sampled at a minimum distance of 5 m to reduce the likelihood of sampling closely related individuals. Samples were labeled and stored in silica bags and GPS records were taken, as well as associated species and herbarium vouchers.

1.7.3 Choice of markers

Chloroplast DNA (cpDNA) is the most widely used and useful marker for analyzing phylogeographic structure in plant populations (Ennos, 1999). This is due to the ease which regions can be amplified and sequenced, and its predominantly maternal mode of inheritance in angiosperms, leading to marked phylogeographic structure. This was the 'marker of choice' for this study. However, one downside of cpDNA is that it can be difficult to find variable sites in some species. In addition, the uniparental mode of inheritance means that it provides information on only one of the modes of dispersal of plants (seeds) and in angiosperms, it does not carry any information about patterns of pollen dispersal. Nuclear markers represent a useful addition to cpDNA as (a) they typically show more variation, (b) they show bi-parental inheritance, and (c) because of recombination they can provide multiple independent markers (compared to the linked regions in the non-recombinant cpDNA molecule). However, accessing nuclear markers is more difficult. At the time when this study was started (2005) the main sources of nuclear markers for non-model organisms were isozymes, SSR and AFLP's. Isozymes are codominant markers which have moderate levels of variation. However, they require fresh material and thus could not be used in my study in which the field sites were remote from laboratory facilities. SSRs (simple-sequence repeats or microsatellites) are powerful, variable co-dominant nuclear markers. However, in the context of the work-load of the project as a whole, these were considered too expensive and too time consuming to develop with the technologies available at the start of this project (something which is changing now rapidly with next-generation sequencing technologies). They also can sometimes be too variable to be useful for range-wide phylogeographic studies. Finally, arbitrary fingerprinting approaches like AFLP's (amplified fragment length polymorphisms) are useful nuclear markers. Although they are dominant, the approach can be a relatively cost-effective method of getting large numbers of molecular markers. Therefore I attempted the use of AFLP's in one of the study species (*G. magellanica*). However, despite trials and optimization attempts with 12 primer pairs, I did not succeed in obtaining useable profiles.

In the end I focused the study on cpDNA sequences. These markers are expected to be maternally inherited in six of the seven species, and paternally inherited in the gymnosperm *Prumnopitys andina.*

Chapter 2: Patterns of phylogeographic structure from chloroplast DNA in five Patagonian plant species

2.1 Introduction

Large scale natural environmental perturbations can have a marked influence on the distribution of intra-specific genetic variation. Major geological events such as mountain uplifts or landmass movement can influence the likelihood of genetic exchange by establishing or removing physical barriers to gene flow and seed dispersal. Likewise, cyclical changes in the earth's climate such as the Pleistocene glaciations can also have a marked influence on patterns of genetic variation. Oscillation between more or less favorable climatic conditions for a given species can result in range expansions during favorable climatic conditions and range contractions when it is less favorable. When populations are restricted to localized refugial patches of optimal climate and habitat they can diverge in isolation. As climatic conditions become more favorable, these refugia serve as a source of colonists for range expansions, and the genetic signature of the source populations can be maintained in the expanding wave of colonist populations (Petit *et al*., 2003).

Understanding how species respond to major environmental perturbations is difficult as it requires reconstruction of a set of past-events, some of which leave no obvious trace in the phenotypes of contemporary populations. However, along with historical climatic reconstruction and the distribution of plant fossils, genetic markers can be a useful tool for gaining insights into how species have responded to environmental change over long time periods (Hewitt, 2001). Phylogeographic studies using genetic makers have proved extremely useful in reconstructing, (a) the source of refugial populations during periods of sub-optimal climatic conditions, (b) the migration pathways of different species as they have expanded from refugia when the climate has been more favourable, and (c) how different features of the landscape such as major mountain chains and water bodies have influenced the colonisation process, and served to further shape the genetic structure of individual species.

Our understanding of the historical dynamics of the European biota has been greatly enhanced by a series of detailed studies on plant and animal taxa (Heuertz *et al.*, 2004; Petit *et al.*, 2002a,b; 2003; Hewitt, 1999; Dumolin-Lapegue *et al.*, 1997). Likewise, phylogeographic studies have provided important insights into historical biogeography and the distribution of genetic diversity patterns in NW America (Galbreath *et al.*, 2010; Gugger *et al.*, 2010; Aubry *et al.*, 2009; Rodriguez-Banderas *et al.*, 2009; Tomimatsu *et al.*, 2009; Godbout *et al.*, 2008; Marr *et al.*, 2008; Spellman and Klicka, 2006; Brunsfeld *et al.*, 2000; Soltis *et al.*, 1997), China, central America (Cavers *et al.*, 2005; 2003; Dutech *et al.*, 2000; Gillies *et al.*, 1999) and tropical South America (Caetano *et al.*, 2008; Simoes *et al.*, 2007). As the density of studies from a given geographical region increases, so does the power of the inferences that can be drawn. The identification of common patterns across multiple species can identify major events or features that have shaped this history of many species. Likewise, examples of species that do not fit these common patterns permit a more fine-tuned understanding of how different species traits and attributes can influence their responses to long term environmental change.

One area of the world that has only recently been the subject of phylogeographic studies is Patagonia. This region is of interest for two main reasons. Firstly, outside of Antarctica, Patagonia is the most heavily glaciated region in the Southern hemisphere, and hence is an obvious model system for examining the influence of glacial cycles on austral biodiversity patterns. Secondly, Patagonia has a complex topography encompassing steep altitudinal and climatic gradients that are likely to have had a major influence on past species distribution patterns. The Andes are orientated on a north-south axis in the region, and in northern Patagonia reach heights of over 3,500 m, whereas in the far south of Patagonia, the mountain chain drops down to sea level as it curves round onto an east-west axis in Tierra del Fuego. A second major mountain chain is the coastal cordillera that runs from the northern limit between Chile and Peru to the Taitao peninsula. Between these two mountain chains is the Central Valley, a low fertile region where the main population centres are located.

From recent studies on the phylogeography of plants and animals in Patagonia, various hypotheses (see below) have been proposed regarding the location of refugial regions, and also the identification of major suture zones across which intra-specific gene flow may be limited. However, the knowledge base on plant species remains relatively restricted, and only seven studies have used chloroplast DNA markers, which are particularly powerful for detecting phylogeographic structure (Ennos *et al.*, 1999).

In this paper, the aim is to expand this knowledge base on the history of the Patagonian biota, and specifically to increase our understanding of the geographical distribution of genetic lineages within plant species to assess whether there is evidence for congruent patterns among species that can be related to topographical features or past climates. By gathering cpDNA data from multiple populations of 5 widely distributed plant species, the following questions have been addressed.

1) Is there evidence for geographically discrete genetic lineages within individual plant species?
2) Do the patterns of genetic diversity and genetic differentiation correlate with previous hypotheses of historical suture zones or glacial refugia, viz:
    a. Distinct north versus south genetic lineages (sensu Acosta and Premoli, 2010; Mathiasen and Premoli, 2010; Azpilicueta *et al.*, 2009; Carrasco *et al.*, 2009; Muellner *et al.*, 2005; Marchelli and Gallo, 2004; Pastorino and Gallo, 2002; Allnutt *et al.*, 2001; Marchelli *et al.*, 1998; Premoli, 1997).
    b. Presence of a major suture zone at 43ºS separating northern versus southern populations (sensu Mathiasen and Premoli, 2010; Tremetsberger *et al.*, 2009).

c. Presence of a suture zone at 40ºS separating northern versus southern populations (sensu Acosta and Premoli, 2010; Mathiasen and Premoli, 2010; Azpilicueta *et al.*, 2009; Marchelli and Gallo, 2006; Nuñez-Avila and Armesto, 2006; Marchelli and Gallo, 2004; Pastorino and Gallo, 2002; Marchelli *et al.*, 1998).

d. Evidence of genetically divergent lineages between the coastal cordillera and the Andes (sensu Mathiasen and Premoli, 2010; Muellner *et al.*, 2005; Bekessy *et al.*, 2002; Allnutt *et al.*, 2001; Premoli, 1997).

e. Evidence of genetically divergent lineages east and west of the Andes (sensu Mathiasen and Premoli, 2010; Marchelli and Gallo, 2006; Muellner *et al.*, 2005; Allnutt *et al.*, 2003; 1999).

f. Evidence of higher differentiation among populations in the north of Patagonia, versus populations in more southerly regions (sensu Acosta and Premoli, 2010; Mathiasen and Premoli, 2010; Nuñez-Avila and Armesto, 2006; Marchelli and Gallo, 2004).

g. Presence of a contact zone from 38ºS to 42ºS (sensu Mathiasen and Premoli, 2010, Pastorino *et al.*, 2009; Azpilicueta *et al.*, 2009; Marchelli and Gallo, 2006).

2.2 Materials and Methods

2.2.1 Study Species

Five species were sampled throughout their entire distribution range within Chile and Argentina in the region between 34°S (at the northern edge of Patagonia) to 54°S (Tierra del Fuego, at the southern tip of Patagonia); this area has been divided into nine informal zones (Figure 2.1). The study species are *Disacaria chacaye, Donatia fascicularis, Escallonia virgata, Tepualia stipularis* and *Weinmannia trichosperma.* All are angiosperms and are endemic to Patagonia with hermaphrodite flowers and insect pollination but have some differences in levels of cold tolerances and seed dispersal (Table 2.2). They have wide latitudinal and altitudinal distributions and occur on both cordilleras (except for *D. fascicularis* which grows only on the Coastal Cordillera). In all species (except for the northern coastal populations of *T. Stipularis* and *W. trichosperma*), there is a change in altitudinal distribution in relation to latitude, such that northern populations are at high elevations, whereas more southerly populations grow at low altitudes (Figure 2.2). This suggests that these species are cold tolerant, although two of the species (*Tepualia stipularis* and *Weinmannia trichosperma*) do not occur south of 49°S, suggesting a more limited tolerance of cold temperatures. Four of the five species have gravity dispersed seeds which are likely to disperse only short distances, and hence are more likely to show clear cut phylogeographic structure. The fifth species, *Weinmannia trichosperma,* has wind dispersed seeds which are likely to be more efficiently dispersed.

Table 2.1. Ecological attributes and species traits of five Patagonian plant species.

Species	Likely pollinators[1]	Fruit type	Seed dispersal	Pollen dispersal	Life history traits	Seed size (mm)	Level of cold tolerance[4]
Discaria chacaye[2]	Bees	Dry capsule	Gravity/ water = restricted	Biotic	Carpet/shrub/tree	2-3	High
Donatia fascicularis[2]	Bees	Dry capsule	Gravity/ splash = restricted	Biotic	Cushion	1	High
Escallonia virgata[2]	Bees	Dry capsule	Gravity = restricted	Biotic	Shrub	4-6	High
Tepualia stipularis[2]	Bees	Dry capsule	Gravity = restricted	Biotic	Tree	2	Low
Weinmannia trichosperma[3]	Bees	Dry capsule	Wind = unrestricted	Biotic	Tree	1	Low

1= Likely pollinators have been checked on field; 2 = species data taken from Moore, 1983; 3 = species data taken from Rodriguez *et al*., 1983; 4 = Levels of cold tolerance are inferred from either (a) upper altitude limits at low latitudes, or (b) restriction southerly latitudinal distribution (e.g. plants not growing on the top of mountain in the north, or not growing at all in the south). Thus Tepualia and Weinmannia are inferred as showing lower cold tolerance than the other species. Detail description of altitudes and latitudes from which this summary was derived are given in Figure 2.2.

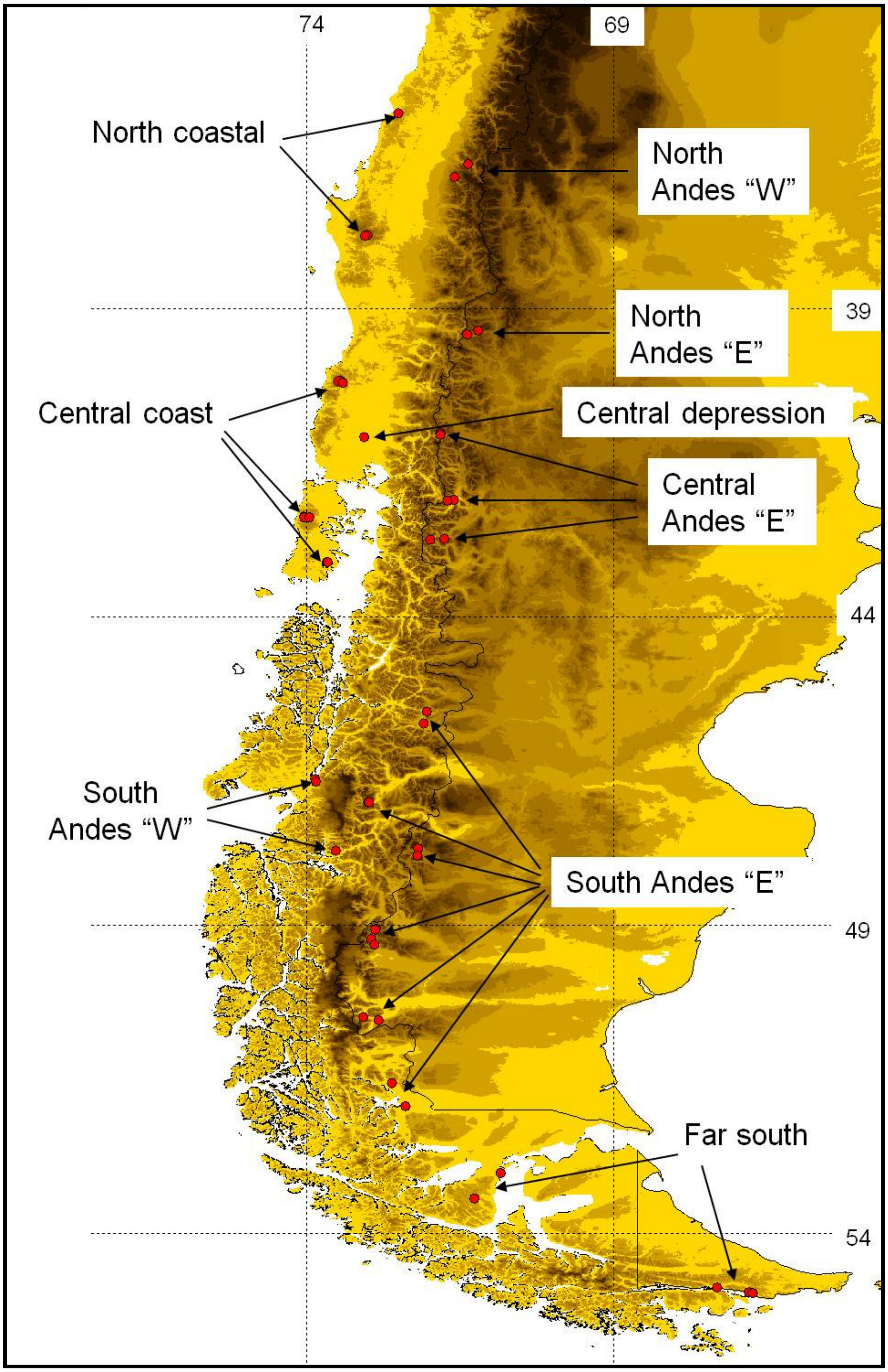


Figure 2.1. Distribution of sample sites and their allocation to nine informal geographic zones.

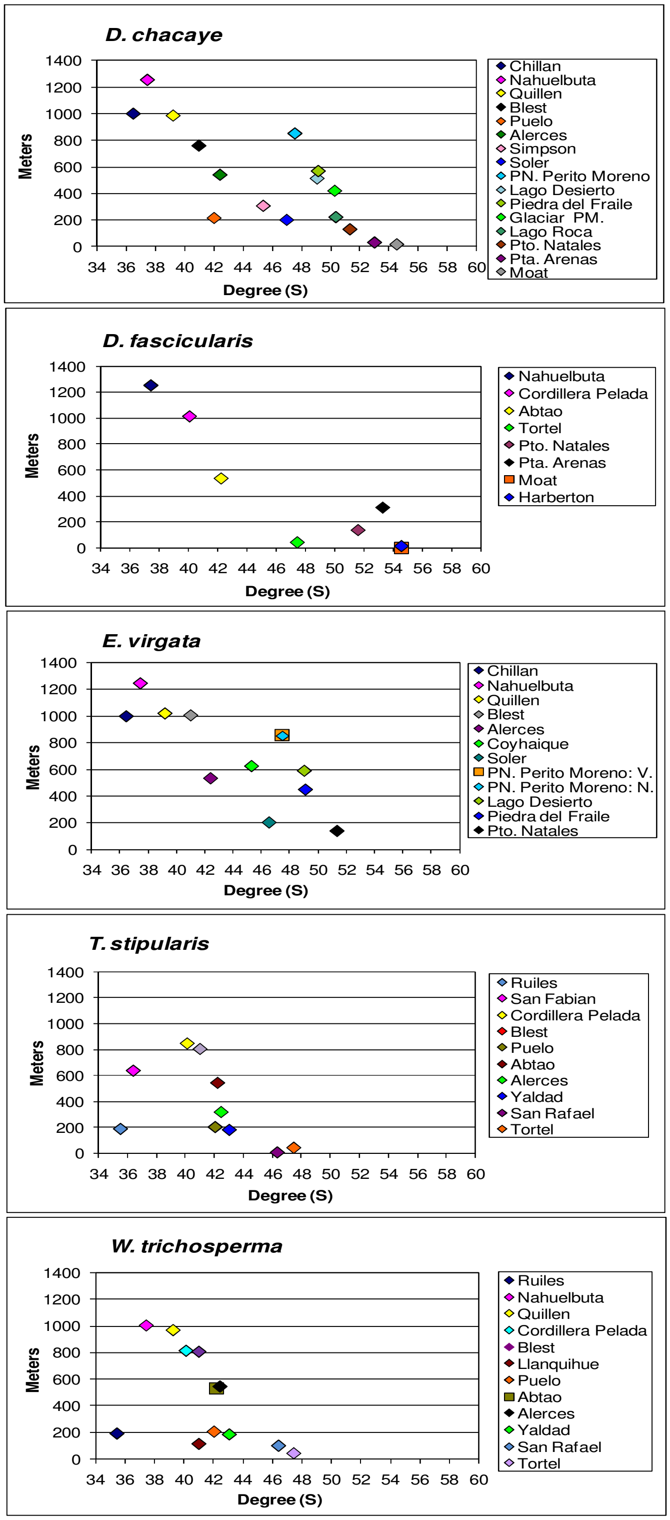


Figure 2.2. Plots of altitude and latitude from sample sites of five Patagonian plant species.

Discaria chacaye (G.Don) Tortosa

Disacaria chacaye belongs to the Rhamnaceae family. It is a very polymorphic species, with many different shapes from a tree up to 8 m tall to a prostrate woody carpet (Figure 2.3). In Chile, it occurs in both Cordilleras and the central valley (Regions: V, IX, XI and XII) (see Figure 1.2 for region numbers). In Argentina it grows in the Andes and in Tierra del Fuego (Provinces of: Chubut, Neuquen, Rio Negro, Sta. Cruz and Tierra del Fuego) (Figure 2.4). It has the typical distribution of a cold tolerant species, varying its altitudinal distribution in relation to latitude (Figure 2.2). It occurs in very variable habitats, normally in wet ground near to rivers or lakes, between rocks or in the margins of *Nothofagus* forest, from sea level up to 2.000 mosl. (Zuloaga *et al.*, 2009; Hoffmann *et al.*, 1998; Hoffmann, 1997; Correa, 1988; Moore, 1983).

No records have been found regarding its historical distribution, although due to its cold temperature tolerance it is likely that this species was widely dispersed during the LGM within the northern Patagonia, while in its southern distribution it will have had a more restricted distribution due to ice cover.

Figure 2.3. *Discaria chacaye,* from top left clockwise: (a) close up of plant morphology, (b) plant growing as a prostrate mat in Perito Moreno glacier, (c) plant growing as a prostrate mat in PN. Perito Moreno, (d) plant forming a small tree in Puelo, (e) plant forming a small tree in Torres del Paine.

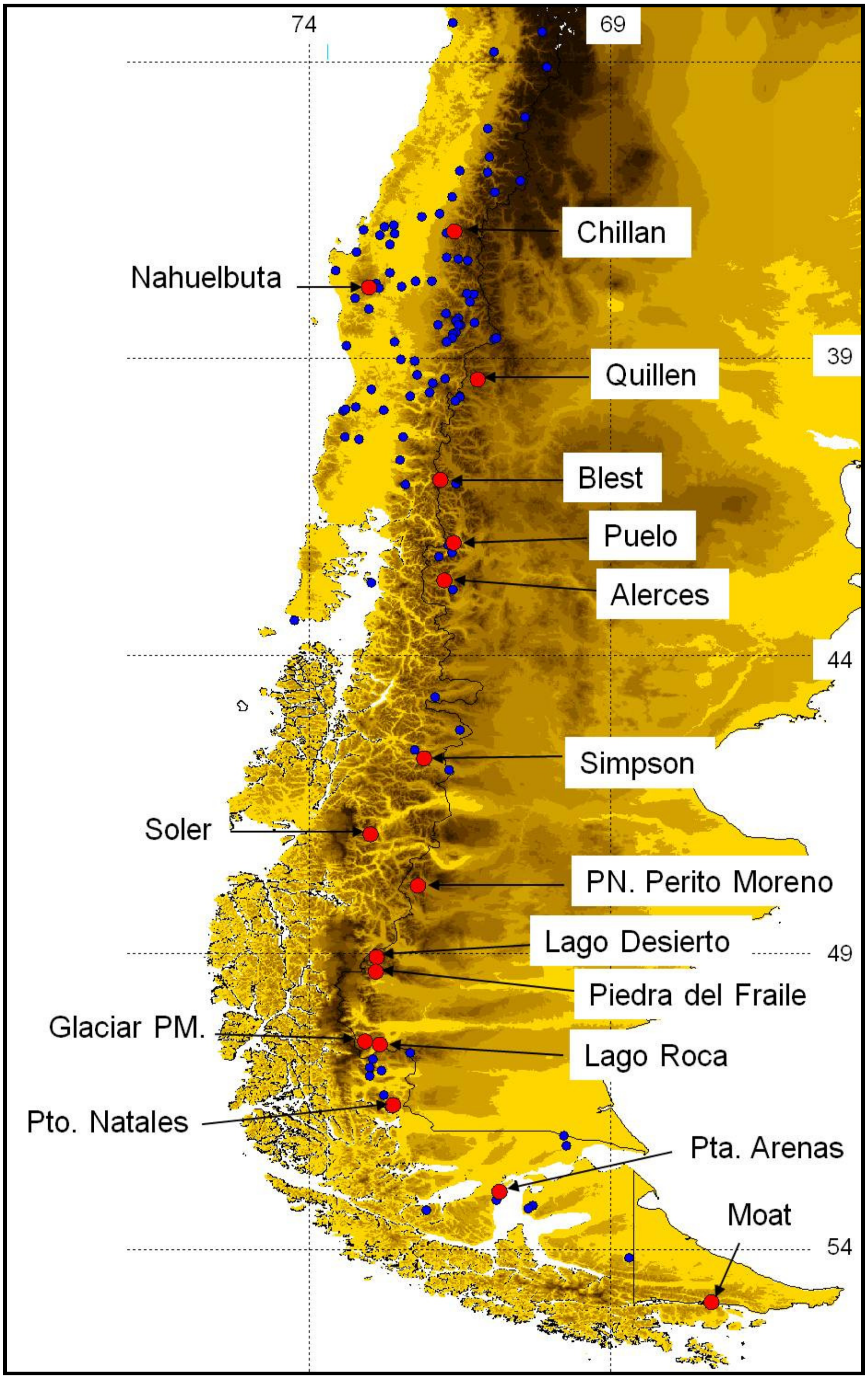


Figure 2.4. Distribution of *Discaria chacaye.* The red dots indicate sampled populations and blue dots are unsampled localities.

Donatia fascicularis J.R. Forst & G. Forst.

This species belongs to the family Doniateae. The family has only one genus with two species *Donatia fascicularis* and *Donatia novae-zalandiae* (Correa, 1984). *D. fascicularis* is one of the major representatives of the South American moorland flora along with *Astelia pumilia*. Is a sub-shrub and it forms distinctive dense cushions, densely covered by green leaves and white flowers (Figure 2.5). Its distribution is centred in southern Chile (Regions: VIII, IX, X, XI and XII) and Tierra del Fuego, mostly in the hyper humid seaward archipelago of southern Patagonia. Its northern distribution corresponds to only a few populations on the top of the higher hills of the Coastal Cordillera (Nahuelbuta (37°S), Cordillera Pelada (40°S) and Chiloe (42°S) (Figure 2.6). It grows from sea level up to 1,000 mosl (Figure 2.2) (Zuloaga *et al.*, 2009; Donoso, 1998; Moore, 1983).

Its historical distribution has been infered from pollen studies. These suggest this species was one of the colonizers of Coastal Cordillera (Nahuelbuta 37°S-73°W) about 6,000 yr. In the Coastal Cordillera near the Lake District it appeared after the deglacial period (9,070 yr) (Villagran, 2001). On the other hand, in the Central Valley, during the LGM period *D. fascicularis* reached its peak distribution (21,000 yr), being one of the major representatives of the glacial vegetation, disappearing within the deglaciation 11,000 yr (Heusser *et al.*, 2006a; Abarzua *et al.*, 2004; Moreno *et al.*, 1999; Moreno, 1997). This pattern has a time shift, going further south within the central depression with an appearance at 16,000 yr and disapearing at the same period (Moreno, 2004; Moreno and Leon, 2003; Heusser *et al.*, 1999). In southern Patagonia *D. fascicularis* appeared at the deglacial period at almost the same period when *D. fascicularis* disappeared from its northern distribution (Haberle and Benett, 2004; Fesq-Martin *et al.*, 2004; Heusser, 1995a). This pollen records suggest that *D. fascicularis* has cold tolerance behaviour due to its wide distribution in the Central Valley between the glacial periods, and also a warm weather intolerance regarding to the disappearance in the Central Valley after the cold weather got warmer.

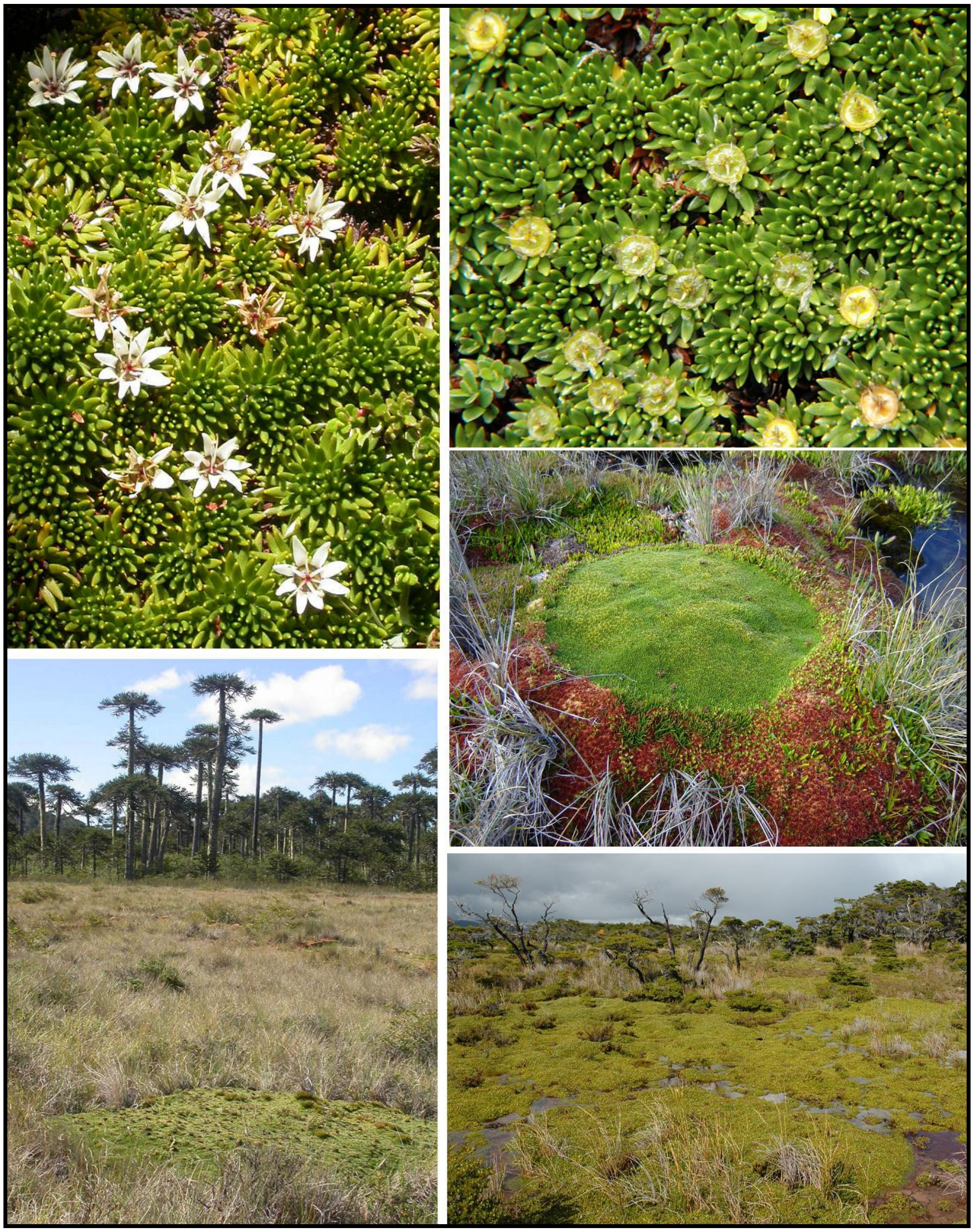


Figure 2.5. *Donatia fascicularis*. From top left clockwise (a) close up of plant flowers from Cordillera pelada, (b) close up of plant habit from Moat, (c) photograph showing an individual cushion of *D. fasicularis*, (d) general habitat shot, from Haberton with *D. fasicularis* in the foreground. (e) general habitat shot, from Nahuelbuta with *D. fasicularis* in the foreground.

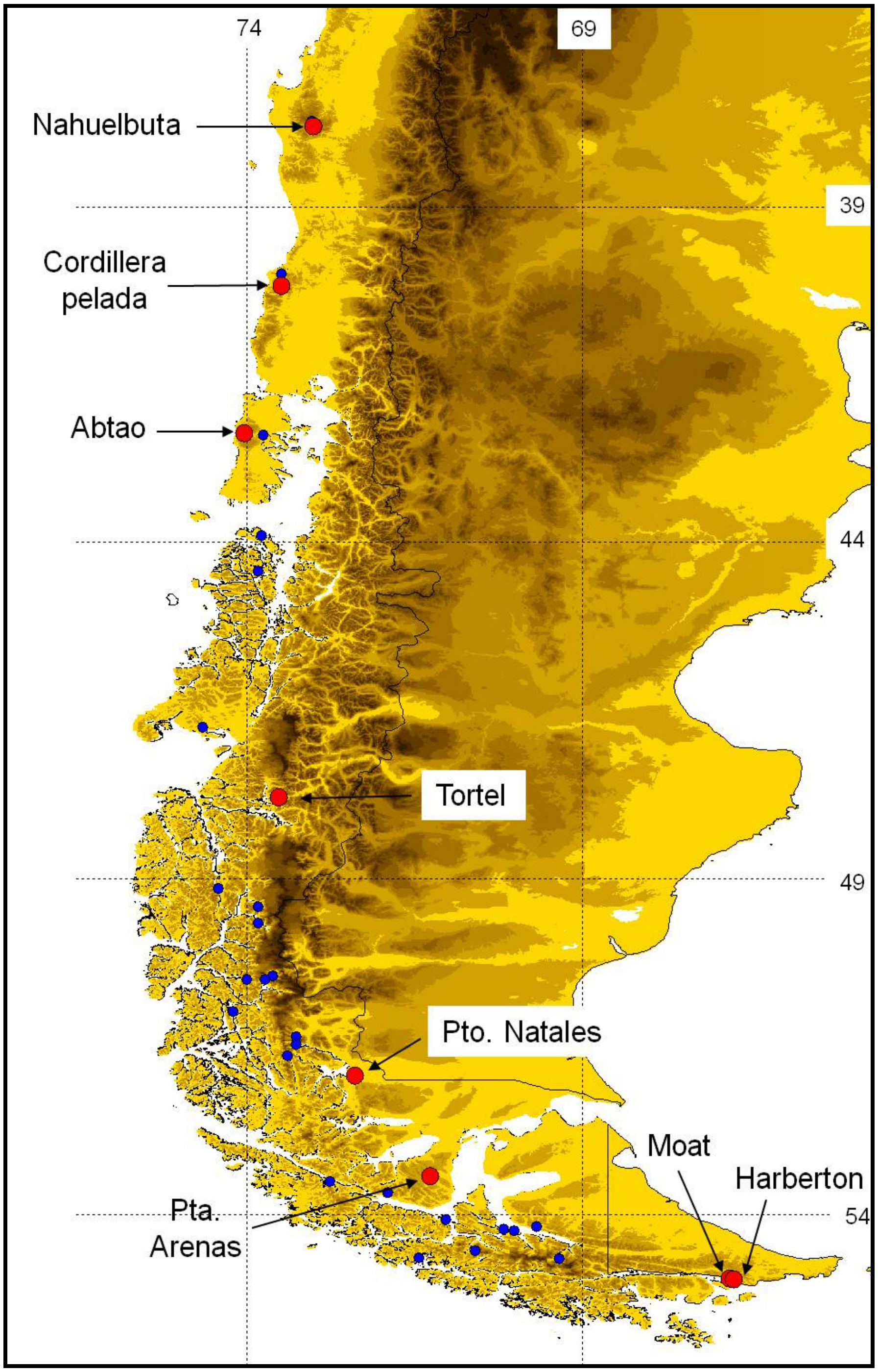


Figure 2.6. Distribution map of *Donatia fascicularis.* The red dots indicate sampled populations and blue dots are unsampled localities.

Escallonia virgata (Ruiz & Pavon) Pers.

Escallonia virgata belongs to the Escalloniaceae family. It is an evergreen shrub reaching approximately 2 m high (Figure 2.7). In Chile it has a wide distribution from sea level and on both Cordilleras reaching altitudes of up to 3,000 m in the Andes (Regions: VII, VIII, XI, X, XI, XII and Metropolitana) (Figure 2.8). In Argentina it grows only in the Andes (Provinces of: Chubut, Neuquen, Rio Negro, Sta. Cruz and Tierra del Fuego). Its altitudinal distribution changes in relation to latitude (Figure 2.2), growing in its northern distribution at the top of the hills and in its southern distribution at sea level. It occurs mostly in wet areas with restricted drainage and near to lake or rivers (Zuloaga *et al.*, 2009; Riedemann and Aldunate, 2003; Hoffman *et al.*, 1998; Hoffmann, 1997; Correa, 1984; Moore, 1983; Kausel, 1953). This species is not included in the national list of species with conservational problems, although it is listed as a rare species for the XII Region (Benoit, 1989).

Its historical distribution has been analyzed by several pollen core studies, although individual species cannot be distinguished. In the Coastal Cordillera of the Lake District, Escalloniaceae had a small appearance after the LGM period (9,000 yr) (Villagran, 2001). In the Central Valley it had a wide distribution between the LGM and the postglacial periods (16,400-6,900 yr) (Abarzua *et al.*, 2004; Moreno, 2004; Moreno and Leon, 2003). Further south (44°S) the species has its appearance between ~13,000 and 11,000 yr. This suggests that species in the Escalloniaceae family can survive with cold and dry weather, but as they also have an extensive post-glacial distribution, it suggests an ability to cope with warmer weather. This variable behaviour to temperature could be related to the fact that multiple species are lumped together in the pollen analyses.

Figure 2.7. *Escallonia virgata*. From top left of figure clockwise (a) close up of flowers and fruits, (b) photograph of flowering plant, (c) habit photograph taken at Coyhaique, (d) habit photograph taken at Coyhaique.

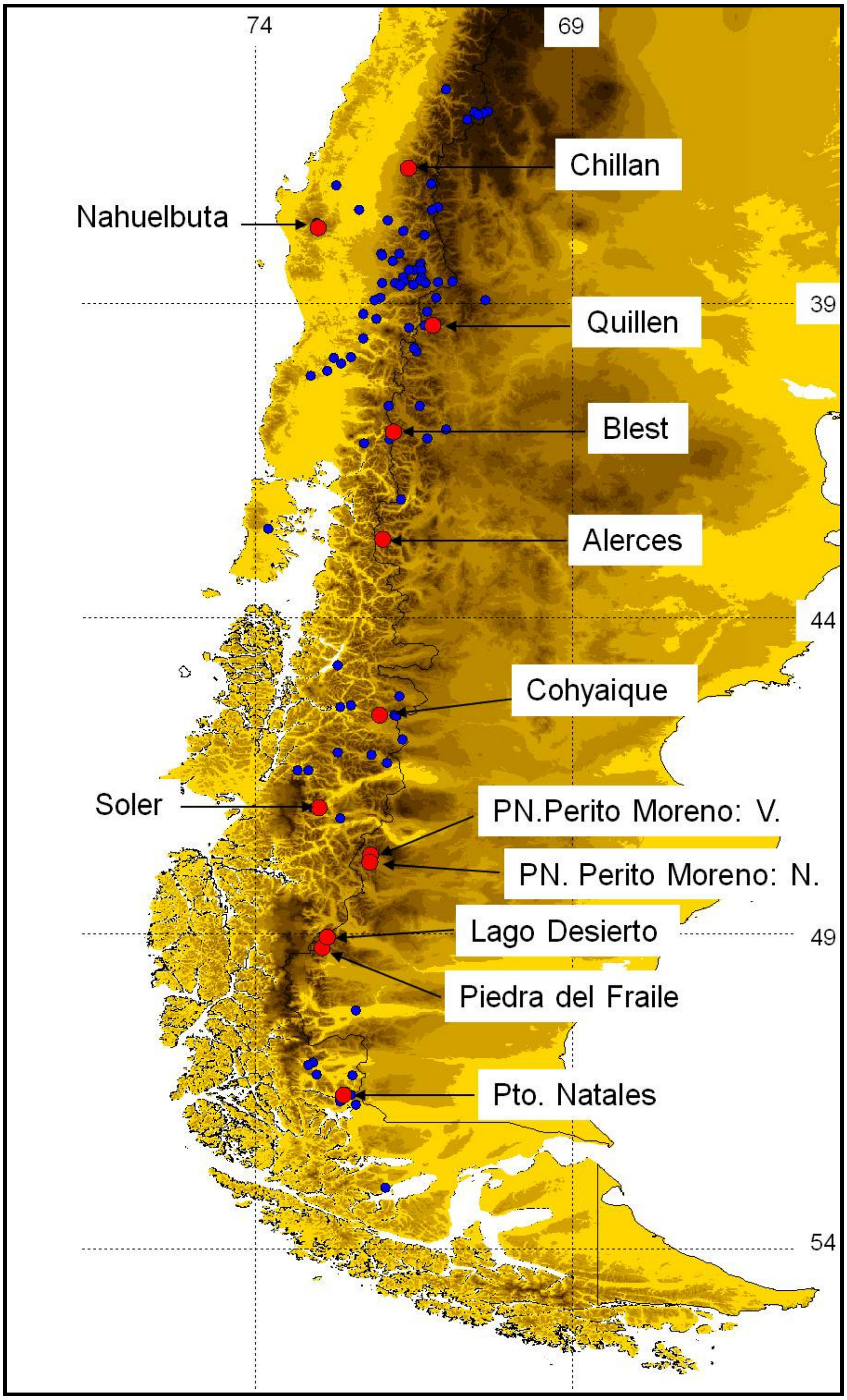


Figure 2.8. Distribution map of *Escallonia virgata.*
The red dots indicate sampled populations and blue dots are unsampled localities.

Tepualia stipularis (Hooker f.)

Tepualia stipularis belongs to the Myrtaceae family. Is the only American representative of the sub family Leptospermoides (characterised by the presence of dry fruit). It varies in habit between a shrub and a tree up to 10 m high (Figure 2.9). It has elliptical to ovate shape leaves, with characteristic conspicuous glands all over the surface. In Chile it occurs in both Cordilleras, and further south grows only on the hyper humid seaward archipelago (Regions: VII, VIII, IX, X, XI and XII) from sea level up to 1,000 mosl (Figure 2.2). In western Argentina it occurs in the Andes (Provinces of: Chubut, Rio Negro and Tierra del Fuego) (Figure 2.10). Its typical habitats are humid areas, often in moorlands, near to rivers or lakes and in margins and clearings of mixed forest dominated by *Nothofagus* species (Zuloaga *et al.*, 2009; Donoso and Escobar, 2006; Riedemann and Aldunate, 2003; Donoso, 1998; Hoffmann, 1997; Landrum, 1988; Moore, 1983). This species is not included in the national conservation list, although it is listed as rare for the VII Region (Benoit, 1989).

Its historical distribution has been studied by many pollen analyses. They suggest that this species had only one peak at the end of the deglacial period in the Coastal Cordillera at 9,000 yr (Lake District) (Villagran, 2001). In the Central Valley it has a small appearance by the end of the LGM at 13,000 yr within the Chiloe island (Abarzua *et al.*, 2004) reaching its maximum distribution, in the central depression, within the post-glacial period at 10,000 yr (Moreno, 2004; Haberle and Bennet, 2004; Moreno and Leon, 2003; Moreno, 2000; Heusser, 1999; Lumley and Switsur, 1993). These studies suggest *T. stipularis* has low cold tolerance behaviour, due to its first appearance at the end of the LGM, and having its peak distribution during the post-glacial periods.

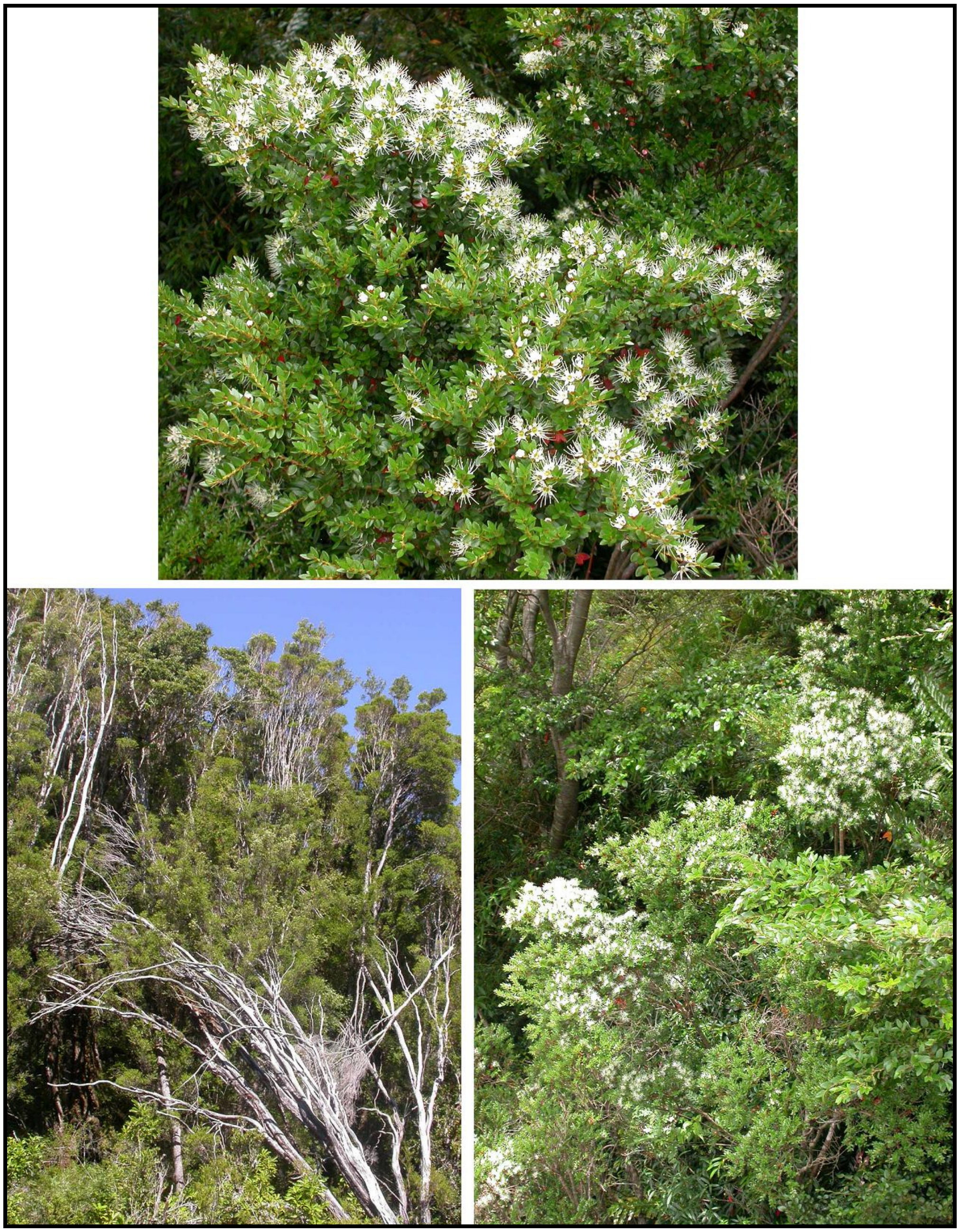


Figure 2.9. *Tepualia stipularis* clockwise from top (a) flowering shrub at Tortel, (b) occurring as a large shrub at Tortel, (c) occurring as a tree at Abtao.

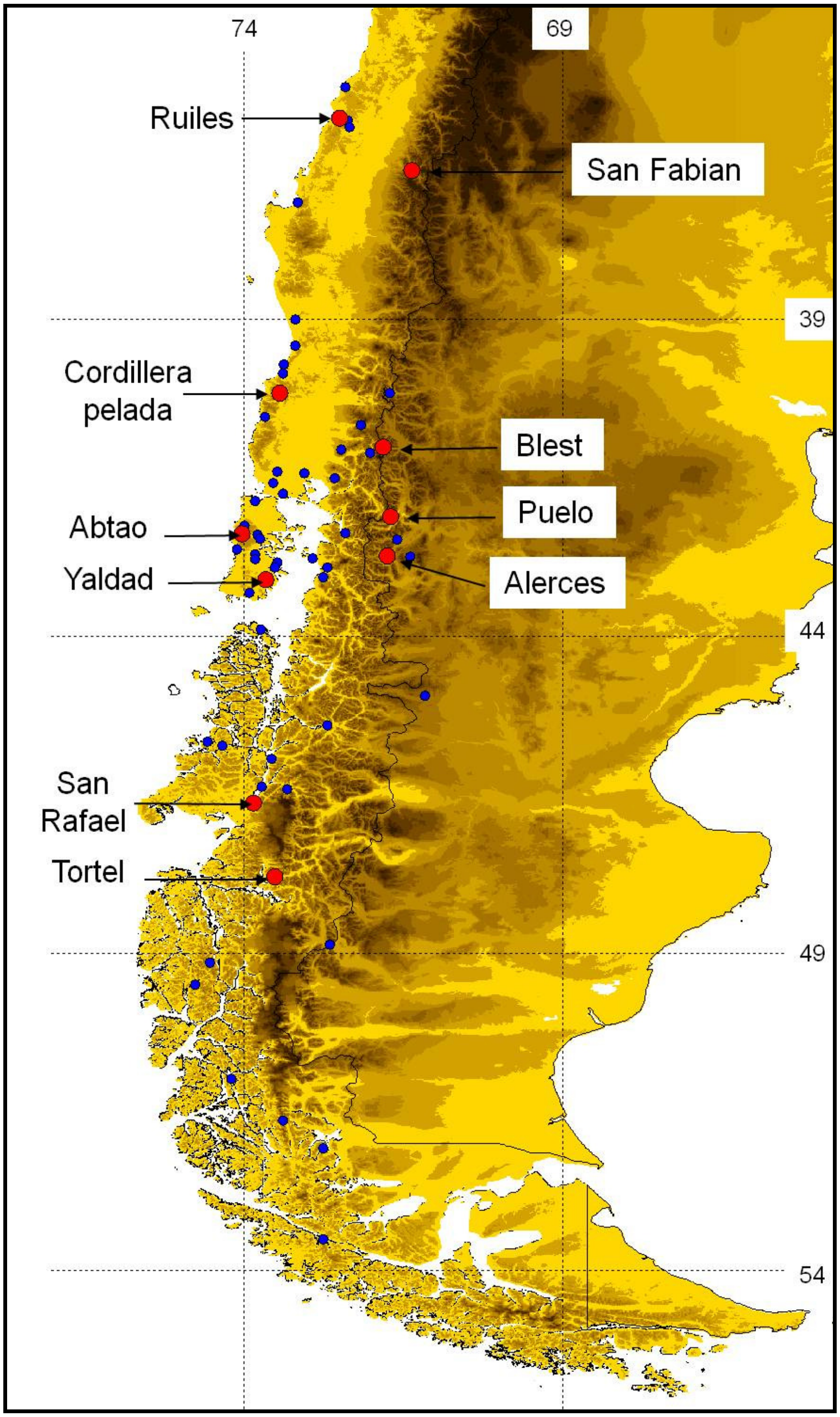


Figure 2.10. Distribution map of *Tepualia stipularis.*
The red dots indicate sampled populations and blue dots are unsampled localities.

Weinmannia trichosperma Cav

Weinmannia trichosperma belongs to the Cunoniacea family. Is a perennial tree up to 30m high with compound and imparipinnate leaves. It has a distinct feature on the leaves of triangular wings in the rachis between each foliole (Figure 2.11). In Chile grows in both Cordilleras (Regions: VII, VIII, IX, X, XI and XII) (Figure 2.12) from sea level up to 1,000 mosl (Figure 2.2). In Argentina it occurs only in the Andes (Provinces of: Chubut, Neuquen, Rio Negro and Sta. Cruz). It occurs in humid forest areas, in ravines and near to rivers, lakes or even in restricted drainage soils (Zuloaga *et al.,* 2009; Donoso, 2006; Riedemann and Aldunate, 2003; Donoso, 1998; Hoffmann, 1997; Rodriguez *et al.*, 1983). This species is not included in the national conservation list, although it is listed as In danger for the VII Region, because there are only few plants left in its northern distribution, due to its over exploitation (Benoit, 1989).

Its historical distribution has been studied by several fossils pollen records. The presence of this species in core pollen studies suggest the occurrence of evergreen forest, therefore warmer climates prevailing. It shows a first appearance at the end of the deglacial period (9,000 yr) in the Coastal Cordillera (Lake District), in the Eastern Andes slope (~41°S) and at Taitao peninsula (known as covered by a thick ice sheet) (Whitlock *et al.*, 2006; Villagran, 2001; Lumley and Switsur, 1993). In the Central Valley it appeared at the end of the LGM reaching its peak distribution by the deglacial period (11,000 yr) (Markgraf *et al.*, 2009; Moreno, 2004; Haberle and Bennet, 2004; Abarzua *et al.*, 2004; Moreno and Leon, 2003; Moreno, 2000; Heusser *et al.*, 1999; 1996). These results suggest this species has low tolerance to cold and dry weather, showing a wide postglacial distribution. The only location which present small amount of pollen traces of the species within the LGM was at Canal de la punilla population, next to lago Llanquihue, with traces at ~20,000 yr with an increase at 14,000 yr persisting until ~11,000 yr (Moreno *et al.*, 1999).

Figure 2.11. *Weinmannia trichosperma*. from top left clockwise (a) close up of plant flowers (b) flowering tree (c) fruiting tree at Tortel (d) general habitat shot, from San Rafael with *W. trichosperma* in the foreground (red canopy, trees with fruits).

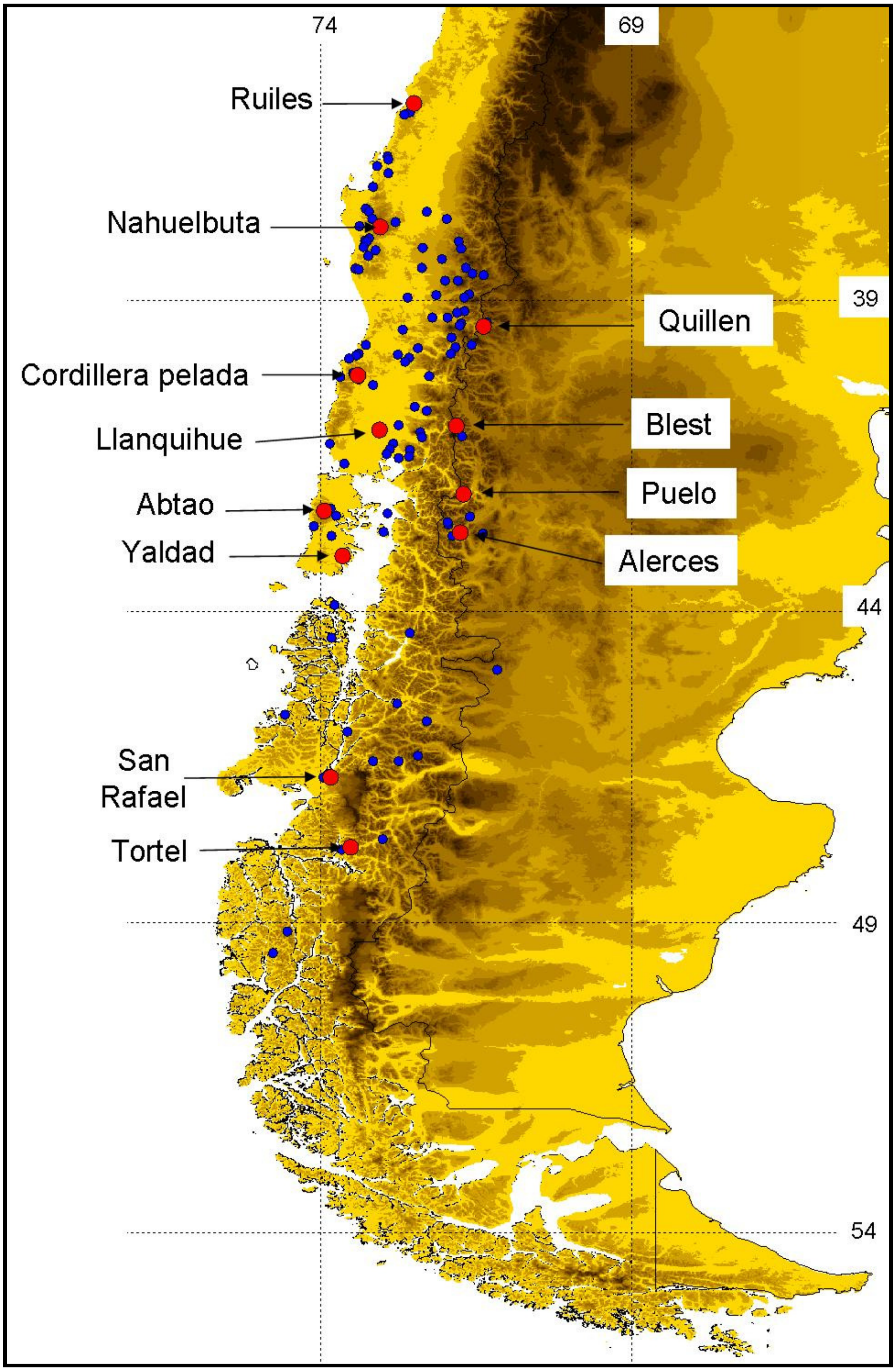


Figure 2.12. Distribution map of *Weinmannia trichosperma.*
The red dots indicate sampled populations and blue dots are unsampled localities.

2.2.2 Sampling Methodology

Species distribution data was obtained from the following herbaria: Royal Botanic Garden Edinburgh (E), Universidad de Concepción (CONC), Instituto de Botánica Darwinion (SI), Universidad Nacional del Comahue (BCRU), Universidad de Magallanes Punta Arenas (HIP), Museo del Fin del mundo Ushuaia and the web page of the Argentinean National parks (APN) http://www.sib.gov.ar/index.php. Sampling was undertaken between 2006 and 2007, and aimed to encompass the geographical range of each species.

The locations of sample populations were classified according to broad geographical regions. We classified samples along a latitudinal gradient as “northern” (north of 40°S), “central” (between 40-45°S), “southern” (consisting of the remainder of southern Patagonia where the Andes follow a N-S axis, namely 45-53°S), and finally “far south” (consisting of populations bordering and south of the Straits of Magellan, where the Andes follow a W-E axis, namely 53-55°S). Populations were further classified as being part of the Coastal range, the central valley, or the Andean mountains, and where possible we recorded whether populations were on the west or eastern flanks of these mountain chains. In places, the latter aspects of this classification scheme is somewhat subjective, particularly in the Southern region where the Coastal range and Andes almost converge, and also where the Andes decrease in height in southern Patagonia, and it can be difficult to classify populations as western or eastern. Populations can be to the east of the higher mountains of the Andean cordillera, but nevertheless in a watershed that drains to the west. Where the classification is subjective, we have indicated this with a “~”.

For each population at least one individual was collected as voucher herbarium specimen. For genetic analyses, up to 5 individuals per population were sampled, with a minimum inter-plant distance of at least 5 m, where possible, to avoid sampling related individuals. Plant samples, which consist of one or two leaves per bag for *W. trichosperma*, a small twig containing several leaves for *D. chacaye*, *E. virgata* and *T. stipularis*, and 2 whole plants for *D. fascicularis*, were either placed in i) zip-sealable plastic bags containing silica gel or ii) a "make-your-own" tea bag surrounded by silica gel. The only species that all samples were stored in 'silica-gel containing plastic bags' was *D. fascicularis* due to its thick leaves, as complete contact with silica gel was necessary to prevent hydration and subsequent degradation of the DNA.

Table 2.2. Distribution of samples of five Patagonian plant species used for phylogeographic studies. Indicating glacial status of each population.

Area	**Population**	**Location**	**Lat (S)**	**Long (W)**	***D. chacaye***	***D. fascicularis***	***E. virgata***	***T. stipularis***	***W. trichosperma***	**Region**
North coastal	Ruiles	RN. Los Ruiles	35°49'	72°30'	-	-	-	Y/c	Y/a	NG
North Andes “W”	San Fabian	San Fabian de Alico	36°39'	71°22'	-	-	-	Y/a	-	NG
North Andes “W”	Chillan	Termas de Chillan	36°51'	71°35'	Y/b	-	Y/c	-	-	NG
North coastal	Nahuelbuta	PN. Nahuelbuta	37°47'-37°48'	73°00’-73°03’	Y/b	Y/b	Y/d	-	Y/c	NG
North Andes “E”	Quillen	PN. Lanin, Lago Quillen	39°21’-39°25’	71°12'-71°23'	Y/e	-	Y/d	-	Y/d	NG
Central coastal	Cordillera pelada	MN. Alerce costero	40°09'-40°12'	73°25'-73°29'	-	Y/c	-	Y/b	Y/d	NG
Central Andes “E”	Blest	PN. Nahuelhuapi, Puerto Blest	41°00'-41°02'	71°48'-71°49'	Y/b	-	Y/c	Y/b	Y/c	G
Central depression	Llanquihue	Llanquihue, Fundo paso la Botella	41°04'	73°03'	-	-	-	-	Y/c	NG
Central Andes “E”	Puelo	PN. Puelo	42°06'	71°36'-71°42'	Y/e	-	-	Y/a	Y/a	-
Central coastal	Abtao	PN. Chiloe, Sector Abtao	42°22'	73°57'-74°02'	-	Y/d	-	Y/e	Y/e	NG
Central Andes “E”	Alerces	PN. Los Alerces	42°43’-42°44'	71°45'-71°59'	Y/d	-	Y/d	Y/d	Y/e	-
Central coastal	Yaldad	Chiloe, road to Yaldad	43°06’	73°39'	-	-	-	Y/e	Y/e	G
South Andes “E”	~Cohyaique	RN. Cohyaique	45°32'	72°02'	-	-	Y/b	-	-	-
South Andes “E”	~Simpson	Coyhaique, Rio Simpson	45°43'	72°05'	Y/b	-	-	-	-	-

Table 2.2. Continued

Area	Population	Location	Lat (S)	Long (W)	*D. chacaye*	*D. fascicularis*	*E. virgata*	*T. stipularis*	*W. trichosperma*	Region
South Andes "W"	San Rafael	PN. Laguna San Rafael	46°36'-46°37'	73°51'	-	-	-	Y/d	Y/e	G
South Andes "E"	~Soler	Puerto Bertrand, Rio Soler	46°59'-47°00'	72°59'	Y/b	-	Y/c	-	-	G
South Andes "E"	PN. Perito Moreno	PN. Perito Moreno	47°45'-47°52'	72°10'-72°12'	Y/b	-	Y^/c	-	-	-
South Andes "W"	Tortel	Aisen, Caleta Tortel	47°47'	73°31'	-	Y/b	-	Y/e	Y/e	G
South Andes "E"	Lago Desierto	Chalten, Lago Desierto	49°03'-49°04'	72°51'-72°52'	Y/c	-	Y/c	-	-	G
South Andes "E"	Piedra del Fraile	PN. Los Glaciares, Piedra del Fraile	49°13'-49°18'	72°56'-72°54'	Y/b		Y/b			G
South Andes "E"	Glaciar PM	PN. Los Glaciares, glaciar Perito Moreno	49°18'	72°54'	Y/c	-		-	-	G
South Andes "E"	Lago Roca	Santa Cruz, Lago Roca	50°32'	72°49'	Y/c	-	-	-	-	G
South Andes "E"	Pto. Natales	Puerto Natales	51°33'-51°56'	72°33'-72°37'	Y/b	Y/c	Y/c	-	-	G
Far South	Pta. Arenas	Punta Arenas	53°00'-53°25'	70°50'-71°15'	Y/c	Y/c	-	-	-	G
Far South	Moat	Tierra del Fuego, Moat road	54°52'-54°57'	67°44'-67°19'	Y/c	Y^/c	-	-	-	G

PN. = National park; MN. = Natural monument; RN. =Natural reserve; ^ = represent the locations with more than one population sampled. G = population location likely to have been glaciated; NG = population location considered not to have been glaciated; - = no data available; ~ = populations difficult to know if they are western or eastern; a = <20 individuals per population; b = 20-50 individuals per population; c = 50-100 individuals per population; d = 100-1000; e = >1000 individuals per population

2.2.3 Molecular Methods

DNA extraction

Approximately 50 mg of dried leaf material was ground to a powder using the TissueLyser system (QIAGEN), for 1 min at 20 Hz, extending the grinding time if necessary. 300 samples were extracted using the DNeasy 96 plant kit, following the manufacturer's recommendations and 150 were extracted using a CTAB buffer, as outlined below. The CTAB method was modified from Doyle and Doyle (1987) with the inclusion of 0.1% insoluble polyvinylpoly-pyrrolidine (PVPP) to the 2x CTAB buffer. Two 24:1 chloroform/ isoamyl alcohol washes were performed and the DNA precipitated overnight in freezer-cold isopropanol. Depending on the size of the resultant pellet, the DNA was dissolved in 50-70 µl of TE (10 mM TRIS-HCl, 1 mM EDTA, pH7.4). The quality and quantity of extracted DNA was checked under UV light on 1.0% agarose gels containing 400 mg of Syber Safe (Invitrogen). From the five species, only *Tepualia stipularis* did not amplify with the extraction of the DNeasy plant kit, so all the individuals from this species were extracted using the CTAB method.

PCR amplification

The reaction mixture (25 *ul*) for all regions tested:

- 16.05 *ul* water (Sigma)
- 2.5 *ul* 10x NH_4 Reaction Buffer (160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCI (pH 8.8 at 25°C), 0.1% stabilizer (Bioline)
- 1.25 *ul* 50 mM $MgCl_2$ Solution (Bioline)
- 2.5 *ul* 10 mM dNTP Mix; dATP, dGTP, dCTP and dTTP
- 0.75 *ul* 10 mM Forward primers
- 0.75 *ul* 10 mM Reverse primers
- 0.2 *ul* BIOTAQ DNA polymerase (5 units/ *ul*) (Bioline)
- 1.0 *ul* template DNA (for some individuals the amount of DNA used, varied between 0.5 to 2 *ul*)

Amplification was carried out using the following PCR profile: 94°C for 4 min. followed by 35 cycles of 94°C for 45 sec, 53°C-5X°C for 45 sec (according to the primer annealing temperature) (Table 2.3), 72°C for 1-X min (according to the length of fragment to be amplified (Table 2.3) and finally 72°C for 10 min. All PCRs were performed using a Tetrad 2 peltier thermal cycler (Biorad). PCR products quality and quantity was assessed by were electrophoresis on 1.0% agarose gels containing 400 mg of Syber Safe (Invitrogen) and checked under UV light, comparing the products with 1kb+standard marker (Invitrogen).

Sequencing PCR

PCR product was purified either by QIAquick PCR Purification Kit (Qiagen) or ExoSAP-IT (GE Healthcare). QIAquick PCR purification was carried out following the manufacturer's recommendations. The protocol for ExoSAP IT is as follows: mix 5 *ul* PCR product and 2 *ul* ExoSAP-IT and incubate at 37°C for 15 minutes followed by deactivation of enzyme at 80°C for 15 min. Sequencing was performed using either DTCS Quickstart sequencing mix on a CEQ 8,000 automated sequencer (Beckman and Coulter) or BigDye V3.1 sequencing mix on an ABI 3,730 system (Perkin Elmer). Protocols for both sequencers are given in Table 2.4. The resulting sequences were manually edited and analysed using Sequencher® 4.7 (Gene Codes Corporation).

Table 2.3. Primers, reaction conditions and usefulness of eleven chloroplast regions for phylogeographic studies in five Patagonian plant species.

Cp region	**Primer sequence 5’-3’ F=Forward R=Reverse**	**Species**	**Annealing temp. °C**	**Extension time (sec)**	**Amplification length (bp)**	**PCR success**	**Sequence polymorphism**
atpB-rbcL	**F:** ACATCKARTACKGGACCAATAA **R:** AACACCAGCTTT RAATCCAA	*D. chacaye*	49	60	850	Yes	No
		D. fascicularis	49	60	850	Yes	No
		E. virgata	49	60	850	Yes	No
		T. stipularis	49	60	850	Yes	Yes
		W. trichosperma	49	60	850	Yes	No
trnC-trnD	**F:** CCAGTTCAAATCT GGGTGTC **R:** GGGATTGTAGTT CAATTGGT	*D. chacaye*	52-58	180	-	No	-
		D. fascicularis	58	180	3000	Yes	Yes
		E. virgata	58	180	3000	Yes	No
		T. stipularis	58-64	180	-	No	-
		W. trichosperma	58	180	3000	Yes	Yes
trnD-trnT	**F:** ACCAATTGAACTA CAATCCC **R:** CTACCACTGAGTTAAAAGGG	*D. chacaye*	54	120	1650	Yes	Yes
		D. fascicularis	52-54	120	-	No	-
		E. virgata	54	120	1450	Yes	Yes
		T. stipularis	54	120	1650	Yes	Yes
		W. trichosperma	52-54	120	-	No	-
trnH-psbA	**F:** ACTGCCTTGATCCACTTGGC **R:** CGAAGCTCCATCTACAAATGG	*D. chacaye*	53	80	400	Yes	Yes
		D. fascicularis	50	80	400	Yes	Yes
		E. virgata	53	80	500	Yes	Yes
		T. stipularis	53	80	650	Yes	Yes
		W. trichosperma	53	80	650	Yes	Yes

A range of annealing temps were tried for PCRs which failed (typically at 2°C differences)

Table 2.3. Continued

Cp region	**Primer sequence 5’-3’** **F=Forward R=Reverse**	**Species**	**Annealing temp. °C**	**Extension time (sec)**	**Amplification lenght**	**PCR success**	**Sequence polymorphism**
trnL-trnF	F: CGAAATCGGTAGACGCTACG R: ATTTGAACTGGTGACACGAG	*D. chacaye*	55	180	2000	Yes	No
		D. fascicularis	55	180	2000	Yes	No
		E. virgata	55	180	2000	Yes	No
		T. stipularis	55	180	2000	Yes	Yes
		W. trichosperma	55	180	2000	Yes	No
trnK-trnQ	**F:** TAAAAGCCGAGTACTCTACCGTTG **R:** ATTTGAACTGGTGACACGAG	*D. chacaye*	47.5	180	-	No	-
		D. fascicularis	55	180	3000	Yes	No
		E. virgata	55	180	4000	Yes	No
		T. stipularis	55	180	4000	Yes	Yes
		W. trichosperma	55	180	4000	Yes	No
trnS-trnFM	**F:** GAGAGAGAGGGATTCGAACC **R:** CATAACCTTGAGGTCACGGG	*D. chacaye*	55-62	120	-	No	-
		D. fascicularis	62	120	1300	Yes	Yes
		E. virgata	62	120	1300	Yes	No
		T. stipularis	62	120	2000	Yes	Yes
		W. trichosperma	62-64	120	-	No	-
trnS-trnG	**F:** GCCGCTTTAGTCCACTCAGC **R:** GAACGAATCACACTTTTACCAC	*D. chacaye*	52	60	1650	Yes	Yes
		D. fascicularis	52	60	1650	Yes	No
		E. virgata	52	60	1650	Yes	Yes
		T. stipularis	52	60	2000	Yes	Yes
		W. trichosperma	52	60	1650	Yes	Yes

Table 2.3 Continued

Cp region	**Primer sequence 5'-3'** **F=Forward R=Reverse**	**Species**	**Annealing temp. °C**	**Extension time (sec)**	**Amplification lenght**	**PCR success**	**Sequence polymorphism**
rpl20-rps12	**F:**TTTGTTCTACGTCTCCGAGC **R:**GTCGAGGAACATGTACTAGG	*D. chacaye*	53	60	850	Yes	No
		D. fascicularis	53	60	850	Yes	No
		E. virgata	53	60	850	Yes	No
		T. stipularis	53	60	850	Yes	Yes
		W. trichosperma	53	60	850	Yes	Yes
rpoC1-trnC	**F:** GCACAAATTCCRCTTTTTATRGG **R:** CGACACCCRGATTTGAACTGG	*D. chacaye*	47.5	300	3000	Yes	No
		D. fascicularis	47.5	300	-	No	-
		E. virgata	47.5	300	3000	Yes	No
		T. stipularis	47.5	300	-	No	-
		W. trichosperma	47.5	300	-	No	-
rps16	**F:** AAACGATGTGGTARAAAGCAAC **R**: AACATCWATTGCAASGATTCGATA	*D. chacaye*	48	60	850	Yes	No
		D. fascicularis	48	60	850	Yes	No
		E. virgata	48	60	850	Yes	No
		T. stipularis	48	60	850	Yes	Yes
		W. trichosperma	48	60	850	Yes	Yes

Table 2.4. Protocols used for DNA sequencing.

	Sequencing Platform	
	Beckman	**ABI**
Fluorescent Dye	DTCS Quickstart mix	BIGDYE mix
Total reaction volume	10 *ul*	10 *ul*
Cycle sequencing profile	96˚C 20sec	95˚C 30sec
	50˚C 20 sec + 60˚C 4 min for 35 cycles	50˚C 20 sec + 60˚C 4 min for 25 cycles

Identification of chloroplast DNA variation

To assess genetic variation in the chloroplast genome, a total of 11 chloroplast regions were initially tested for PCR amplification on 4 samples per species (Table 2.3). These samples were selected from the pool of available material from the first field trip to broadly represent the geographical range of each species, and hence to maximize the chances of detecting variation (Table 2.5). Details of the amplified chloroplast regions, primers and annealing temperature for each primer set are given in Table 2.3.

Table 2.5. Samples used in initial assessment of chloroplast sequence variation and their associated sample identifiers.

Region	Species	Population name	Sample number EDNA	Collection number CM
North	*D. chacaye*	Chillan	EDNA06-0551	CM6-1233
	D. fascicularis	Nahuelbuta	EDNA06-1307	CM6-1354
	E. virgata	Chillan	EDNA06-0815	CM6-1197
	T. stipularis	Ruiles	EDNA06-1089	CM6-1001
	W. trichosperma	Ruiles	EDNA06-1217	CM6-836
East	*D. chacaye*	Blest	EDNA06-0509	CM6-1173
	D. fascicularis	Abtao	EDNA06-1277	CM6-149
	E. virgata	Blest	EDNA06-0778	CM6-1368
	T. stipularis	Blest	EDNA06-1030	CM6-828
	W. trichosperma	Blest	EDNA06-1168	CM6-1165
South	*D. chacaye*	Pta Arenas	EDNA06-0479	CM6-750
	D. fascicularis	Pto Natales	EDNA06-1253	CM6-344
	E. virgata	Pto Natales	EDNA06-0758	CM6-627
	T. stipularis	Yaldad	EDNA06-0988	CM6-290
	W. trichosperma	Yaldad	EDNA06-1107	CM6-278
West	*D. chacaye*	Nahuelbuta	EDNA06-0547	CM6-1359
	D. fascicularis	Cordillera Pelada	EDNA06-1285	CM6-6
	E. virgata	Nahuelbuta	EDNA06-0788	CM6-802
	T. stipularis	Cordillera Pelada	EDNA06-1137	CM6-54
	W. trichosperma	Cordillera Pelada	EDNA06-1053	CM6-22

For a distribution of these samples see maps in Figure 2.4-2.12.

From the initial screen of eleven chloroplast regions per species, three regions were particularly polymorphic for the five analysed species: *trnD-trnT*, *trnH-psbA* and *trnS-trnG* (these showed variations in at least three species) (Table 2.6). The species that showed the highest level of polymorphism was *T. stipularis* with eight variable regions; the next most variable species was *W. trichosperma* with five variable regions, the rest of the species showed variation in 2-3 regions (Table 2.6).

To finalize the choice of cpDNA regions for the full study, three of the most variable chloroplast regions were screened on one individual per population for the whole sample set. From this screen, the best two regions were selected for each species. The regions used varied from species to species although the regions *trnH-psbA* and *trnS-trnG* were the most often used and most often showed informative variation (Table 2.6). In *T. stipularis* almost all the regions showed variation, although many regions were redundant in terms of resolving new haplotypes (e.g. they showed variation which resolved the same haplotypes as other loci) (Table 2.6). In the case of *W. trichosperma*, *trnS-trnG* showed a high level of variation but only as indels, and priority was given to selecting regions where the mutational changes were nucleotide substitutions. Another criterion used in the final selection process was the ease of amplification and sequencing.

Table 2.6. Sequencing success and levels of polymorphism in five Patagonian plant species, based on a single individual from each sampled population.

Chloroplast region	***D. chacaye***	***D. fascicularis***	***E. virgata***	***T. stipularis***	***W. trichosperma***
atpB-rbcL	NV	NV	NV	NU(2sub)	NV
trnC-trnD	F	1SSR*	NV	F	NU(1SSR)
trnD-trnT	1SSR*	F	1sub,1ind,1SSR*	7sub,2ind,2SSr	F
trnH-psbA	1sub	1ind*	1sub	5sub,4ind*	2sub,1ind*
trnK-trnQ	F	NV	NV	NV	F
trnL-trnF	NV	NV	NV	NU(2sub,1ind)	NV
trnS-trnFM	F	NU(1SSR)	NV	NU(4sub,1ind,1SSR)	F
trnS-trnG	2ind,2SSR*	NV	1sub*	7sub,3ind,1SSR*	10ind
rpl20-rps12	NV	NV	NV	NU(1sub)	NU(2sub,1SSR)
rpoC1-trnC	NV	F	NV	F	F
rps16	NV	NV	NV	NU(3sub,2 SSR)	2sub*

F = failed initial screen; NV = no variation in initial screen; NU = not used (showed variation in initial screen, but few informative markers and no new haplotypes were resolved); sub = substitution; ind = indel; * represent loci chosen for the full population screen.

Following the selection of two regions for each species, these regions were then analyzed in up to five individuals per population for the final analysis.

The dataset for each species consisted of between 40-76 individuals, with an average of twelve populations per species (total N = 280 individuals). In all but one cases this involved bi-directional sequencing of two regions using the protocols described above (Table 2.7). The one exception was for *trnS-trnG* in *E. virgata*, in which variation was assayed by means of an RFLP (restriction fragment length polymorphism). The RFLP protocol is listed below.

Table 2.7. Chloroplast regions used to screen the full sample set of five Patagonian plant species.

Species	**Region 1**	**Region 2**
D. chacaye	*trnS-trnG*	*trnD-trnT*
D. fascicularis	*trnH-psbA*	*trnC-trnD*
E. virgata	*trnS-trnG**	*trnD-trnT*
T. stipularis	*trnH-psbA*	*trnS-trnG*
W. trichosperma	*trnH-psbA*	*rps16*

*Restriction fragment length polymorphisms (RFLP's)

Substitutions and indels were checked for suitability for RFLP-based assays using Web Cutter 2.0 and Sequencher ® 4.7 (Gene Codes Corporation). A base substitution in *E. virgata* was assayed using the following reaction mixture (25 *ul*):

- 2.5 *ul* Buffer No 3
- 0.5 *ul* BSPE I restriction enzyme (Invitrogen)
- 2 *ul* water (Sigma)
- 20 *ul* DNA PCR

Digestion was carried out using the following profile: 37°C for 30 min. followed by an inactivation step of 65°C for 20 min. performed using a Tetrad 2 peltier thermal cycler (Biorad). Digestion product quality and quantity was assessed by an electrophoresis on 2.5% agarose gels containing 400 mg of Syber Safe (Invitrogen) and checked under UV light, comparing the products with 1kb+standard marker (Invitrogen). The RFLP profile consisted of a single band pattern in samples with a T at 603 Bp, versus a double band pattern in samples with a C at the same site.

2.2.4 Molecular Data Analysis

To define chloroplast haplotypes and to format the molecular data for population genetic software packages, the RFLP and sequence variation (substitutions, indels and variation in chloroplast microsatellite length) from each analysed region was scored and analysed using a Chloroplast Excel macro (French pers.com.). Substitutions and indels were quantified by coding the most common type as 0 and the rarer character state as 1. Microsatellites (SSR) variants were coded according to the length of the repeats.

The number of haplotypes per population was recorded, as was the distribution of private haplotyes (haplotypes found only in a given population or geographical regions). Quantification of genetic diversity and differentiation was undertaken using Nei's gene diversity estimators including total gene diversity (Ht), gene diversity present within populations (Hs) and gene diversity between populations (Gst) using Haplodiv (Pons and Petit, 1995). Pairwise population differentiation (Fst) was estimated using Arlequin ver. 2.000 (Schneider *et al.*, 2000). To assess whether there is evidence for phylogeographic structure in the data set, Nst was estimated using Haplonst (Pons and Petit, 1996). This metric takes into account mutational differences between haplotypes as well as frequency differences among haplotypes (as opposed to Fst and Gst which treat distinct haplotypes as being equidistant from one another). To test whether Nst was significantly different from Gst, a randomisation test was undertaken using Permut (Pons and Petit, 1996). A minimum spanning network (MSN) tree was generated to visualise relationships and levels of mutational change between haplotypes using Arlequin ver. 2.000 (Schneider *et al.*, 2000). In species where the polymorphisms contained a mixture of indels, substitutions and SSRs, MSN trees were created using (a) all characters, (b) using only substitutions to create the tree backbone, with indels and SSR's then used to differentiated among haplotypes defined by a given set of substitutions, or (c) using substitutions and indels to create the tree backbone, with SSR's used to differentiated among haplotypes defined by a given set of substitutions and indels. This approach was adopted to explore if characters suspected of high levels of homoplasy (particularly SSR's) influence tree topologies.

2.3 Results

2.3.1 General patterns of sequence and haplotype diversity

Levels of haplotype diversity ranged from 10 haplotypes in *Discaria chacaye* to only three haplotyes in *Donatia fasicularis*. In terms of amount of sequence diversity, the highest levels of variation were obtained from *W. trichosperma* which showed a total of 20 mutations including substitutions, indels and SSR's. The species with lowest levels of sequence variability was *D. fascicularis* with only two polymorphic characters (from Table 2.8 to Table 2.12).

Table 2.8. Chloroplast haplotypes detected in *Discaria chacaye.*

Haplotype name	***trnD-T***	***trn S-G***			
	Bp 318 SSR	**Bp 334 Indel**	**Bp 377 Indel**	**Bp 448 SSR**	**Bp 532 SSR**
A	10	+19 bp	+11 bp	11	12
B	11	+19 bp	+11 bp	11	12
C	11	+19 bp	+11 bp	10	13
D	11	+19 bp	+11 bp	11	14
E	12	+19 bp	+11 bp	11	12
F	12	+19 bp	+11 bp	11	14
G	12	+19 bp	+11 bp	11	15
H	10	+19 bp	-11 bp	12	12
I	10	-19 bp	-11 bp	11	12
J	11	-19 bp	-11 bp	11	12

Bp= base pair; + = insertion; - = deletion

Table 2.9. Chloroplast haplotypes detected in *Donatia fascicularis.*

Haplotype name	***trnC-D***	***trnH-psbA***
	Bp 395 SSR	**Bp 258 Indel**
A	17	-14 bp
B	16	-14 bp
C	16	+14 bp

Bp= base pair; + = insertion; - = deletion

Table 2.10. Chloroplast haplotypes detected in *Escallonia virgata.*

Haplotype name	***trnS-G***	***trnD-T***		
	Bp 623 Sub	**Bp 667 SSR**	**Bp 781 Indel**	**Bp 879 Sub**
A	C	8	+5 bp	A
B	T	8	-5 bp	A
C	T	7	-5 bp	A
D	T	8	-5 bp	T

Bp= base pair; + = insertion; - = deletion; Sub = substitution

Table 2.11. Chloroplast haplotypes detected in *Tepualia stipularis*.

Haplotype name	***trnH-psbA***									***trnS-G***								
	Bp 113 Sub	**Bp 207 Indel**	**Bp 241 Indel**	**Bp 301 Indel**	**Bp 365 Sub**	**Bp 386 Sub**	**Bp 391 Indel**	**Bp 509 Sub**	**Bp 510 Sub**	**Bp 164 Sub**	**Bp 167 Sub**	**Bp 217 Indel**	**Bp 247 Indel**	**Bp 412 Sub**	**Bp 488 SSR**	**Bp 571 Sub**	**Bp 628 Indel**	**Bp 760 Sub**
A	G	+5 bp	+14 bp	+5 bp	A	T	-5 bp	T	A	T	A	-6 bp	+5 bp	C	8	T	-1 bp	C
B	A	+5 bp	-14 bp	-5 bp	C	A	+5 bp	G	A	G	C	+6 bp	-5 bp	A	8	T	-1 bp	C
C	A	+5 bp	-14 bp	-5 bp	C	A	-5 bp	G	A	G	C	+6 bp	-5 bp	A	8	T	-1 bp	C
D	G	-5 bp	+14 bp	+5 bp	A	T	-5 bp	T	A	T	A	+6 bp	-5 bp	C	8	G	+1 bp	G
E	G	-5 bp	+14 bp	+5 bp	A	T	-5 bp	T	T	T	A	+6 bp	-5 bp	C	8	G	+1 bp	G
F	G	-5 bp	+14 bp	+5 bp	A	T	-5 bp	T	A	T	A	+6 bp	-5 bp	C	9	G	+1 bp	G

Bp= base pair; + = insertion; - = deletion; Sub = substitution

Table 2.12. Chloroplast haplotypes detected in *Weinmannia trichosperma*.

Haplotype name	***trnH-psbA***			***RPS 16***																
	Bp 308 Sub	**Bp 343 Indel**	**Bp 514 Sub**	**Bp 148 Sub**	**Bp 177 Sub**	**Bp 178 Sub**	**Bp 185 Sub**	**Bp 199 Sub**	**Bp 231 Sub**	**Bp 234 SSR**	**Bp 362 Sub**	**Bp 364 Sub**	**Bp 375 Sub**	**Bp 371 Sub**	**Bp 399 Sub**	**Bp 406 Sub**	**Bp 425 Sub**	**Bp 457 Sub**	**Bp 523 Indel**	**Bp 548 Sub**
A	C	+8 bp	C	A	T	T	A	T	C	9	A	A	T	C	A	A	C	C	-20 bp	T
B	T	+8 bp	C	A	T	T	A	C	C	9	A	A	T	C	A	A	C	C	-20 bp	T
C	C	-8 bp	T	A	T	T	A	C	C	9	A	A	T	C	A	A	C	C	-20 bp	T
D	C	+8 bp	C	A	T	T	A	C	C	9	A	A	T	C	A	A	C	C	-20 bp	T
E	C	+8 bp	C	A	T	T	A	C	C	9	A	A	T	C	A	C	C	C	-20 bp	T
F	T	+8 bp	C	A	T	T	A	C	A	9	A	A	T	C	A	A	C	C	-20 bp	T
G	C	+8 bp	C	A	T	T	A	C	A	9	A	A	T	C	A	A	C	C	-20 bp	T
H	T	+8 bp	T	A	T	T	A	C	A	9	A	A	T	C	A	A	C	C	-20 bp	T
I	T	+8 bp	C	G	C	G	G	C	C	8	A	A	G	A	G	A	T	G	+20 bp	C
J	C	-8 bp	C	A	T	T	A	C	C	9	G	G	T	C	A	A	C	C	-20 bp	T

Bp= base pair; + = insertion; - = deletion; Sub = substitution

2.3.2 Diversity and distribution of genetic variation for all five species

Before analyzing each species separatedly, genetic diversity and differentiation levels for each species are summarized on Table 2.13. Number of haplotypes per population, Number of private haplotypes per latitude and comparison of mean parwise Fst for each species are shown on Figure 2.13 to Figure 2.15.

Table 2.13. Diversity and differentiation of chloroplast haplotypes in five Patagonian plant species

Species	No. pop	No. ind	Region	Number and types of mutations per region			No. haplotypes	Mean number of haplotypes/ pop	H_S (s.e.)	V_S (s.e.)	H_T (s.e.)	V_T (s.e.)	G_{ST} (s.e.)	N_{ST} (s.e.)
				Subs	Indels	SSRs								
D. chacaye	16	76	*trnD-trnT*	0	0	1	10	0.625	0.398 (0.077)	0.335 (0.076)	0.757 (0.051)	0.760 (0.128)	0.474 (0.101)	0.559 (0.087)
			trnS-trnG	0	2	2								
D. fascicularis	8	40	*trnC-trnD*	0	0	1	3	0.375	0.200 (0.100)	0.184 (0.092)	0.476 (0.155)	0.478 (0.178)	0.580 (0.222)	0.614 (0.209)
			trnH-psbA	0	1	0								
E. virgata	12	58	*trnD-trnT*	1	1	1	4	3	0.000 (0.000)	-	0.514 (0.155)	-	1.000 (0.000)	-
			*trnS-trnG***	1	0	0								
T. stipularis	10	49	*trnH-psbA*	5	4	0	6	0.6	0.120 (0.061)	0.000 (0.000)	0.439 (0.175)	0.450 (0.303)	0.727 (0.133)	1.000 (0.000)
			trnS-trnG	5	3	1								
W. trichosperma*	11	56	*trnH-psbA*	2	1	0	9	0.82	0.442 (0.076)	0.453 (0.241)	0.677 (0.093)	0.676 (0.250)	0.347 (0.100)	0.329 (0.115)
			RPS16	15	1	1								

Pop = populations; ind = individual; * = analisys not using Ruiles population; ** = region analysed using RFLP's; Hs = Population diversity; Ht = Total gene diversity; Gst = Coeficient of differentiation amog populations; Vs = Population phylogenetic diversity; Vt = Total phylogenetic diversity

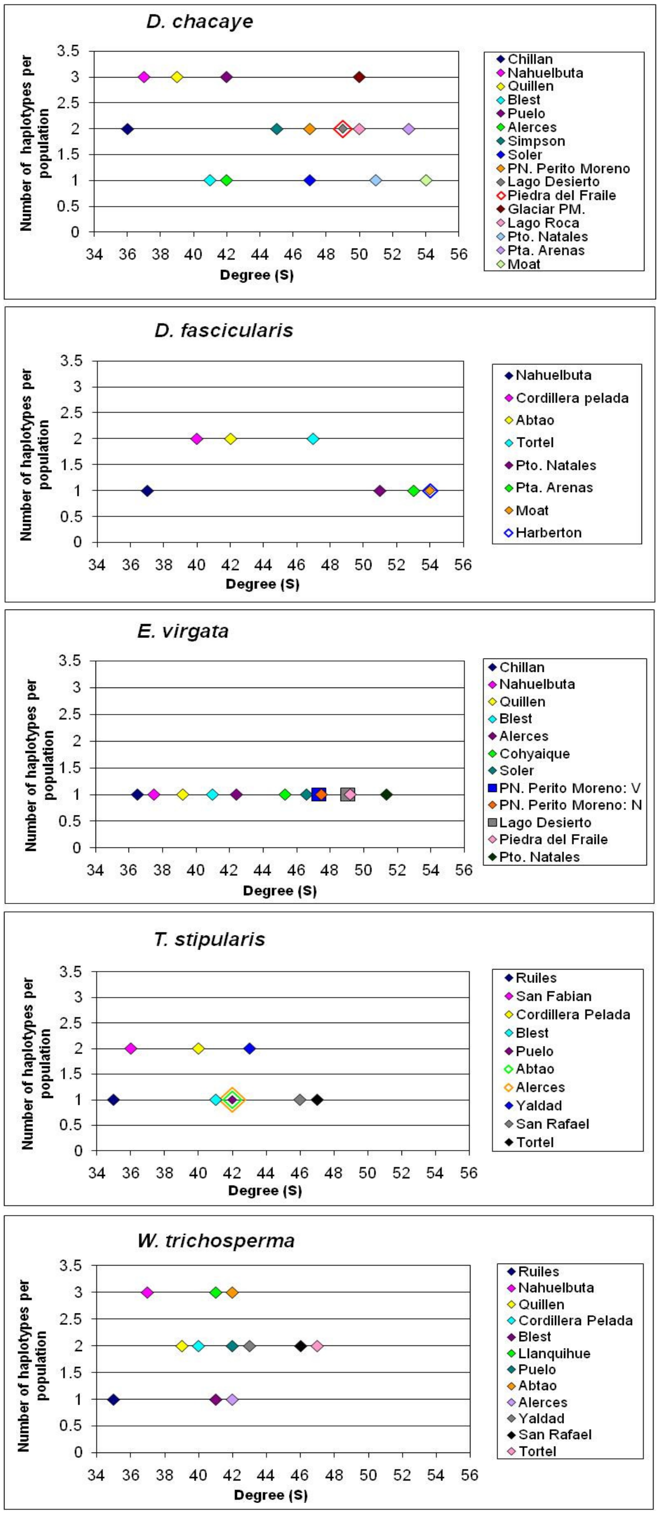


Figure 2.13. Number of haplotypes per population, plotted according to latitude for five Patagonian plant species.

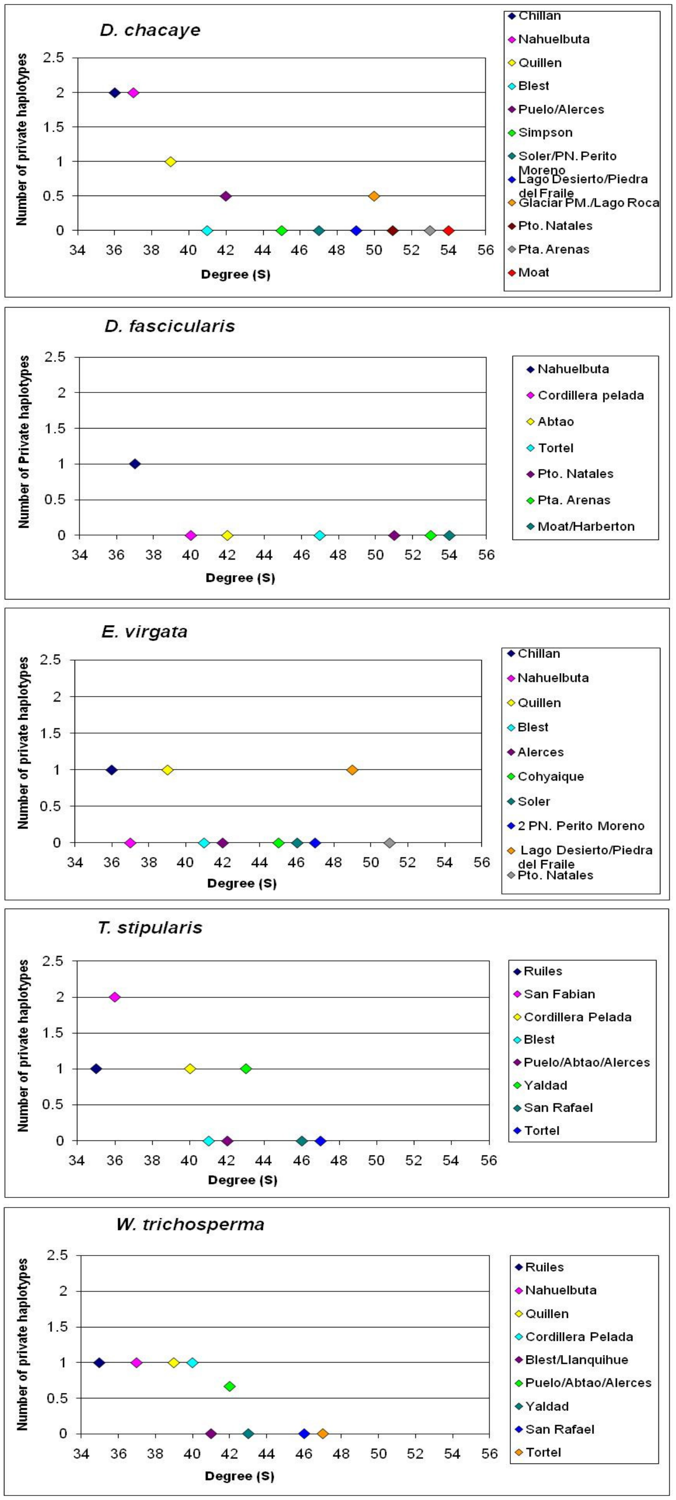


Figure 2.14. Number of private haplotypes plotted according to latitude for five Patagonian plant species.

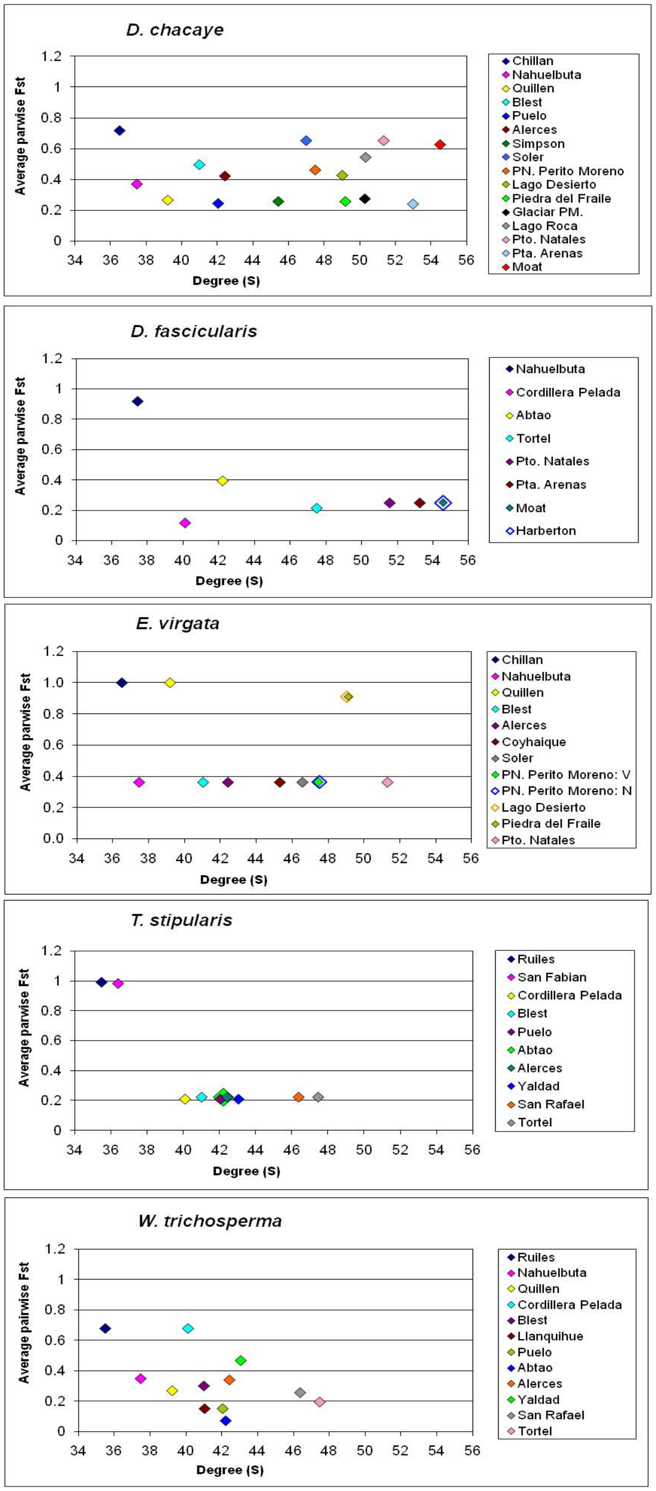


Figure 2.15. Comparison of the mean pairwise Fst for anyone population plotted against all others, according to latitude.

2.3.3 Diversity and distribution of genetic variation in individual species

Discaria chacaye

A total of 10 haplotypes were detected based on a combination of indel and cpSSR variation (Table 2.13; Table 2.14). The number of haplotypes recovered from individual populations range from 1-3, with 11/16 (69%) of populations showing haplotype polymorphism, and levels of gene diversity range from 0-0.7 (mean Hs = 0.398) (Table 2.13; Table 2.14; Figure 2.13). Of the 10 haplotypes, 7 are private to individual populations (haplotypes A and I are private to Chillan, haplotypes H and J are private to Nahulbuta, haplotye C is private to Quillen, haplotype E is private to Puelo and haplotype G is private to Lago Roca) (Table 2.14; Figure 2.14). The population with the highest frequency of private haplotypes is Chillan, where 4/4 individuals have private haplotypes, followed by Nahuelbuta (3/5), Lago Roca (2/5), and then Puelo and Quillen (both 1/5). All other populations shared all of their haplotypes with at least one other population. In terms of the proportion of populations that are polymorphic, there is no clear cut association with latitude (Table 2.14; Figure 2.13).

Three haplotypes were widespread, haplotype B occurred in 10 populations from 37°S to 53°S, haplotype F occurred in 9 populations from 42°S to 53°S, and haplotype D occurred in 5 populations from 39°S to 54°S. Mean Gst = 0.474, and Nst = 0.559, both significantly different from zero. There was a significant difference between Gst and Nst, with Nst significantly greater than Gst ($p < 0.01$) (Table 2.13)

Table 2.14. Distribution of haplotype variation within and between populations of *Disacaria chacaye* in Patagonia.

Population	**Region**	**Latitude (S)**	**N**	**Number of individuals with each haplotype**										**Number of private haplotypes**	**Proportion of populations polymorphic per region**	**He**
				A	B	C	D	E	F	G	H	I	J			
Chillan	North Andes "W"	36°51'42.81"	4	1								3		2	3 of 3	0
Nahuelbuta	North coastal	37°47'59.85"	5		2						2		1	2		0.6
Quillen	North Andes "E"	39°22'23.6"	5		3	1	1							1		0
Blest	Central Andes "E"	41°01'21.50"	5		5									0	1 of 3	0.7
Puelo	Central Andes "E"	42°06'02.4"	5		3			1	1					1		0.7
Alerces	Central Andes "E"	42°43'46.7"	3		3									0		0
Simpson	South Andes "E"	45°43'28.8"	5		4				1					0	6 of 8	0.4
Soler	South Andes "E"	47°00'11.1"	5						5					0		0
PN. Perito Moreno	South Andes "E"	47°52'17.1"	5		1		4							0		0.4
Lago Desierto	South Andes "E"	49°04'25.8"	3				1		2					0		0.7
Piedra del Fraile	South Andes "E"	49°18'47.4"	5		4				1					0		0.4
Glaciar PM.	South Andes "E"	50°29'38.4"	5		1		2		2					0		0.4
Lago Roca	South Andes "E"	50°32'31.8"	5						3	2				1		0.667
Pto. Natales	South Andes "E"	51°33'51.21"	5						5					0		0
Pta. Arenas	Far South	53°00'52.68"	5		4				1					0	1 of 2	0.7
Moat	Far South	54°52'37.9"	4				4							0		0

Populations are classified into geographical regions (see Materials and Methods for classification scheme). N = sample size. He = gene diversity. Haplotype designations correspond to those illustrated in the associated minimum spanning tree (Figure 2.16) and defined in Table 2.8. Colour coding of groups of haplotypes corresponds to 'clades' highlighted in the minimum spanning tree (Figure 2.17)

A minimum spanning tree constructed using indels to establish the backbone of the tree is shown in Figure 2.17, and using indels and SSR's together is shown in Figure 2.16. Constructing trees using just the indels to form the network backbone serves to visualise the restriction of the major indels to the northern populations, with all of the more southern haplotypes resulting from SSR variation based on the + 19 bp and + 11 bp haplotype combination. This distinction is lost when all data are used to form the network backbone, as there are more 'alternative' connections in the network. In this case, the use of the indels to form the tree backbone is my preferred method of constructing the network. Although both trees show shallow divergences among haplotypes, with no long interior branches. Of the 60 individuals sampled from 13 populations south of 40°S, all but three individuals have one of three closely related haplotypes (B, D, F) which differ by only two mutational changes. Of the remaining three individuals, one at Puelo has another closely related haplotype (E), and 2 individuals from Lago Roca share another closely related haplotype (G). In contrast, the 14 individuals from the three northern most populations (north of 40°S) collectively contain 7/10 of the detected haplotypes, and using the tree which constrains the back-bone to non-SSR characters, the three northern most populations contain representatives of the three clades (separated by different combinations of two indels), whereas the 13 populations south of 40°S all fall into just 'clade 1'. Considering only the northern populations, representatives of all three 'clades' occur to the west of the Andes, whereas 'clades 2-3' are not found to the east of the Andes. When SSRs are used to contribute towards the interior structure of the tree, the correlation of tree-topology and geography is somewhat less clear cut (Figure 2.16).

There is no evidence for a phylogenetic suture zone north and south of 43°S, although there is a difference in levels of diversity with 4/10 haplotypes found south of 43°S (one not found further north), whereas 9/10 haplotypes are found north of this line (6 not found further south) (Table 2.14). The isolation of the northern most populations (Chillan) is also evident from a comparison between the mean pairwise Fst associated with anyone population and all others, and latitude (Figure 2.15), with a decrease in mean pairwise Fst south of this population until ~47°S where three populations (Soler, Pto. Natales and Moat) present high values.

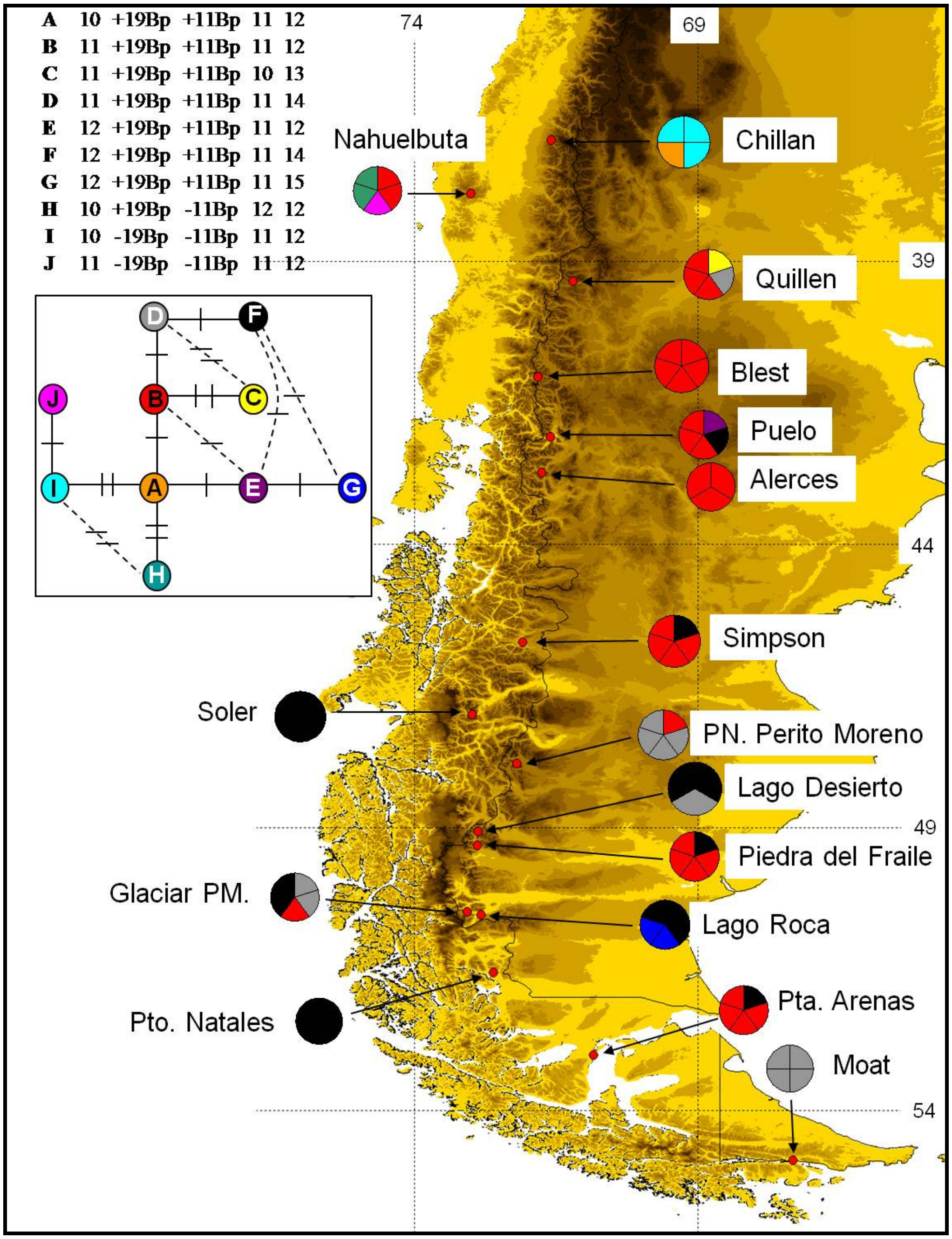
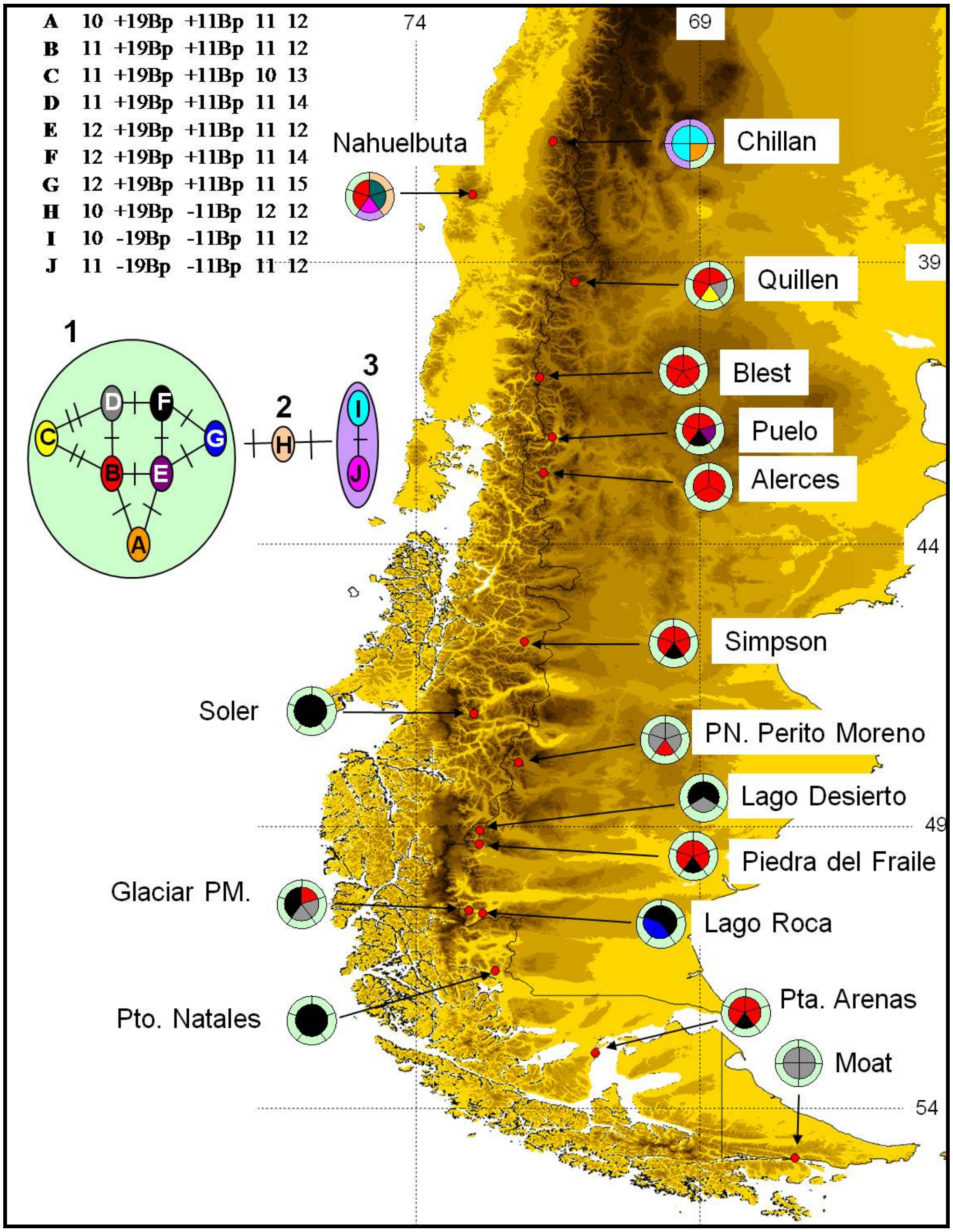


Figure 2.16. MSN and haplotype distribution map for *Discaria chacaye*, using all data to construct the backbone.



A	10	+19Bp	+11Bp	11	12
B	11	+19Bp	+11Bp	11	12
C	11	+19Bp	+11Bp	10	13
D	11	+19Bp	+11Bp	11	14
E	12	+19Bp	+11Bp	11	12
F	12	+19Bp	+11Bp	11	14
G	12	+19Bp	+11Bp	11	15
H	10	+19Bp	-11Bp	12	12
I	10	-19Bp	-11Bp	11	12
J	11	-19Bp	-11Bp	11	12

Figure 2.17. MSN and haplotype distribution map for *Discaria chacaye*, using indels only to construct the backbone. Haplotype group 1 all haplotype have +19bp and +11 bp; haplotype group 2 all haplotypes have +19bp and -11bp and Haplotype group 3 all haplotypes have -19bp and -11bp.

Donatia fascicularis

A total of 3 haplotypes was detected based on an indel and a single cpSSR locus (Table 2.13; Table 2.15). The number of haplotypes recovered from individual populations range from 1-2, with 3/8 (38%) of populations showing haplotype polymorphism, and levels of gene diversity range from 0-0.6 (mean Hs = 0.2) (Figure 2.13). Of the 3 haplotypes, 1 is private to Nahuelbuta (Table 2.13; Table 2.15; Figure 2.14). The other haplotypes are more widespread, with haplotype B occurring in 7 populations from 40°S to 54°S, and haplotype C occurring in three populations from 40°S-47°S. Mean Gst = 0.580, and Nst = 0.614, both significantly different from zero. There was a significant difference between Gst and Nst, with Nst being significantly greater than Gst ($p<0.01$).

Table 2.15. Distribution of haplotype variation within and between populations of *Donatia fascicularis* in Patagonia.

Population	**Region**	**Latitude (S)**	**N**	**Number of individuals with each haplotype**			**Number of private haplotypes**	**Proportion of populations polymorphic per region**	**He**
				A	B	C			
Nahuelbuta	North coastal	37°47'44.74"	5	5			1	0 of 1	0.000
Cordillera Pelada	Central coastal	40°10'13.70"	5		4	1	0	2 of 2	0.400
Abtao	Central coastal	42°22'34.85"	5		2	3	0		0.600
Tortel	South coastal	47°47'46.5"	5		3	2	0	1 of 2	0.400
Pto. Natales	South Andes "E"	51°56'27.56"	5		5		0		0.000
Pta. Arenas	Far South	53°25'41.89"	5		5		0	0 of 3	0.000
Moat	Far South	54°57'07.9"	5		5		0		0.000
Harberton	Far South	54°57'58.0"	5		5		0		0.000

Populations are classified into geographical regions (see Materials and Methods for classification scheme). N = sample size. He = gene diversity. Haplotype designations correspond to those illustrated in the associated minimum spanning tree (Figure 2.18) and defined in Table 2.9. Colour corresponds to haplotypes highlighted in the minimum spanning tree (Figure 2.18).

Haplotype B occupies the interior node in the minimum spanning tree, with haplotypes A and C connected to this by a single step each (Figure 2.18). The major phylogeographic signal in the data set is that the northern most population has a distinct haplotype (A), and that south of 40°S two other haplotypes occur (B, C). The three coastal populations in the central region are polymorphic for haplotypes B and C, and further south all populations are fixed for haplotype B.

The isolation of the northern most populations (Nahuelbuta) is also evident from a comparison between the mean pairwise Fst associated with anyone population and all others, and latitude (Figure 2.15), with a decrease in mean pairwise Fst south of this population.

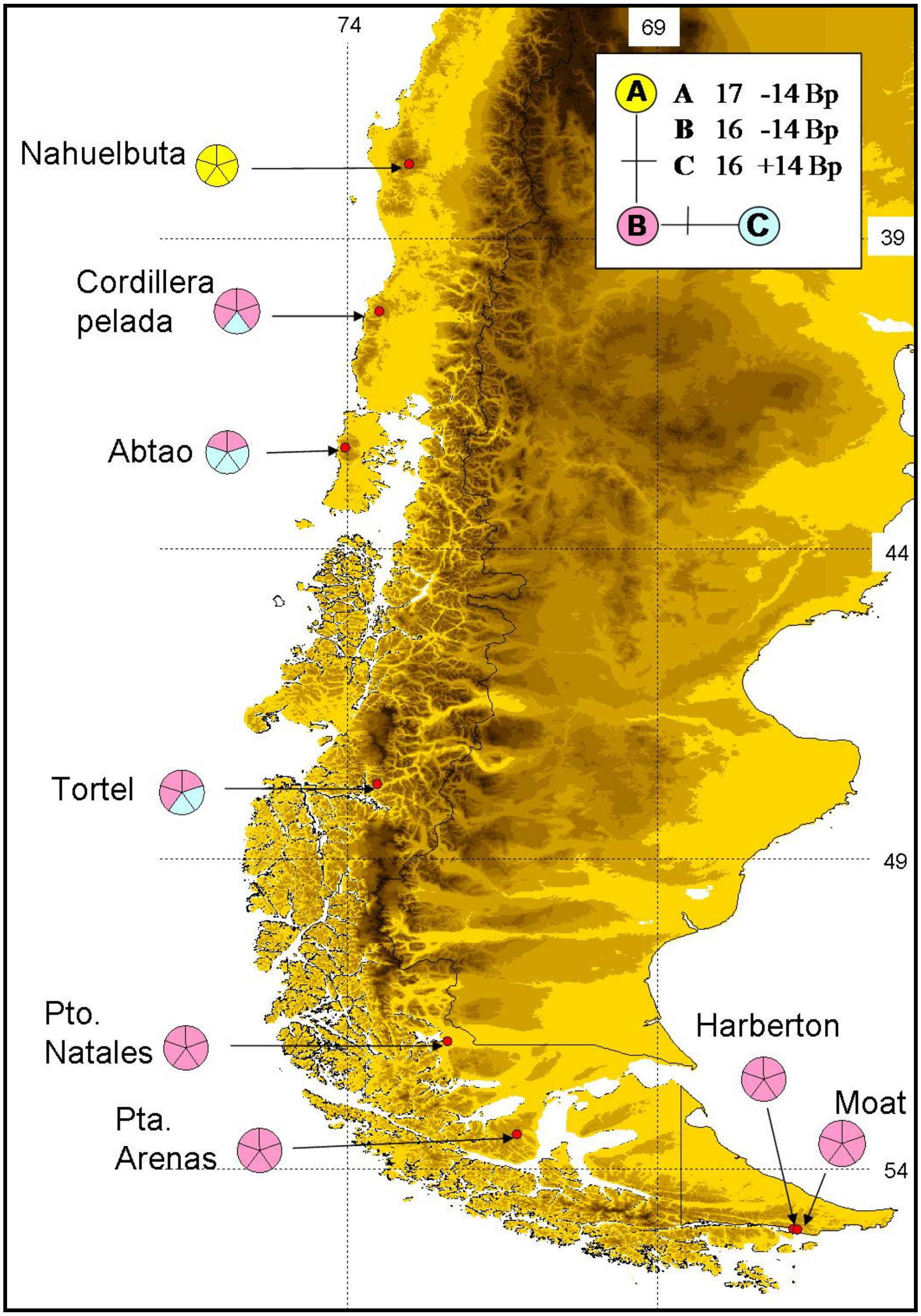


Figure 2.18. MSN and haplotype distribution map for *Donatia fascicularis.*

Escallonia virgata

A total of 4 haplotypes was detected based on two nucleotide substitutions, one indel and one cpSSR locus (Table 2.13; Table 2.16). No within population variation was detected. Of the 4 haplotypes, A is private to Chillan, and C is private to Quillen (Figure 2.14). Both other haplotypes occurred in more than one population, although haplotype D is restricted to two neighbouring populations from around the Fitzroy massif. Haplotype B is by far the most widespread, occurring from Nahuelbuta (37°S) to Pto. Natales (51°S). The mean Gst = 1.0. (Table 2.13).

The widespread haplotype B occupies the interior node in the minimum spanning tree, with haplotypes C and D each connected to this by a single step, and haplotype A by two steps (Table 2.6; Figure 2.19). The major phylogeographic signal in the data set is that the northern-most population has the most distinct haplotype (A), and that one of the two other private haplotypes is also found at the north of the distributional range. The population in the northern coastal range (Nahuelbuta) has an identical haplotype (B) to the common type found along the Andes (Figure 2.19).

The isolation of the northern and central andes “E” populations (Chillan and Quillen) is also evident from a comparison between the mean pairwise Fst associated with anyone population and all others, and latitude (Figure 2.15), with decrease in mean pairwise Fst south of this population, until a rise at ~ 49°S.

Table 2.16. Distribution of haplotype variation within and between populations of *Escallonia virgata* in Patagonia.

Population	**Region**	**Latitude**	**N**	**Number of individuals with each haplotype**				**Number of private haplotypes**	**Proportion of populations polymorphic per region**	**He**
				A	**B**	**C**	**D**			
Chillan	North Andes "W"	S36°51'38.81"	5	5				1	0 of 3	0
Nahuelbuta	North coastal	S37°48'02.19"	5		5			0		0
Quillen	North Andes "E"	S39°21'03.9"	5			5		1		0
Blest	Central Andes "E"	S41°02'09.30"	5		5			0	0 of 2	0
Alerces	Central Andes "E"	S42°44'25.6"	5		5			0		0
Cohyaique	South Andes "E"	S45°32'09.4"	5		5			0	0 of 7	0
Soler	South Andes "E"	S46°59'59.7"	5		5			0		0
PN. Perito Moreno: V.	South Andes "E"	S47°45'03.4"	5		5			0		0
PN. Perito Moreno: N.	South Andes "E"	S47°52'18.9"	3		3			0		0
Lago Desierto	South Andes "E"	S49°03'16.7"	5				5	0		0
Piedra del Fraile	South Andes "E	S49°13'48.2"	5				5	0		0
Pto. Natales	South Andes "E"	S51°33'55.17"	5		5			0		0

Populations are classified into geographical regions (see Materials and Methods for classification scheme). N = sample size. He = gene diversity. Haplotype designations correspond to those illustrated in the associated minimum spanning tree (Figure 2.19) and defined in Table 2.10. Chloroplast haplotypes detected in *Escallonia virgata*.. Colour corresponds to haplotypes highlighted in the minimum spanning tree (Figure 2.19)

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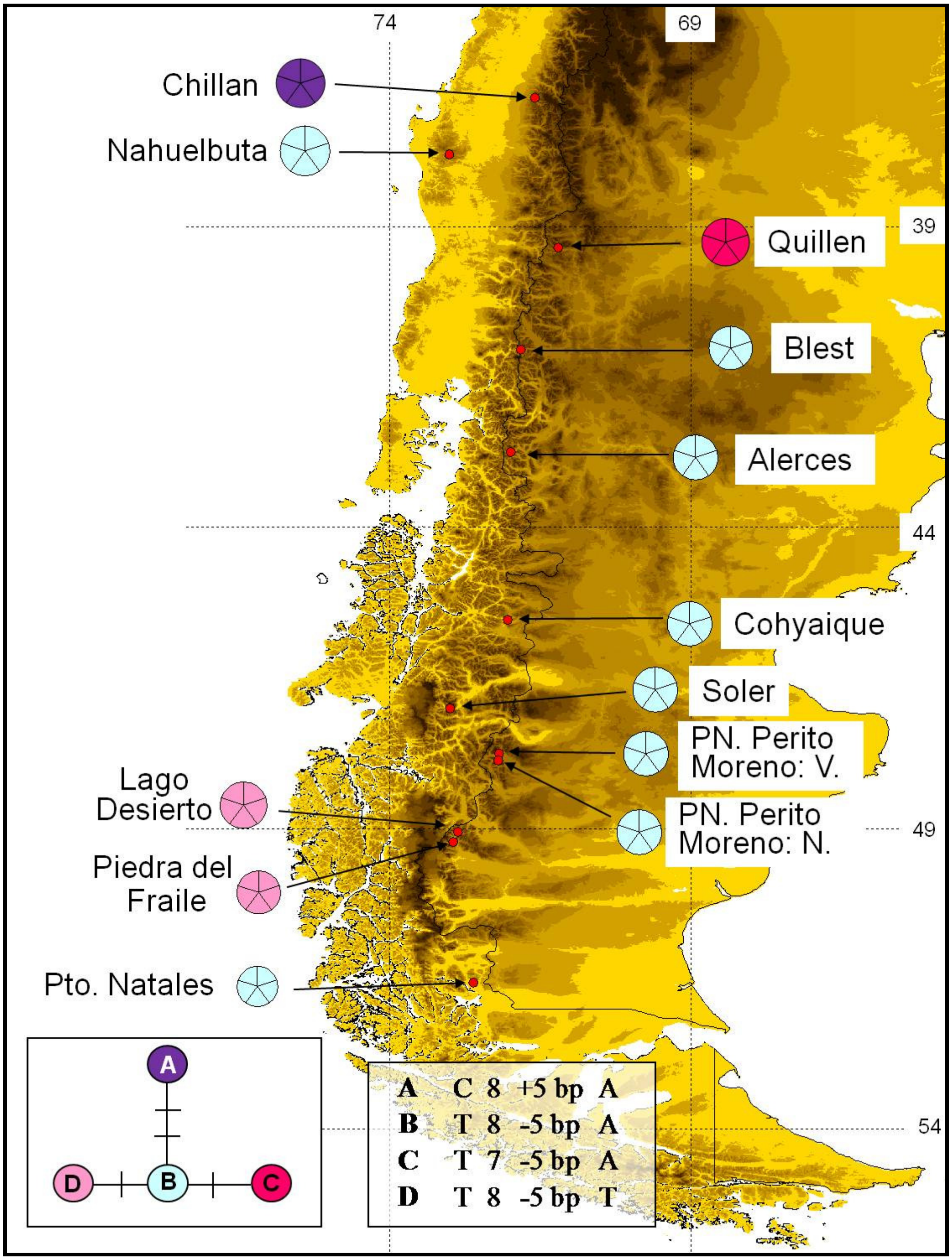


Figure 2.19. MSN and haplotype distribution map for *Escallonia virgata.*

Tepualia stipularis

A total of 6 haplotypes were detected based on a combination of nucleotide substitutions, indels and cpSSR variation (Table 2.13; Table 2.17). The number of haplotypes recovered from individual populations range from 1-2, with 3/10 (30%) of populations showing haplotype polymorphism (San Fabian, Cordillera pelada and Yaldad) (Figure 2.13). Levels of gene diversity range from 0-0.4 (mean Hs = 0.120) (Table 2.13; Table 2.17). Of the 6 haplotypes, 5 are private to individual populations (haplotype A is private to Ruiles, haplotypes B and C are private to San Fabian, haplotye E is private to Cordillera pelada and haplotype F is private to Yaldad). The populations with the highest frequency of private haplotypes are San Fabian and Ruiles where 5/5 individuals have private haplotypes in each case, followed by Cordillera Pelada and Yaldad (1/5 in each case) (Figure 2.14). All other populations are fixed for a single haplotype (D). This haplotype is the most widespread and is present in 8 populations from 40°S to 47°S (Table 2.17). Mean Gst = 0.727, and Nst = 1.0, both significantly different from zero. There was a significant difference between Gst and Nst, with Nst significantly greater than Gst ($p<0.01$) (Table 2.13).

A minimum spanning tree is shown in Figure 2.20, and this consists of three haplotype ‘clades’ separated by multiple mutations, with two of these clades containing some closely related haplotypes. The two haplotypes (B, C) present in San Fabian constitute one ‘clade’ with a single step branch between them, and haplotype A from Ruiles constitutes another ‘clade’. Finally, all individuals south of 40°S occur in the third ‘clade’, with the most common haplotype B occupying the interior node of this clade, and low frequency haplotype variants from Cordillera Pelada and Yaldad each being separated from this by single-step branches.

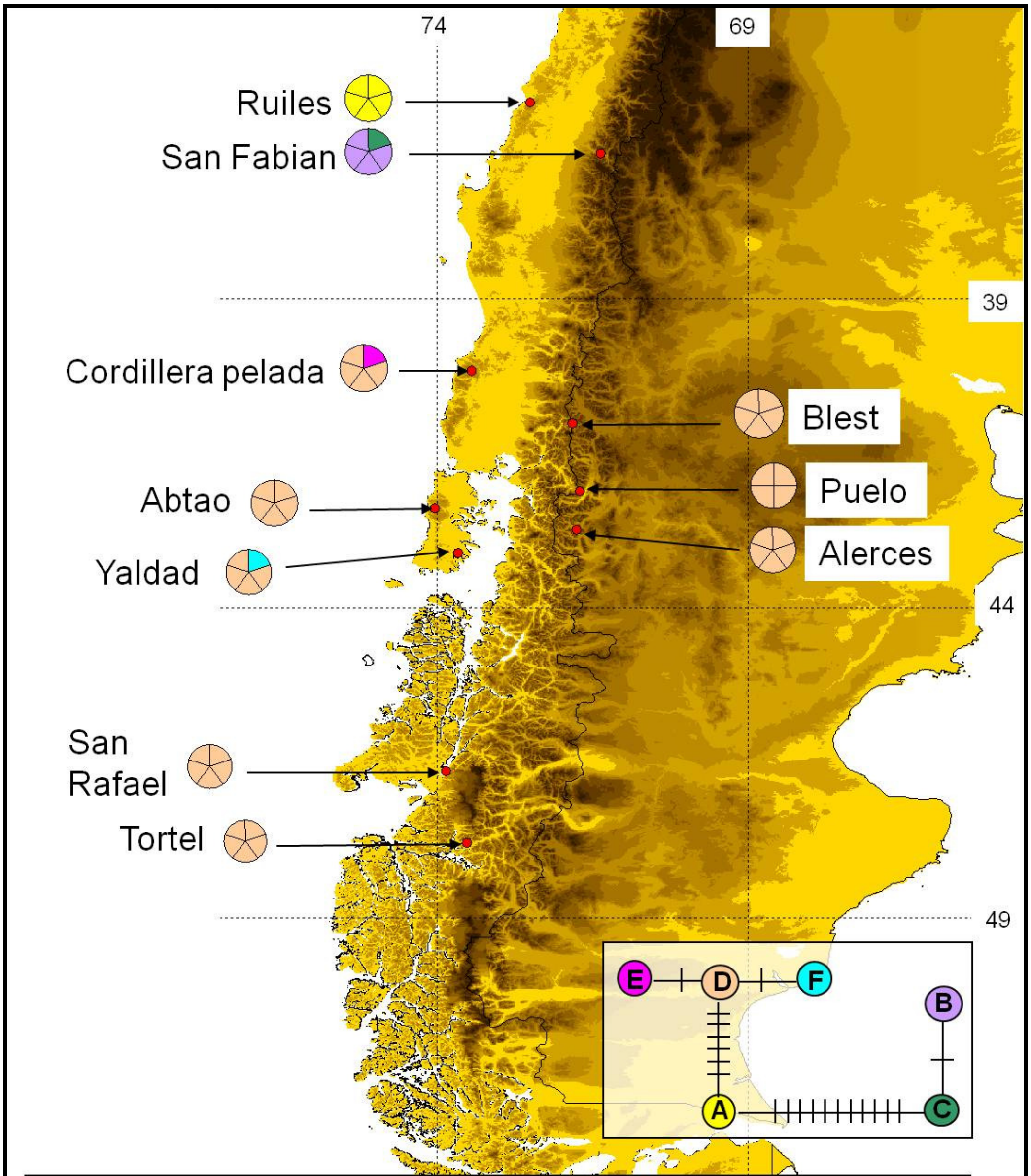
The major phylogeographic signal in the data, is the phylogenetic separation of northern population versus thus south of 40°S. In addition, in the north the Andean and coastal populations share no haplotypes in common.

The isolation of the northern populations (Ruiles and San Fabian) is also evident from a comparison between the mean pairwise Fst associated with anyone population and all others, and latitude (Figure 2.15), with decrease in mean pairwise Fst south of these populations.

Table 2.17. Distribution of haplotype variation within and between populations of *Tepualia stipularis* in Patagonia.

Population	**Region**	**Latitude (S)**	**N**	**Number of individuals with each haplotype**						**Number of private haplotypes**	**Proportion of populations polymorphic per region**	**He**
				A	B	C	D	E	F			
Ruiles	North coastal	35°49'52.53"	5	5						1	1 of 2	0
San Fabian	North Andes "W"	36°39'01.55"	5		4	1				2		0.4
Cordillera Pelada	Central coastal	40°09'48.53"	5				4	1		1	2 of 6	0.4
Blest	Central Andes "E"	41°01'00.53"	5				5			0		0
Puelo	Central Andes "E"	42°06'46.9"	4				4			0		0
Abtao	Central coastal	42°22'39.50"	5				5			0		0
Alerces	Central Andes "E"	42°43'49.1"	5				5			0		0
Yaldad	Central coastal	43°06'13.89"	5				4		1	1		0.4
San Rafael	South Andes "W"	46°37'30.4"	5				5			0	0 of 2	0
Tortel	South Andes "W"	47°47'47.5"	5				5			0		0

Population are classified into geographical regions (see Materials and Methods for classification scheme). N = sample size. He = gene diversity. Haplotype designations correspond to those illustrated in the associated minimum spanning tree (Figure 2.20) and defined in Table 2.11. Colour corresponds to haplotypes highlited in Figure 2.20.



A	G	+5 bp	+14 bp	+5 bp	A	T	-5 bp	T	A	T	A	-6 bp	+5 bp	C	8	T	-1 bp	C
B	A	+5 bp	-14 bp	-5 bp	C	A	+5 bp	G	A	G	C	+6 bp	-5 bp	A	8	T	-1 bp	C
C	A	+5 bp	-14 bp	-5 bp	C	A	-5 bp	G	A	G	C	+6 bp	-5 bp	A	8	T	-1 bp	C
D	G	-5 bp	+14 bp	+5 bp	A	T	-5 bp	T	A	T	A	+6 bp	-5 bp	C	8	G	+1 bp	G
E	G	-5 bp	+14 bp	+5 bp	A	T	-5 bp	T	T	T	A	+6 bp	-5 bp	C	8	G	+1 bp	G
F	G	-5 bp	+14 bp	+5 bp	A	T	-5 bp	T	A	T	A	+6 bp	-5 bp	C	9	G	+1 bp	G

Figure 2.20. MSN and haplotype distribution map for *Tepualia stipularis.*

Weinmannia trichosperma

A total of 10 haplotypes was detected based on a combination of nucleotide substitutions, indels and cpSSR variation (Table 2.13; Table 2.18). The number of haplotypes recovered from individual populations range from 1-3, with 9/12 (75%) of populations showing haplotype polymorphism, populations with highest levels are: Nahuelbuta, Llanquihue and Abtao (Figure 2.13), and levels of gene diversity range from 0-0.7 (mean Hs = 0.44) (Table 2.13; Table 2.18). Of the 10 haplotypes, 6 are private to individual populations (haplotypes A is private to Ruiles, haplotype C is private to Nahuelbuta, haplotype E is private to Quillen, haplotype G is private to Cordillera pelada, haplotypes I and J are private to Abtao (Figure 2.14). The Ruiles population has only one sampled individual, and this is a unique haplotype. Otherwise, the population with the highest frequency of private haplotypes is Abtao, where 2/5 individuals have private haplotypes, whereas Nahuelbuta, Cordillera Pelada, and Quillen each have 1/5 individuals with a private haplotype (Table 2.18).

All other populations share their haplotypes with at least one other population. Two haplotypes are common and widespread, haplotype F occurs in 8 populations from 40°S to 47°S; haplotype B occurred in 6 populations from 37°S to 47°S. The remaining haplotypes were shared among populations but rare (e.g. haplotype D found in Nahuelbuta 37°S and Quillen 39°S; haplotype H found in Llanquihue 41°S and San Rafael 46°S). Mean Gst = 0.347, and Nst = 0.329, both significantly different from zero. There was no significant difference between Gst and Nst. Note that the Ruiles population was excluded from this analysis as only a single individual was sampled.

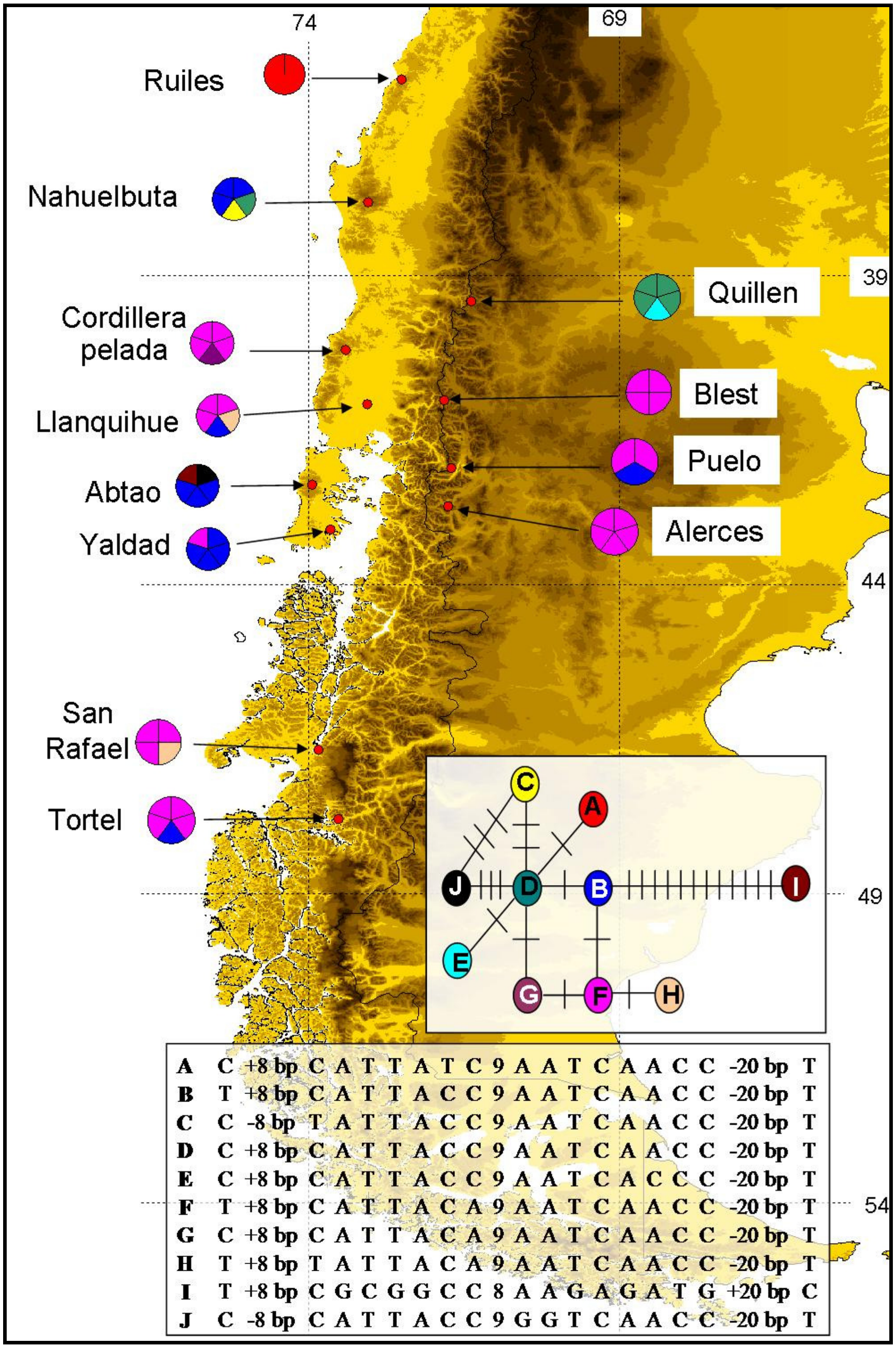
Table 2.18. Distribution of haplotype variation within and between populations of *Weinmannia trichosperma* in Patagonia.

Population	**Region**	**Latitude (S)**	**N**	**Number of individuals with each haplotype**										**Number of private haplotypes**	**Proportion of populations polymorphic per region**	**He**
				A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**			
Ruiles	North coastal	35°49'52.53"	1	1										1	2 of 2 (where n > 1)	1,000
Nahuelbuta	North coastal	37°48'25.06"	5		3	1	1							1		0.700
Quillen	North Andes "E"	39°25'10.4"	5				4	1						1		0.400
Cordillera Pelada	Central coastal	40°12'00.03"	5						4	1				1	5 of 7	0.400
Blest	Central Andes "E"	41°00'44.81"	4						4					0		0.000
Llanquihue	Central depression	41°04'33.43"	5		1				3		1			0		0.700
Puelo	Central Andes "E"	42°06'46.3"	3		1				2					0		0.667
Abtao	Central coastal	42°22'59.59"	5		3							1	1	2		0.700
Alerces	Central Andes "E"	42°43'48.2"	5						5					0		0.000
Yaldad	Central coastal	43°06'28.12"	5		4				1					0		0.400
San Rafael	South Andes "W"	46°39'55.0"	4						3		1			0	2 of 2	0.500
Tortel	South Andes "W"	47°47'46.5"	5		1				4					0		0.400

Populations are classified into geographical regions (see Materials and Methods for classification scheme). N = sample size. He = gene diversity. Haplotype designations correspond to those illustrated in the associated minimum spanning tree (Figure 2.21) and defined in Table 2.11. Colour corresponds to haplotypes highlighted in the minimum spanning tree (Figure 2.21).

A minimum spanning tree is shown in Figure 2.21. In general, haplotypes are separated from one another on short branches by one or two mutations. However, a single individual from Abtao has a haplotype (I) separated from all other samples by 12 mutations. The most common haplotype (F) is separated from the other two haplotypes which are more/less widespread (B, H) by single mutational changes. The remaining private (A, C, E, G, J) and narrowly distributed (D) haplotypes are all interconnected on the haplotype tree, although homoplastic mutations creating alternative connections in the MSN makes precise inference of relationships uncertain.

The major phylogeographic signal in the data is the prevalence of private haplotypes towards the northern, and/or coastal populations. No private haplotypes are present in the central Andes populations or the south coastal populations (Figure 2.14). In terms of the proportion of populations that are polymorphic, there is no clear cut association with latitude (Table 2.18). The isolation of the northern populations (Ruiles and Cordillera pelada) is also evident from a comparison between the mean pairwise Fst associated with anyone population and all others, and latitude (Table 2.15), with a decrease in mean pairwise Fst south of these population.



A	C	+8 bp	C	A	T	T	A	T	C	9	A	A	T	C	A	A	C	C	-20 bp	T
B	T	+8 bp	C	A	T	T	A	C	C	9	A	A	T	C	A	A	C	C	-20 bp	T
C	C	-8 bp	T	A	T	T	A	C	C	9	A	A	T	C	A	A	C	C	-20 bp	T
D	C	+8 bp	C	A	T	T	A	C	C	9	A	A	T	C	A	A	C	C	-20 bp	T
E	C	+8 bp	C	A	T	T	A	C	C	9	A	A	T	C	A	C	C	C	-20 bp	T
F	T	+8 bp	C	A	T	T	A	C	A	9	A	A	T	C	A	A	C	C	-20 bp	T
G	C	+8 bp	C	A	T	T	A	C	A	9	A	A	T	C	A	A	C	C	-20 bp	T
H	T	+8 bp	T	A	T	T	A	C	A	9	A	A	T	C	A	A	C	C	-20 bp	T
I	T	+8 bp	C	G	C	G	G	C	C	8	A	A	G	A	G	A	T	G	+20 bp	C
J	C	-8 bp	C	A	T	T	A	C	C	9	G	G	T	C	A	A	C	C	-20 bp	T

Figure 2.21. MSN and haplotype distribution map for *Weinmannia trichosperma.*

Table 2.19. Summary of phylogeographic patterns in five Patagonian plant species.

Species	**Distribution**	**No haplotypes**	**Phylogenetic divergence among haplotypes?**	**High diversity in part of range?**	**Unique northern haplotypes ?**	**Unique central haplotypes ?**	**Unique southern haplotypes ?**	**Unique far southern haplotypes ?**	**Comments**
Disacaria chacaye	North- Far South (Andes and coastal)	10	minimal	Yes	Yes (Andes and coastal)	Yes (Andes, but rare)	Yes (Andes but rare)	No	North and coastal populations encompass most diversity of haplotypes. Relatively even distribution of haplotypes throughout rest of the range
Donatia fascicularis	North- Far South (coastal and south Andes only)	3	No	Yes (ish)	Yes (in the coastal range)	No (but one haplotype restricted to central-southern regions)	No (but one haplotype restricted to central-southern regions)	No	Far south uniform, most northerly coastal population is unique, polymorphism in central/southern coastal populations.
Escallonia virgata	North-South (Andes and coastal)	4	No	No	Yes (in the Andes)	No	Yes	-	No intra-population variation, both northern Andes populations have unique haplotypes, two neighbouring populations in south share a unique haplotype

Table 2.19. Continued

Species	**Distribution**	**No haplotypes**	**Phylogenetic divergence among haplotypes?**	**High diversity in part of range?**	**Unique northern haplotypes ?**	**Unique central haplotypes ?**	**Unique southern haplotypes ?**	**Unique far southern haplotypes ?**	**Comments**
Tepualia stipularis	North-South (Andes and coastal)	6	Yes	Yes	Yes (Andes and coastal)	Rare variants in coastal range	No	-	Divergent haplotypes north, elsewhere dominated by one haplotype, coastal populations contain this and other low frequency variants
Weinmannia trichosperma	North-South (Andes and coastal)	10	Yes	Yes	Yes (Andes and coastal)	Yes (but rare coastal and central)	Yes (but rare)	-	Populations with private haplotypes are northern/coastal; One plant from Chiloe very divergent

2.4 Discussion

2.4.1 Is there evidence for geographically discrete genetic lineages within individual plant species?

If populations had experienced long periods of historical isolation within parts of their ranges, this should lead to the presence of highly divergent lineages within individual species, such that chloroplast haplotypes in parts of the species range would be separated by many mutations from those in other parts of the range. The clearest evidence for this comes from *Tepualia stipularis* which has a distinct lineages with non-overalapping geographical distributions. *Weinmannia trichosperma* also has a 'long branch' haplotype, but this haplotype is just associated with a single individual, in a population containing haplotypes from the main 'clade' for this species. Although clear-cut phylogenetic structure is uncommon in this data set (in the form of highly divergent lineages with discrete distributions), high levels of population differentiation are found in all species, with Gst values ranging from Gst = 0.347 in *W. trichosperma* to Gst = 1.00 in *Escallonia virgata*. In all of the data sets, excluding *W. trichosperma*, there is at least some component of geographical structure to this partitioning of genetic diversity. Thus some regions contain either (a) high frequencies of private haplotypes, and /or (b) higher regional-level haplotype diversity, than other regions.

2.4.2 Do the observed patterns of genetic diversity and genetic differentiation correlate with previous hypotheses of historical suture zones or glacial refugia?

Escallonia virgata

There is clear cut partitioning of haplotype diversity in *Escallonia virgata*, with no variation at all within populations (Table 2.13). The major phylogeographic signal is the presence of distinct haplotypes in the north. Thus the two most northerly populations in the Andes are fixed for unique haplotypes not found elsewhere (Table

2.16). This is a somewhat different signal to the other study species in that all have at least one private haplotype from coastal populations.

The most northern population contains the most divergent haplotype, although this just relates to a 2-step, rather than a 1-step branch. These data are consistent again with some form of northern refugium, in that these two haplotypes from the north of the range are not found further south. However, the population in the northern Coastal Cordillera at Nahuelbuta, shares a haplotype with the majority of the populations from further south, making the north vs ‘the rest’ split less clear cut than in other species such as *Tepualia stipularis*. The other interesting observation is the presence of distinct haplotypes from two neighbouring populations in the south near the Mt Fitzroy massif (Piedra del Fraile, Lago Desierto) (Figure 2.19). Private haplotypes in this area also occur in *Discaria chacaye* (Lago Roca), and also in previously published studies *Nothofagus pumilio* (Mathiassen and Premoli, 2010) and *Pilgerodendron uviferum* (Allnutt *et al.,* 2003).

These results can be used to make some inferences on the history of this species. Firstly, the absence of long-branches in the MS trees corresponding to mutational differences between haplotypes, suggest that there has been no ancient intra-specific divergence between these populations, and that they share a relatively recent common ancestor. In the absence of a robust calibration method it is not possible to quantify the timing of this with any reasonable level of precision, other than to make the observation that the ancestry is more recent compared to species such as *Tepualia stipularis.* The presence of unique haplotypes in the north is consistent with the presence of a northern refugium for this species. The shared common haplotype between the northern coastal population and further south would be consistent with southern populations being derived from northern refugia, but equally there is no clear evidence to rule out that populations with haplotype B originated from more southern refugia. That a southerly refugium may have been important for this species receives some support due to the presence of unique haplotypes in the southern populations near Mt Fitzroy.

Donatia fascicularis

The restriction of *Donatia* to coastal locations along the western flank of Chile, gives this species a somewhat different distribution to the other species examined here. However, consistent with results from other species, the northern most populations contain a haplotype not found further south. Thus the population at Nahuelbuta is fixed for private haplotype. As with *Escallonia*, the differences between haplotypes are small, suggesting relatively recent common ancestory. The only polymorphic populations detected in this species are in the middle of its range, with populations in the far south being fixed for a single haplotype.

Previous workers have suggested that Magallanic moorlands (the habitat for this species) have persisted in refugia in islands off the west coast of Chile. Other possibilities are that refugia existed in the far south/east of Tierra del Fuego, on land which is currently submerged. Logistical difficulties prevented exhaustive sampling along the western Chilean islands. Nevertheless, it is interesting that the only polymorphic populations are those that are closest to these putative west coast refugia. The far south populations on Tierra del Fuego could be explained by colonisation from these central-southern populations, but the reverse is not true, as our Fuegian sampling did not detected one of the haplotypes that is found in the central/southern region. Thus the data are consistent with the hypothesis of refugia in the western islands, but provide no support for the hypothesis of a refugium in Tierra del Fuego for this species.

The fixed unique haplotype on Nahuelbuta suggests some level of isolation for this northern population. The northern population of *Donatia* is restricted to a high altitude location, and thus the population can be thought of as occupying a habitat island surrounded by areas of unsuitable (e.g. too warm) terrain. This genetically divergent population in the north could be classified as currently being in a refugium, as suitable habitat conditions in this northern area are likely to be more common in colder climates. The current data suggest that the populations south of 40°S has a low probability of been derived from the northern population in Nahuelbuta, although the low levels of genetic divergence between populations make phylogeographic inference difficult.

Donatia shows a similar pattern to that of with the North American species *Acaena*, which did not show significant phylogeographic pattern due to its survival as a metapopulation during the LGM (King *et al.*, 2009).

Discaria chacaye

The data set for *Discaria* shows a high number of haplotypes (10), three of which are widespread. Most of the variation among haplotypes is due to length variants at 3 cpSSR loci, but two indel characters also occur. Interestingly, variation in these indel characters is restricted to northern populations. Three (out of four) individuals from the most northern population at Chillan have a deletion in the *trn S-G* region that is found only there and in Nahuelbuta. A second deletion (in the *trn S-G* region) is found only in Nahuelbuta. All populations south of these two lack these deletions. When the three cpSSR loci are included to define the haplotypes, all 4 individuals from Chillan have unique haplotypes but Nahuelbuta shares one haplotype with more southern populations. In general, in this species, the high level of haplotype diversity in relation to the sample density makes clear-cut phylogeographic inference difficult. However, the presence of distinct haplotypes (and ‘mini clades’) in the north is consistent with observations from the other species about the persistence of populations in northern refugia, and that at least some of the diversity in the north, remains in the north and does not contribute towards populations further south. There are some rare private haplotypes in the south, such as at Lago Roca and also in the central region at Puelo. These could be interpreted as evidence for more southerly (than 40°S) refugia, although a greater sampling density would be desirable to support this suggestion.

The similarity of haplotype occurrence and population differentiation with *W. trichosperma* could be related not to its cold tolerance, but to its seed dispersion system. *W. thichosperma* has wind pollination system which covers large areas and *D. chacaye* is known as having poorly dispersed seeds although it has been seen to possess floating fruits which could suggest a long range seed dispersal system. Even though current populations don't all grow next to water bodies, flotation in streams this could have been an easy seed dispersal mechanism during the LGM and the deglacial. There would have been less seed dispersal and therefore higher isolation within the northern distribution, due to the lower amount of water bodies and ice sheets distributed within that area.

Tepualia stipularis

The clearest phylogeographic signal in the current data set is from *Tepualia stipularis*. In the north of its distribution, we recovered divergent haplotypes between the sampled populations from the north western Andes and the north Coastal Cordillera. The rest of the species range southwards consisted entirely of a separate clade of haplotypes, consisting of one widespread haplotype, and two rare variants restricted to the Coastal Cordillera in the central region. These data suggest that populations in the north of the range have been isolated from those further south for some time, and the southern populations (some of which occupy previously glaciated areas) were not derived from the sampled northern populations.

The area around Chiloe was known to have escaped glaciations and it has been suggested that this region may have served as a refugia for some plant species during past-glaciations (Villagran, 2001). There is some tentative evidence in our data set to support this observation, in that the rare variants in the southern clade (strictly speaking, non-northern clade) are found exclusively in this area. Obviously further sampling is desirable to test the robustness of this observation, but these data are at least consistent with populations below 40°S having originated from a non-northern refugium, possibly from the area around Chiloe.

The current isolated and strongly differentiated nature of the northern populations is of interest. In part the differentiation between the north-coastal and north Andean populations of *Tepualia* is not surprising, given that they are separated by 140 km across the central valley. However, one interesting observation is that the coastal northern populations do not match the general trend of increasing altitude with decreasing latitude (Figure 2.2). This population occur at lower elevation than a simple extrapolation from the distribution of the other populations would predict. One possible contributing factor is its genetic isolation, from populations further south, is that this population is adapted to a somewhat different set of climatic conditions. Thus seed dispersal (and hence cpDNA dispersal) from this population to the south would be limited by reduced establishment probabilities (due to adaptive differences) as well as the inherent seed dispersal limitations for this species. Retrospective examination of the herbarium specimens from these two northern populations shows a tendency for the leaves to be large (more espatulated), wider and thinner than populations from further south suggesting adaptive differences (Figure 2.22). Taxonomic confusion with other known species is unlikely as this species is distinct in being the only dry-fruited member of the Mrytaceae in Chile. However, further investigation into the nature of the differences between these northern populations and those further south would be of interest.

Weinmannia trichosperma

Similar to *Discaria,* a high level of haplotype diversity is found in *Weinmannia* and there is no clear cut phylogeographic signal. In general, the geographically restricted haplotypes are associated with coastal and northern populations, with the central and south Andean populations containing haplotypes that occur elsewhere. Thus the single individual sampled from the most northern population has a unique haplotype (which like the situation in *T. stipularis* is also from a low altitude population for its given latitude; Figure 2.2) and all of the northern populations contain at least one private haplotype. The other particularly interesting population is on Abtao on Chiloe which contains two private haplotypes (one of which is restricted to a single individual and shows remarkable divergence compared to other haplotypes). A tentative interpretation of these data is that the evidence supports refugia in the north of the Andes and also along the Coastal Cordillera as far south as Chiloe, with populations south and east of these areas being derived from these. The fact that this species has a wide seed dispersion mechanism could be the reason why there is no clear phylogeographic pattern, and this species has the lowest Gst value of all sampled species (Table 2.13).

Figure 2.22. Morphological differences between northern and southern populations of *T. stipularis*.

2.5 General Conclusions

The key points that emerge from this investigation of 5 widespread but endemic Patagonian plant species are:

1) There is a wide range in levels of intra-specific diversities and divergences in the study species.
2) Despite the heterogeneity of the patterns, the high density of private haplotypes in the north is consistent among all of the species, without exception, and suggests that refugia for all of these species occur in the northern region (north of 40°S; Figure 2.16-Figure 2.21).
3) Coastal populations (from the Coastal Cordillera to Chiloe for *Tepualia* and *Weinmannia,* in Nahuelbuta for *Discaria,* and more generally along the central western coast for *Donatia*) contain high levels of diversity/private haplotypes consistent with long-term refugial persistence in these regions.
4) These species show limited evidence for important southern or far southern refugia. In general, the haplotype distributions can be explained by colonization's from the north and coastal regions, rather than the south. In the total data set, taken together, it is clear there are proportionately far fewer private haplotypes in southern populations compared to those in the north (Figure 2.14).
5) Previous studies have identified a major suture zone at 43°S and at 40°S. Our data are neither contradictory nor supportive of this hypothesis, in part because the density of sampling does not allow a precise estimate of the geographical location of the differences between 'northern' and non-northern diversity in this study. Certainly, at somewhere around the 40°S latitude, there are more private haplotypes to the north than to the south, and within some species a suture zone. Further sampling is required to pinpoint the exact boundaries of this difference and hence assess the relative importances of climatic, topographical or geological features.

6) Our sampling of species on the west and east of the main Andean cordillera is not comprehensive enough for us to provide a robust test of evidence for the main Andean cordillera as a barrier to gene flow. However, in the species where our sampling encompasses western and eastern populations (*Tepualia* and *Weinmannia*), the fact that haplotypes are shared between the western side of the Andean Cordillera and the eastern Andes/coastal regions does not provide any evidence for a major east-west divide in these species.
7) Northern populations form coast and Andean regions show higher differentiation between populations than southern populations. This could be related to higher altitudes of the Andes cordillera north of 37°S.

Chapter 3: The complex case of the phylogeographic history of *Gentianella magellanica*, an endemic annual Patagonian plant species

3.1 Introduction

Patagonia (Argentina and Chile) is formed by the subduction of 4 different geological plates, making this area seismically and volcanically active (Corbella and Lara, 2008; Ramos and Ghiglione, 2008). This has led to a varied topography with two high north-south mountain ranges: the coastal and Andes cordillera. These affect Patagonian plant species by influencing the climate (producing a strong rain shadow) and also isolating plant species from one side of mountain ranges to another (Armesto *et al.*, 1995a,b).

During the Pleistocene, southern South America experienced many climatic changes due to glaciations. Patagonia was the only area in the southern hemisphere except for Antarctica which had a continuous thick layer of ice (Rabassa, 2008; Hewitt, 2000). Several Pleistocene ice extensions are known, but the most studied is the last glacial maximum (LGM) or the Llanquihue glaciation. The ice covered almost all the territory south of ~42°S, the Andes cordillera south of ~36°S and the estern side of the central depression (Hulton and Sudgen, 1994). In this area under ice, plant populations will have become extinct. However several studies have suggested that plant species survived in many ice free areas, because of their presence within glacial periods and their fast colonization after deglaciation (eg. Manzini *et al.*, 2008; Villagran and Armesto, 2005; Markgraf *et al.*, 1995; Veit and Garleff, 1995; Villagran *et al.*, 1995).

Phylogeographic, palynological and geo-climatic studies can give an insight in the responses of plant species to past glaciations. Pollen studies show the areas and species types growing during the LGM. Reconstructions of the distribution of glaciers show the areas covered by ice, and phylogeographic studies giving information on the distribution of genetic diversity can be used to infer the presence of refugial and post-glacial colonist populations.

In Patagonia almost all plant phylogeographic studies have focused on long lived taxa, mainly woody species such as *Nothofagus* (Acosta and Premoli, 2010; Mathiasen and Premoli, 2010; Azpilicueta *et al*., 2009; Marchelli and Gallo, 2006) and conifers (Quiroga and Premoli, 2010; Marchelli *et al*., 2009; Pastorino and Gallo, 2002).Very little is known about perennial herbs (Tremetsberger *et al*., 2009, Muellner *et al*., 2005), and no studies to date have been published on annual taxa. Short lived plant species may show different patterns of phylogeographic diversity compared to species with life history traits like long lived trees. Annual plant species are frequently selfers (Barrett, 1998; Barret *et al*., 1997), and this and/or the short generation time can lead to high levels of population differentiation compared to long lived woody plant species (Hamrick and Godt, 1996). In addition, the failure to reproduce in any one year as a result of adverse conditions may have a strong influence on the demography of a population of annuals, and hence short-lived species may be more readily subject to genetic divergence due to genetic drift.

Studies of herbs in other parts of the world often show clear cut phylogeographic signal and have been useful in inferring the location of refugia and post-glacial colonization routes (Abbott and Comes, 2003). A phylogeographic study of a Gentianaceae within Tibet, showed high differentiation among populations due to the isolation by mountain ranges and suggested the presence of refugium in the Hengduan Mountains (Chen *et al*., 2008). However, in general there is a shortage of phylogeographic studies on annual plants.

There are many herbs in Patagonia with a wide distribution range, mainly growing on the eastern slopes of the Andes and southern Patagonia. It is possible that some of these species that are cold tolerant had more extensive distributions during the LGM than their present distributions. Nevertheless, even these cold tolerant species will be eliminated from regions under ice, and there is an expectation that their current distributions may contain a signature of diversity associated with long term presence in an area, and divergence associated with long term isolation between areas (Hewitt, 2000).

The aim of this study is to document the phylogeographic pattern shown by a cold tolerant annual herb with a wide distribution within Patagonia. We investigate whether the high degree of population differentiation expected of an annual species is found, and whether this provides clues to the pattern of fragmentation suffered by the species during the LGM. We look for cases of high diversity and divergent lineages among populations in areas where long term persistence has been likely, and conversely look for areas with genetically similar populations consistent with recent common ancestry and recent colonisation.

3.2 Materials and Methods

3.2.1 Study Species

The focus of this study is *Gentianella magellanica* (Gaudich.) Fabris ex D.M. Moore. The genus *Gentianella* Moench was segregated from the Gentians by Moench in 1974. At present, the genus *Gentianella* has ca. 200 species with a world wide distribution, although its majority of species grow in the temperate regions of South America and New Zealand (Filippa and Barboza, 2006; Ho and Liu, 1993). In South America there are thirty two *Gentianella* species of which 27 are endemic to Argentina, two to Chile and three species share its distribution between both countries (Table 3.1). They grow mainly in the Andean regions, from sea level up to 4,500 m (Filippa and Barboza, 2006).

Table 3.1. *Gentianella* species growing in Argentina and Chile.

Specie	Argentina	Chile
Gentianella benedictae	x	
Gentianella bromifolia	x	
Gentianella cabrerae	x	
Gentianella claytonioides	x	
Gentianella coquimbensis		x
Gentianella cosmantha	x	
Gentianella erythrochrysea	x	
Gentianella fabrisii	x	
Gentianella Fiebrigii	x	
Gentianella florida	x	
Gentianella gageoides	x	
Gentianella helianthemoides	x	
Gentianella hieronymi	x	
Gentianella imbersis	x	
Gentianella kurtzii	x	
Gentianella magellanica	x	x
Gentianella meyeniana	x	
Gentianella multicaulis	x	x
Gentianella multiflora	x	
Gentianella myrantha	x	
Gentianella oranesis	x	
Gentianella ottonis	x	x
Gentianella parviflora	x	
Gentianella pseudocrassula	x	
Gentianella pulla	x	
Gentianella punensis	x	
Gentianella riojae	x	
Gentianella silenoides var. Silenoides	x	
Gentianella silenoides var. Striticalyx	x	
Gentianella tarapacana		x
Gentianella thiosphera	x	
Gentianella tubulosa	x	

Gentianella magellanica is a very morphologically variable species (Fabris, 1953). It is an annual herb, with an erect habitat and is 2 to 30 cm tall. Its shape can be simple or multistemed, and if multistemmed the central steam is typically the thickest. The basal leaves form a lax rosette and are shriveled during flowering. It has sessile and cauline leaves which can be of an elliptic or ovate shape with 3 venations. The flowers are terminal and hermaphrodite and vary from white to purple (Figure 3.1). They are insect pollinated (mainly by bees) and also self-compatible (Moore, 1983). The species flowers from January to April; this long flowering season is due to the wide distribution of the species which spans many different climates. The seeds are dry and approximately 7 mm long, and are considered to be poorly dispersed (Arroyo *et al*., 2004).

Figure 3.1. *Gentianella magellanica* (Gaudich.) Fabris ex D.M. Moore.

Distribution and habitat

G. magellanica is the only *Gentianella* species that grows in southern Patagonia, and is widely distributed throughout Chile, Argentina and the Falkland Island. Its distribution range covers in Argentina the provinces of Chubut, Mendoza, Neuquen, Rio Negro, Santa Cruz and Tierra del Fuego (34°S- 50°S). In Chile it occurs in the

Regions: VII, VIII, IX, X, XI, XII (36°S- 50°S) (Zuloaga *et al.*, 2009). Within this distribution it overlaps with *Gentianella ottonis* and *Gentianella multicaulis*. However *G. magellanica* is very easy to distinguish from its neighbouring species (Fabris, 1953) because *G. magellanica* has flower lobes which are equal or a little longer than the corolla tube. The other species have the flower lobes three times longer than the corolla tube (Filippa and Barboza, 2006). This large distribution and morphological variability has led to taxonomic confusion and the presence of a large number of synonyms. To double check the identity of samples used in this project, voucher specimens from all sampled populations were sent to the expert of the Patagonian *Gentianella*, Eva Filippa. All were confirmed as being *G. magellanica* by this authority (Filippa pers.com. Oct-2009).

In the north of its range, *G. magellanica* grows at high altitude in the Coastal Cordillera (~1,250 m) and even higher in the Andes Cordillera (~2,000 m). In the southern edge of its distribution it occurs at sea level and normally grows in damp areas, grassland, open shrub community and stream margins (Figure 3.2). It is a cold tolerant species, growing in areas covered by snow during winter and sometimes experiencing snow falls during its growing season. Due to its wide distribution (Figure 3.3), it can be associated with many different species, the most common of which include: *Gaultheria mucronata*, *Berberis microphylla*, *Nothofagus pumilio, Nothofagus anctartica*, *Euphrasia spp*, *Chiliotrichum diffusum*, *Gunnera magellanica*, *Drimys winteri* and *Embothrium coccineum.*

In some areas of its distribution mainly on top of the coastal cordillera this species is associated with the moorland flora and within this distribution it occurs in isolated high elevation populations, having a more continuous distribution south of 46°S. The species also shows a disjunction between the Coastal and Andean cordilleras, separated by the central depression zone occupied by more thermophilic species.

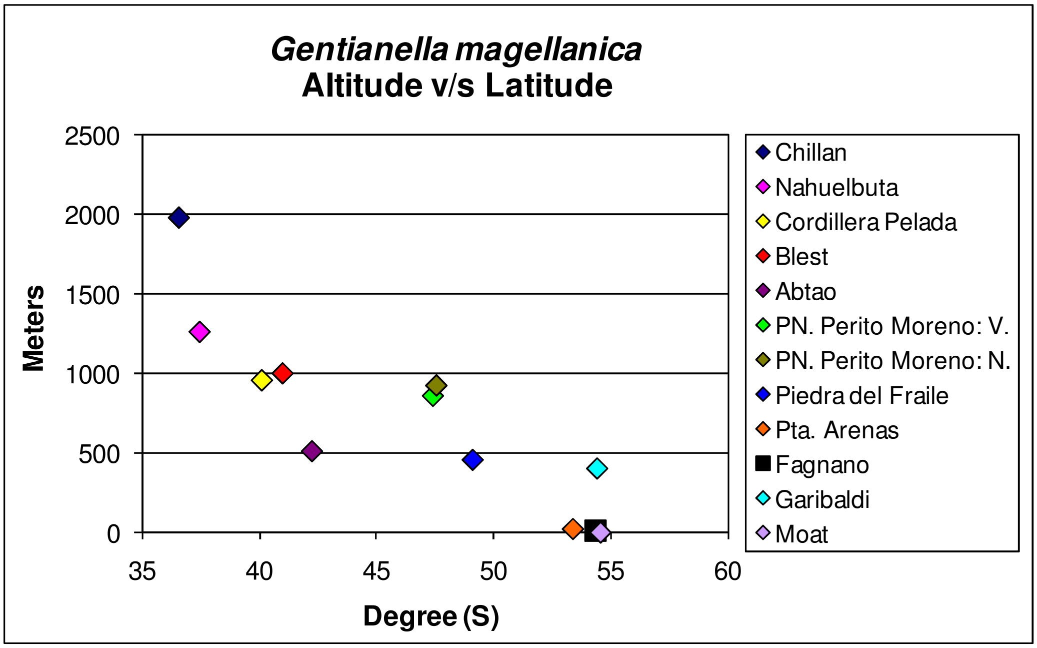


Figure 3.2. Distribution of *Gentianella magellanica* showing the relationship between population altitude and latitude.

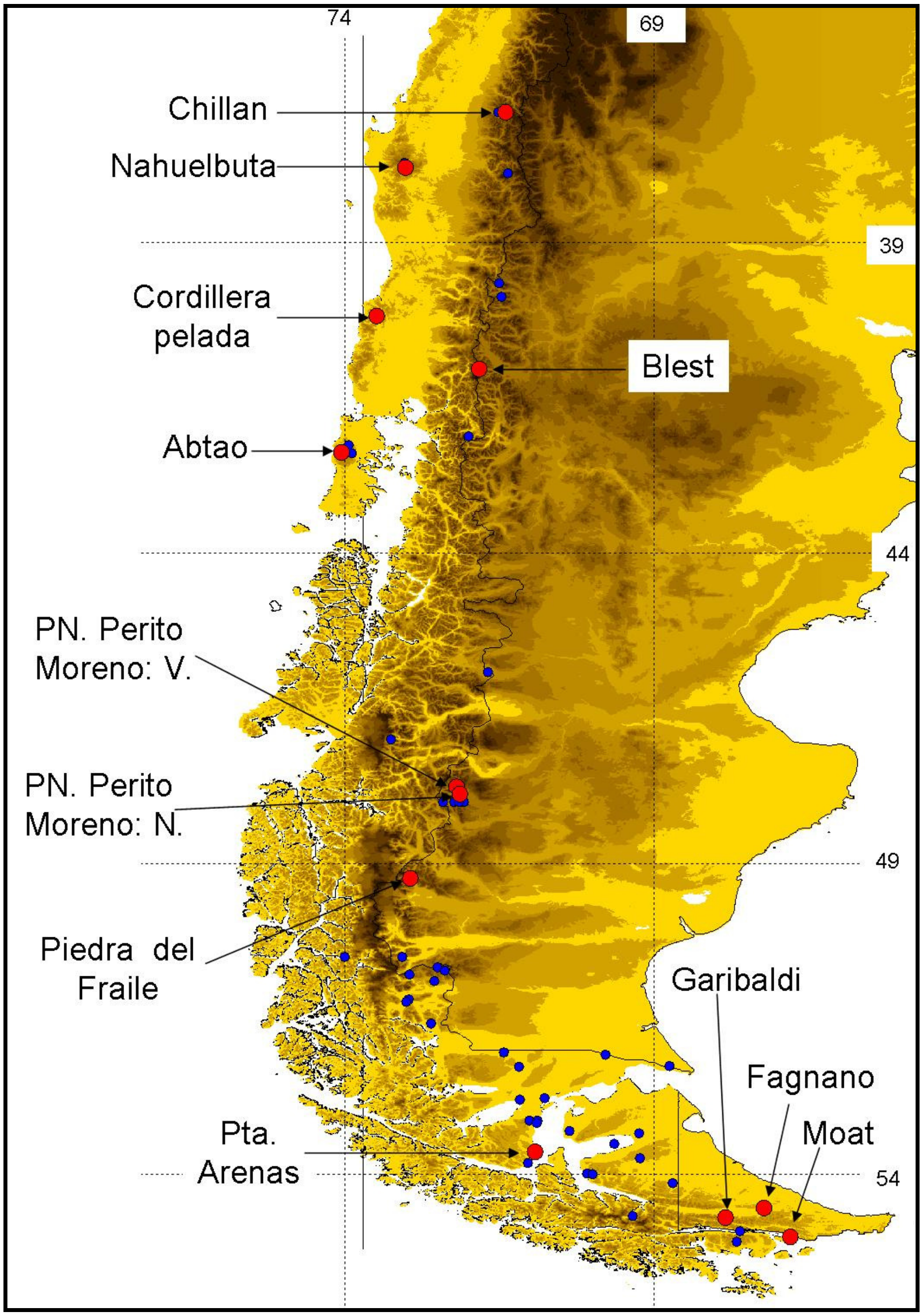


Figure 3.3. *Gentianella magellanica* distribution map.

The dots represent distributional records derived from herbarium specimens and literature review. Red dots represent sampled populations for the current study

Historical background

No records have been found for the species during the LGM, although there are pollen records from Altos del Pabilo, Coastal Cordillera (40°S), which show traces of *Gentianella* around 6,000 yr. This suggests a colonization of the higher peak of the coastal cordillera after the glacial retreat, and it has been suggested that the source came from refugias at lower slopes in the central depression, as proximal refugia fits best this observation of rapid post-glacial recolonisation (Villagran and Armesto, 2005).

There are several studies that describe the fluctuation of the moorland species during the LGM, *Gentianella* belongs to this group of species in some areas of its distribution. Based on these studies (see chapter 1) Villagran (2001) has created a forest distribution model, which is relevant to the survival of these species. The moorland is hypothesized to have survived during the LGM on the lower slopes of the coastal cordillera or in the central depression, in disjunct populations around 40-42°S. During the Lateglacial (13,000-10,000 yr), just after the retreat of the ice sheets, it then showed a rapid advance southwards. At the same time, these moorland species showed an elevational migration in the north, colonizing the previously glaciated and denuded higher peaks of the coastal cordillera producing the currently disjunct and isolated populations on different mountains.

3.2.2 Plant Materials

Sampling methodology

Distributional data for *Gentianella magellanica* (Figure 3.3) was obtained from the following herbaria: Royal Botanic Garden Edinburgh (E), Herbario Universidad de Concepcion (CONC), Instituto de Botanica Darwinion (SI), Universidad Nacional del Comahue (BCRU), Universidad de Magallanes Punta Arenas (HIP) and Museo del Fin del mundo Ushuaia and the web page of the Argentinean National parks (APN) http://www.sib.gov.ar/index.php. Sampling was undertaken between 2006 and 2007 and aimed to encompass the geographical range of *G. magellanica*.

Twelve populations of *Gentianella magellanica* were sampled throughout its entire range. This includes Chilean and Argentinean Patagonia, from 35°S to Tierra del Fuego (Figure 3.3; Table 3.2). Populations were classified as being part of the Coastal range or the Andean mountains, and where possible were recorded as being on the west or eastern flanks of these mountain chains (Figure 3.4; Table 3.2). In places the latter aspects of this classification scheme is somewhat subjective, particularly in the Southern region where the Coastal range and Andes almost converge, and also where the Andes decrease in height in southern Patagonia. Here it can be difficult to classify populations as western or eastern (Figure 3.4). Populations can be to the east of the higher mountains of the Andean cordillera, but nevertheless in a watershed that drains to the west.

For each population at least two individuals were collected as voucher herbarium specimens. For genetic analyses, fresh plant tissue was collected from the wild. Up to 5 individuals per population were sampled, with a minimum inter-plant distance of at least 5 m, where possible, to avoid sampling related individuals. Plant samples, which consisted of an entire Gentianella plant, were placed in either (i) silica-gel in zip-sealable plastic sample bags, which dries the sample very quick but requires a large amount of silica or (ii) a “make-your-own” tea bag surrounded by silica gel. As the leaves of *Gentianella magellanica* dry quickly, both methods worked perfectly preventing the hydration and subsequent degradation of the DNA.

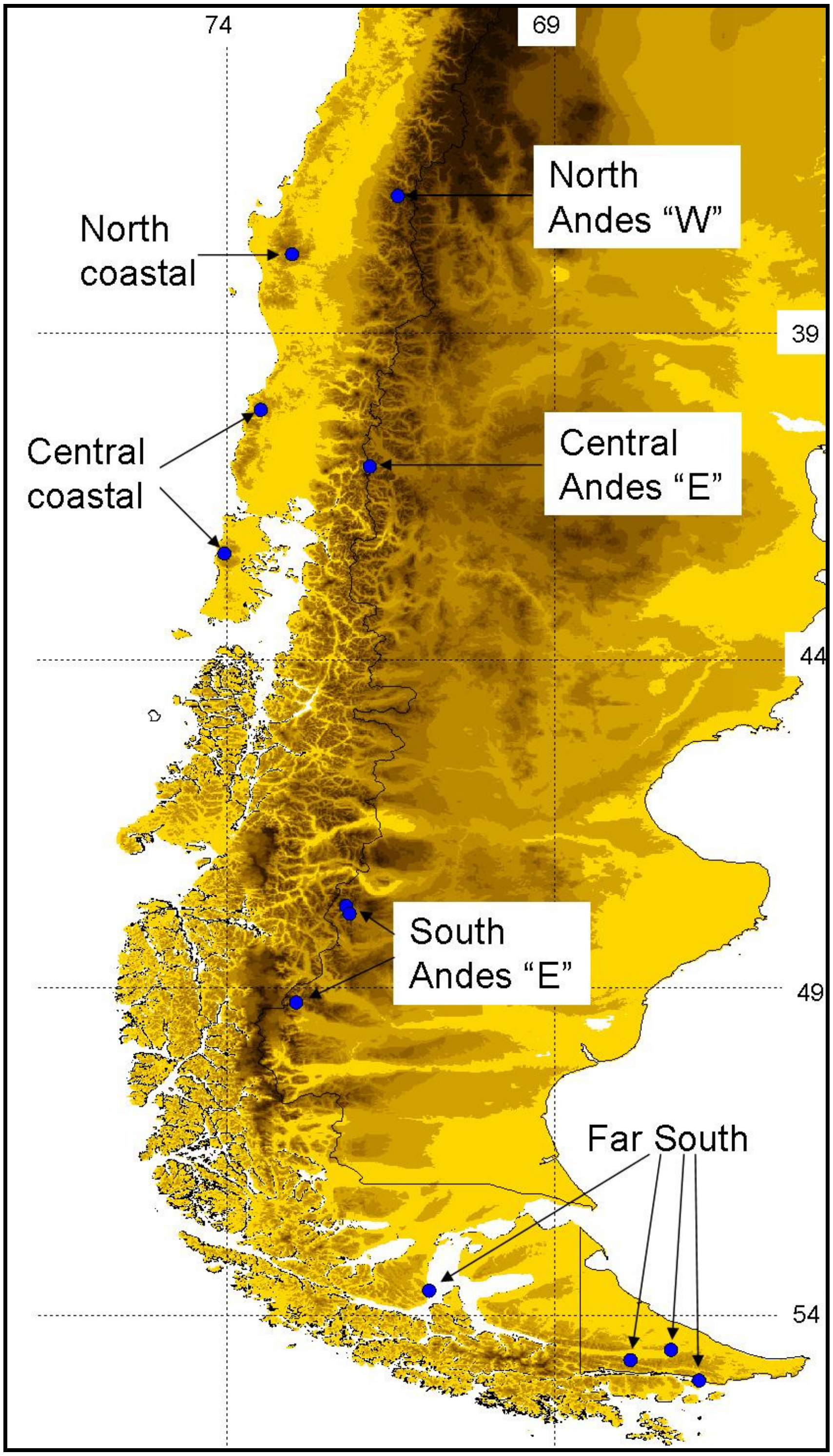


Figure 3.4. Allocation of sampled populations into the informal geographical categories referred to in the text (see Table 3.2).

Table 3.2. Distribution of populations of *Gentianella magellanica* sampled for phylogeography analyses.

Area	**Population**	**Location**	**Country**	**Latitude**	**Longitude**	**Altitude**	**Region**
North Andes "W"	Chillan	Termas de Chillan, Valle de las nubes	CL	S36° 54' 13"	W71° 23' 44"	1976 m	G
North coastal	Nahuelbuta	Malleco, PN. Nahuelbuta	CL	S37° 47' 48"	W73° 00' 53"	1262 m	NG
Central coastal	Cordillera pelada	La Union, Cordillera Pelada ,MN. Alerce costero	CL	S40° 10' 11"	W73° 29' 16"	955 m	NG
Central Andes "E"	Blest	Bariloche, PN. Nahuelhuapi, Puerto Blest	CL	S41° 02' 9"	W71° 49' 21"	1006 m	G
Central coastal	Abtao	Chiloe, PN. Chiloe, Abtao	CL	S42° 22' 59"	W74° 01' 49"	517 m	NG
South Andes "E"	PN. Perito Moreno: V.	PN. Perito Moreno, Lago Volcanes	AR	S47° 45' 14"	W72° 11' 8"	863 m	G
South Andes "E"	PN. Perito Moreno: N.	PN. Perito Moreno, Puesto del Nueve	AR	S47° 53' 47"	W72° 05' 42"	927 m	G
South Andes "E"	Piedra del Fraile	Chalten, PN. Nacional Los Glaciares, Piedra del Fraile	AR	S49° 13' 48"	W72° 56' 28"	453 m	G
Far South	Pta. Arenas	Punta Arenas, Fuerte Bulnes	CL	S53° 37' 43"	W70° 55' 3"	18 m	NG
Far South	Fagnano	Tierra del Fuego, Lago Fagnano	AR	S54° 32' 59"	W67° 14' 42"	13.8 m	G
Far South	Garibaldi	Tierra del Fuego, Paso Garibaldi	AR	S54° 41' 29"	W67° 50' 54"	399 m	G
Far South	Moat	Tierra del Fuego, road form Moat to Harberton	AR	S54° 57' 15"	W66° 48' 27"	1.81 m	G

PN., National park; MN., Natural monument; NG = region presumed not to have been glaciated; G = region considered to have been glaciated based on geo-climatic studies

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3.2.3 Molecular Methods

DNA extraction

Approximately 50 mg of dried leaf material was ground using the TissueLyser system (QIAGEN), for 1 min at 20 Hz, extending the grinding time if necessary. 30 of the samples were extracted using the DNeasy 96 plant kit, following the manufacturer's recommendations and 30 were extracted using a CTAB buffer, as outlined below. The CTAB method was modified from Doyle and Doyle (1987) with the inclusion of 0.1% insoluble polyvinylpoly-pyrrolidine (PVPP) to the 2x CTAB buffer. Two 24:1 chloroform/isoamyl alcohol washes were performed and the DNA precipitated overnight in freezer-cold isopropanol. Depending on the size of the resultant pellet, the DNA was dissolved in 50-70 µl of TE (10 mM TRIS-HCl, 1 mM EDTA, pH7.4). The quality and quantity of extracted DNA was checked under UV light on 1.0% agarose gels containing 400 mg of Syber Safe (Invitrogen).

PCR amplification

The reaction mixture (25 *ul*) for PCR amplification of chloroplast DNA genes contained

- 16.05 *ul* Sigma water
- 2.5 *ul* 10x NH_4 Reaction Buffer (160 mM $(NH_4)_2SO_4$, 670mM Tris-HCI (pH 8.8 at 25°C), 0.1% stabilizer (Bioline)
- 1.25 *ul* 50 mM $MgCl_2$ Solution (Bioline)
- 2.5 *ul* 10 mM dNTP Mix; dATP, dGTP, dCTP and dTTP as lithium salts (pH 7.5)
- 0.75 *ul* 10 mM Forward primers
- 0.75 *ul* 10 mM Reverse primers
- 0.2 *ul* BIOTAQ DNA polymerase (Bioline)
- 1.0 *ul* template DNA (for some individuals the amount of DNA used, varied between 0.5 to 2 *ul*)

Amplification was carried out using the following PCR profile: 94°C for 4 min. followed by 35 cycles of 94°C for 45 sec. (Table 3.4), 52°C-5X for 45 sec.

(according to the primer annealing temperature), 72°C for 1,2-X min. (according to the length of fragment to be amplified) (Table 3.4) and finally 72°C for 10 min. All PCRs were performed using a Tetrad 2 peltier thermal cycler (Biorad). PCR products quality and quantity was assessed by were electrophoresis on 1.0% agarose gels containing 400 mg of Syber Safe (Invitrogen) and checked under UV light, comparing the products with 1kb+ standard markers.

Sequencing PCR

PCR product was purified either by QIAquick PCR Purification Kit (Qiagen) or ExoSAP-IT (GE Healthcare). QIAquick PCR purification was carried out following the manufacturer's recommendations. The protocol for ExoSAP-IT was as follows: mix 5 *ul* PCR product and 2 *ul* ExoSAP-IT and incubate at 37°C for 15 minutes followed by a deactivation of enzyme at 80°C for 15 min. Sequencing was performed using either DTCS Quickstart sequencing mix on a CEQ 8000 automated sequencer (Beckman and Coulter) or BigDye V3.1 sequencing mix on an ABI 3730 system (Perkin Elmer). The resulting sequences were manually edited and analysed using Sequencher® 4.7 (Gene Codes Corporation).

Identification of chloroplast DNA variation

To assess genetic variation in the chloroplast genome, a total of 11 chloroplast regions were initially tested for PCR amplification on 4 samples. These samples were selected from the pool of available material from the first field trip to broadly represent the geographical range of the species, and hence to maximise the chances of detecting variation (Table 3.3). Details of the amplified chloroplast regions, primers and annealing temperature for each primer set are given in Table 3.4.

Table 3.3. Samples used in initial assessment of chloroplast sequence variation and their associated sample identifiers. For the distribution of these samples Figure 3.2.

Region	Population name	Sample number EDNA	Collection number CM
North Andes "W"	Chillan	EDNA06-0942	CM6-797
Central coastal	Cordillera pelada	EDNA06-0896	CM6-80
Central Andes "E"	Blest	EDNA06-0881	CM6-1366
Far South	Pta. Arenas	EDNA06-0838	CM6-531

From the initial screen of eleven chloroplast regions of *G. magellanica* all regions were polymorphic excluding regions *trnK-trnQ* and *rpoC1-trnC*. At least five regions showed more than four polymorphisms (Table 3.5).

To finalize the choice of cpDNA regions for the full study, three of the most variable chloroplast regions were screened on one individual per population for the whole sample set. The regions were: *trnD-trnT*, *trnH-psbA* and *trnS-trnG* (Table 3.5). From this screen, the best two regions were selected. These had to show informative variation (not redundant in terms of resolving the same haplotypes as other loci) and mutational changes of nucleotide substitutions were favored over indels or simple sequence repeats (SSR). Another criterion used in the final selection process was the ease of amplification and sequencing.

Table 3.4. Primers, reaction conditions and usefulness of eleven chloroplast regions of *Gentianella magellanica*

Chloroplast region	**Primer sequence 5'-3' F=Forward R=Reverse**	**Annealing temperature °C**	**Extension time (sec)**	**Amplification lengh**	**PCR success**	**Sequence polymorphism**
atpB-rbcL	F: ACATCKARTACKGGACCAATAA R: AACACCAGCTTTRAATCCAA	49	60	850	Yes	Yes
trnC-trnD	F: CCAGTTCAAATCTGGGTGTC R: GGGATTGTAGTTCAATTGGT	58	180	3000	Yes	Yes
trnD-trnT	F: ACCAATTGAACTACAATCCC R: CTACCACTGAGTTAAAAGGG	54	120	1450	Yes	Yes
trnH-psbA	F: ACTGCCTTGATCCACTTGGC R: CGAAGCTCCATCTAC AAATGG	53	80	500	Yes	Yes
trnK-trnQ	F: TAAAAGCCGAGTACTCTACCGTTG R: CTATTCGGAGGTTCGAATCCTTCC	47.5	180	300	Yes	No
trnL-trnF	F: CGAAATCGGTAGACGCTACG R: ATTTGAACTGGTGACACGAG	55	180	2000	Yes	Yes
trnS-trnFM	F: GAGAGAGAGGGATTCGAACC R: CATAACCTTGAGGTCACGGG	62	120	1300	Yes	Yes
trnS-trnG	F: GCCGCTTTAGTCCACTCAGC R: GAACGAATCACACTTTTACCAC	52	60	1450	Yes	Yes
rpl20-rps12	F: TTTGTTCTACGTCTCCGAGC R: GTCGAGGAACATGTACTAGG	53	60	850	Yes	Yes
rpoC1-trnC	F: GCACAAATTCCRCTTTTTATRGG R: CGACACCCRGATTTGAACTGG	47.5	300	-	No	-
rps16	F: AAACGATGTGGTARAAAGCAAC R: AACATCWATTGCAASGATTCGATA	48	60	850	Yes	Yes

Table 3.5. Sequencing success and levels of polymorphism of *Gentianella magellanica*, based on a single individual from each sampled population.

Primer region	**Polymorphisms**
atpB-rbcL	NU (5 sub, 4 ind)
trnC-trnD	NU (10 sub, 1 ind)
trnD-trnT	6 sub, 6 ind*
trnH-psbA	5 sub, 3 ind, 2 SSR*
trnK-trnQ	F
trnL-trnF	NU (1 sub, 1SSR)
trnS-trnFM	NU (2 sub, 2 ind, 1 SSR)
trnS-trnG	NU (1 sub, 4 ind, 1 SSR)
rpl20-rps12	NU (3 sub)
rpoC1-trnC	F
rps16	NU (5 sub)

F = failed initial screen; NU = not used (showed variation in initial screen, but few informative markers and no new haplotypes were resolved); sub = substitution; ind = indel; * represent loci chosen for the full population screen.

Following the selection of two regions, these regions were then analysed in up to five individuals per population for the final analysis. The dataset consisted of 60 individuals of twelve populations (Table 3.2). This involved bi-directional sequencing of the *trnD-trnT* and *trnH-psbA* regions, using the protocols described above.

3.2.4 Molecular data analysis

To define chloroplast haplotypes and to format the molecular data for population genetic software packages, the sequence variations (substitutions, indels and variation in chloroplast microsatellite length) from each analyzed region was scored and analyzed using Chloroplast Excel macros (Graham French, unpublished). Substitutions and indels were quantified by coding the most common base as 0 and the rarer character state as 1. Microsatellites (SSR) variants were coded according to the length of the repeats.

The number of haplotypes per population was recorded, as was the distribution of private haplotypes (haplotypes found only in a given population or geographical regions). Quantification of genetic diversity and differentiation was undertaken using Nei's gene diversity estimators including total gene diversity (Ht), gene diversity present within populations (Hs) and gene diversity between populations (Gst) using Haplodiv (Pons and Petit, 1995). Pairwise estimated of population differentiation (Fst) were undertaken using Arlequin ver. 2.000 (Schneider *et al.*, 2000).

To assess whether there is evidence for phylogeographical structure in the data set, Nst was estimated using Haplonst (Pons and Petit, 1996). This metric takes into account mutational differences between haplotypes as well as frequency differences among haplotypes (as opposed to Fst and Gst which treat distinct haplotypes as being equidistant from one another). To test whether Nst was significantly different from Gst, a permutation test was implemented using Permut (Pons and Petit, 1996). A minimum spanning tree was generated to visualise relationships and levels of mutational change between haplotypes using Arlequin ver. 2.000 (Schneider *et al.*, 2000).

In *Gentianella magellanica* the polymorphisms contained a mixture of indels, substitutions and SSR's, so the MSN tree was created using (a) all characters, (b) using only substitutions to create the tree backbone, with indels and SSR's then used to differentiate among haplotypes defined by a given set of substitutions. This approach was adopted to explore if characters suspected of high levels of homoplasy (particularly SSR's) influence tree topologies.

3.3 Results

3.3.1 General patterns of sequence and haplotype diversity

In total 11 haplotypes were detected, based on a total of 22 mutations including substitutions, indels and SSR's (Table 3.6).

Table 3.6. Chloroplast haplotypes detected in *Gentianella magellanica* for *trnD-trnT* and *trnH-psbA* regions.

Haplotype name	***trnD-trnT***												***trnH-psbA***									
	Bp 313	Bp 330	Bp 567	Bp 600	Bp 691	Bp 725	Bp 748	Bp 765	Bp 776	Bp 796	Bp 837	Bp 982	Bp 109	Bp 225	Bp 241	Bp 250	Bp 353	Bp 375	Bp 380	Bp 426	Bp 433	Bp 450
A	C	+9	G	A	+18	T	+13	C	-12	G	-18	+13	G	A	C	-12	T	8	8	T	+13	-8
B	A	-9	T	A	-18	T	+13	A	-12	T	-18	+13	G	A	A	-12	T	9	8	A	-13	-8
C	A	-9	T	A	-18	T	+13	A	-12	T	-18	+13	G	A	A	-12	T	8	8	A	-13	-8
D	A	+9	T	C	-18	T	-13	C	-12	G	-18	-13	G	C	A	-12	T	7	8	A	+13	+8
E	A	+9	T	A	-18	G	+13	A	-12	T	+18	-13	G	A	A	+12	G	8	7	A	-13	-8
F	A	+9	T	A	-18	G	+13	A	-12	T	+18	-13	G	A	A	+12	G	8	8	A	-13	-8
G	A	+9	T	C	-18	T	-13	C	-12	G	-18	+13	T	C	A	-12	T	8	8	A	-13	+8
H	A	+9	T	A	-18	G	+13	A	-12	T	-18	-13	G	A	A	-12	G	8	10	A	-13	-8
I	A	+9	T	A	-18	G	+13	A	-12	T	-18	-13	G	A	A	-12	G	8	10	A	+13	-8
J	A	+9	T	A	-18	G	+13	A	+12	T	-18	-13	G	A	A	-12	G	8	9	A	+13	-8
K	A	+9	T	A	-18	G	+13	A	-12	T	-18	-13	G	A	A	-12	G	8	9	A	-13	-8

+= indicate insertion; -= indicate deletion; numbers indicate numbers of base pairs

3.3.2 Diversity and distribution of genetic variation on G. magellanica

The number of haplotypes recovered from individual populations range from 1-2, with 5/12 (41.7%) of populations showing haplotype polymorphism, and levels of gene diversity range from 0-0.7 (mean Hs = 0.222) (Table 3.7; Table 3.8). Of the 11 haplotypes, 7 are private to individual populations (haplotype A is private to Chillan, haplotypes B and C are private to Nahuelbuta, haplotye D is private to Cordillera pelada, haplotypes E and F are private to Blest and haplotype G is private to Abtao) (Table 3.7, Figure 3.5). Populations with the highest frequency of private haplotypes are the northern (Northern Andes "W" and North coastal) and central (Central coastal and Central Andes "E") populations. Chillan had 4/4 individuals with private haplotypes, and Nahuelbuta, Cordillera pelada, Blest and Abtao all have 5/5 individuals with private haplotypes (Figure 3.5; Table 3.7). All populations from the south and far south lack private haplotypes. They share all of their haplotypes with at least one other population (Table 3.7). Haplotype K was widespread in the far south from 53°S to 54°S. Haplotypes H, I and J were shared in five populations between southern and the far southern areas (47°S-54°S). When populations are grouped into clusters of populations from the same latitude, the populations from Perito Moreno national park (PN. Perito Moreno) collectively have one private haplotype H (Figure 3.5; Table 3.7), and when all populations from far south are considered as a group, they share private haplotype K for the region, between 53°S and 54°S (Table 3.7).

There is slight evidence for a phylogenetic suture zone north and south of 43°S, as there is a difference in levels of diversity with 4/11 haplotypes found south of 43°S (none of them found further north), whereas 7/11 haplotypes are found north of this line (none found further south). In terms of the proportion of populations that are polymorphic, there is no clear cut association with latitude, although there is a slightly higher amount of polymorphism in populations of central Andes "E", south Andes "E" and Far south with 4/8 of the populations polymorphic whereas only 1/4 populations corresponding to the north Andes "W", north coastal and central coastal are polymorphic (Figure 3.6; Table 3.7).

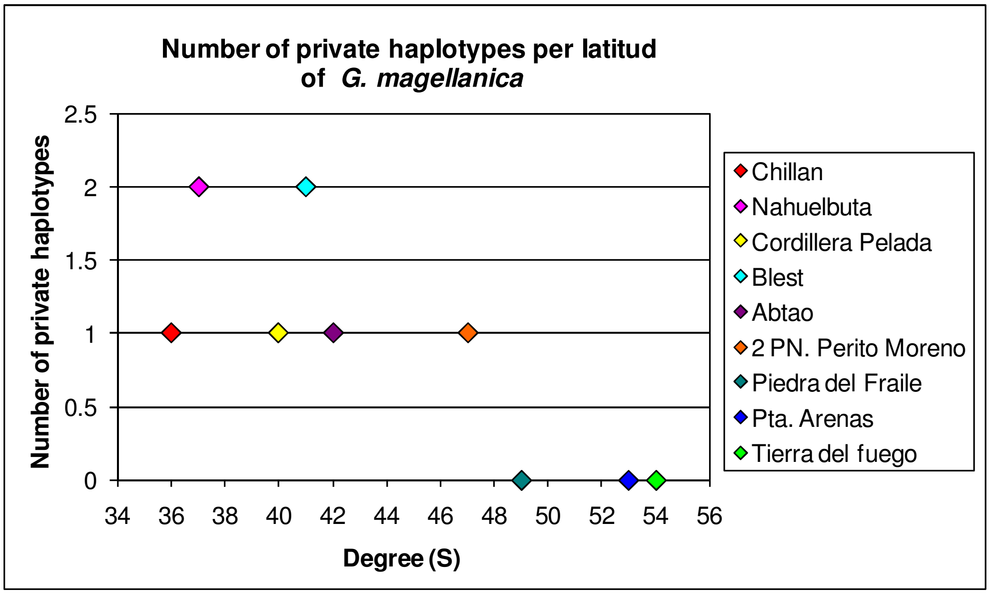


Figure 3.5. Number of private haplotypes in relation to latitude. Population clusters from very similar latitudes have been amalgamated

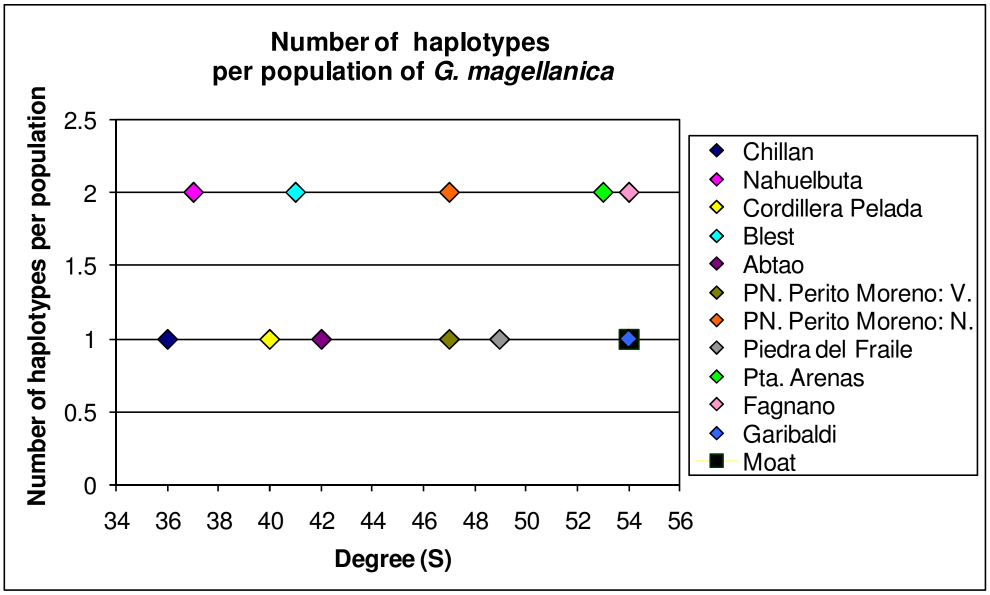


Figure 3.6. Number of haplotypes per population, plotted according to latitude.

Overall, there is marked differentiation of populations, with Gst = 0.760, and mean Nst = 0.951, both significantly different from zero (Table 3.8). There was also a significant difference between Gst and Nst, with Nst being significantly greater than Gst ($p<0.01$). Within individual regions, population differentiation is also high, although this differentiation between populations drops markedly in the far south. The isolation of the northern populations (both north Andes "W" and north coastal) and central populations (including coastal and Andean) is also evident from a comparison between the mean pairwise Fst associated with anyone population and all others, and latitude (Figure 3.7), with a decrease in mean pairwise Fst from north to south.

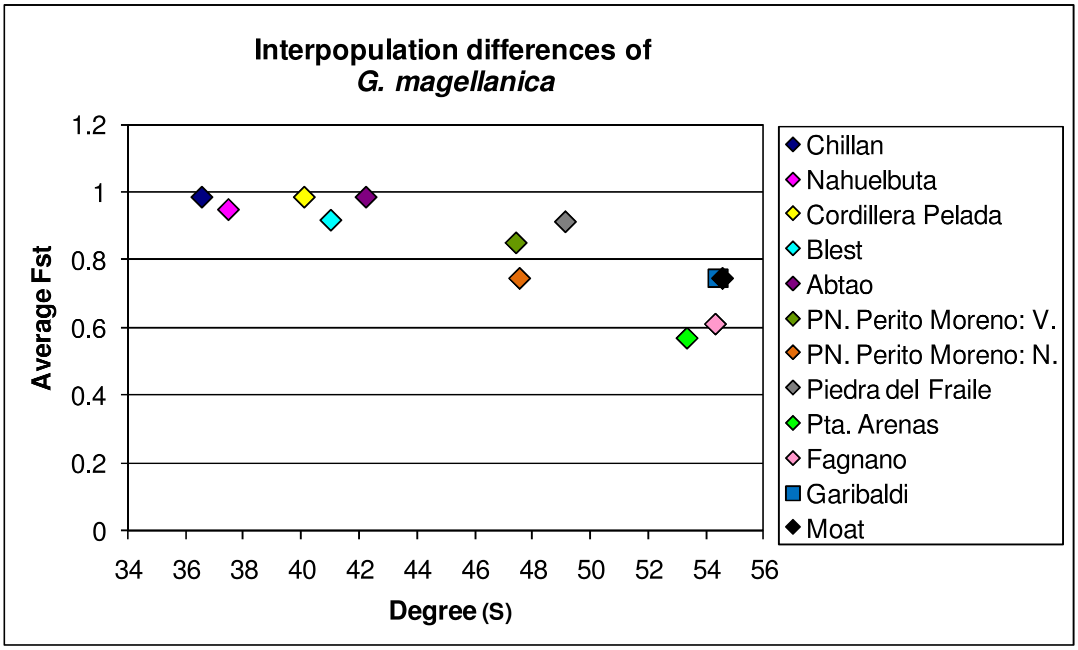


Figure 3.7. Comparison of the mean pairwise Fst for anyone population plotted against all others, according to latitude.

Table 3.7. Distribution of haplotype variation within and between populations of *Gentianella magellanica* in Patagonia

Population	**Region**	**Latitude**	**N**	**Number of individuals with each haplotype**											**Number of private haplotypes**	**Proportion of populations polymorphic per region**	He
				A	B	C	D	E	F	G	H	I	J	K			
Chillan	North Andes "W"	S36°54'13.79"	4	4											1	1of 2	0
Nahuelbuta	North coastal	S37°47'48.17"	5		4	1									2		0.4
Cordillera Pelada	Central coastal	S40°10'11.70"	5				5								1	1 of 3	0
Blest	Central Andes "E"	S41°02'9.30"	5					1	4						2		0.4
Abtao	Central coastal	S42°22'59.51"	5							5					1		0
PN. Perito Moreno:V.	South Andes "E"	S47°45'14.1"	5								5				0	1 of 3	0
PN. Perito Moreno:N.	South Andes "E"	S47°53'47.1"	4								2	2			0		0.7
Piedra del Fraile	South Andes "E"	S49°13'48.8"	5										5		0		0
Pta. Arenas	Far South	S53°37'43.24"	4									2		3	0	2 of 4	0.6
Fagnano	Far South	S54°32'59.1"	5										2	3	0		0.6
Garibaldi	Far South	S54°41'29.9"	5											5	0		0
Moat	Far South	S54°57'15.8"	5											5	0		0

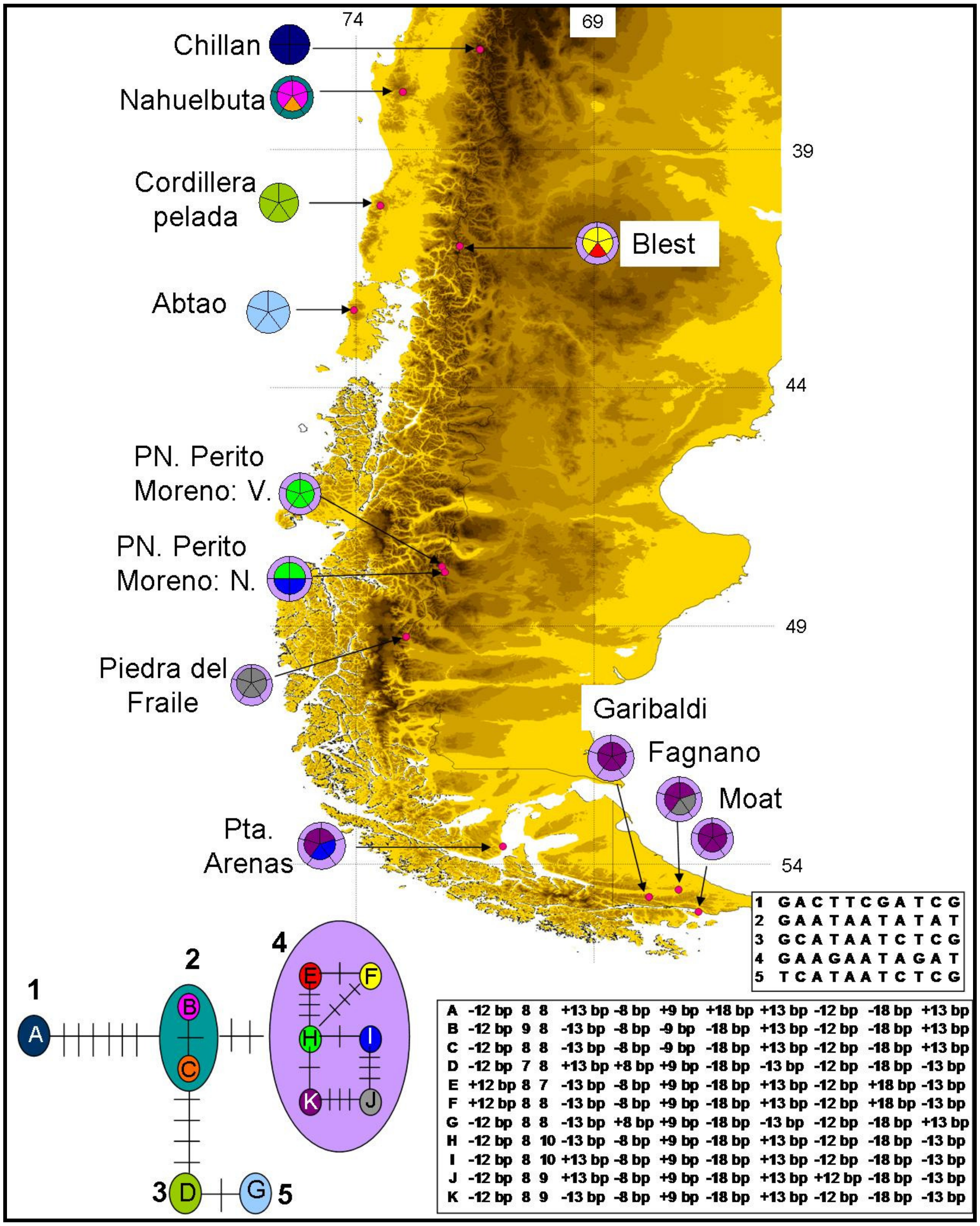
N = sample size. He = gene diversity. Haplotype designations correspond to those illustrated in the associated minimum spanning tree (Figure 3.8; Figure 3.9) and defined in Table 3.9. Colour coding of groups of haplotypes corresponds to 'clades' highlighted in the minimum spanning tree (Figure 3.8)

Table 3.8. Diversity and differentiation of chloroplast haplotypes in Gentianella magellanica for all populations.

No. pop	**No. ind**	**Number and type of mutations per region**			**Haplotypes number**	**Mean number of haplotypes/pop**	**Harmonic mean number of haplotypes/pop**	**HS (s.e.)**	**VS (s.e.)**	**HT (s.e.)**	**VT (s.e.)**	**GST (s.e.)**	**NST (s.e.)**
		Sub	**Indel**	**SSRs**									
12	58	11	9	2	11	0.91	4.8	0.222 (0.08)	0.046 (0.02)	0.926 (0.04)	0.939 (0.20)	0.760 (0.09)	0.951 (0.03)

Pop = population; ind = individuals; Sub = substitutions; Indel = insertions and deletions; Hs = Population diversity; Ht = Total gene diversity; Gst = Coeficient of differentiation amog populations; Vs = Population phylogenetic diversity; Vt = Total phylogenetic diversity.

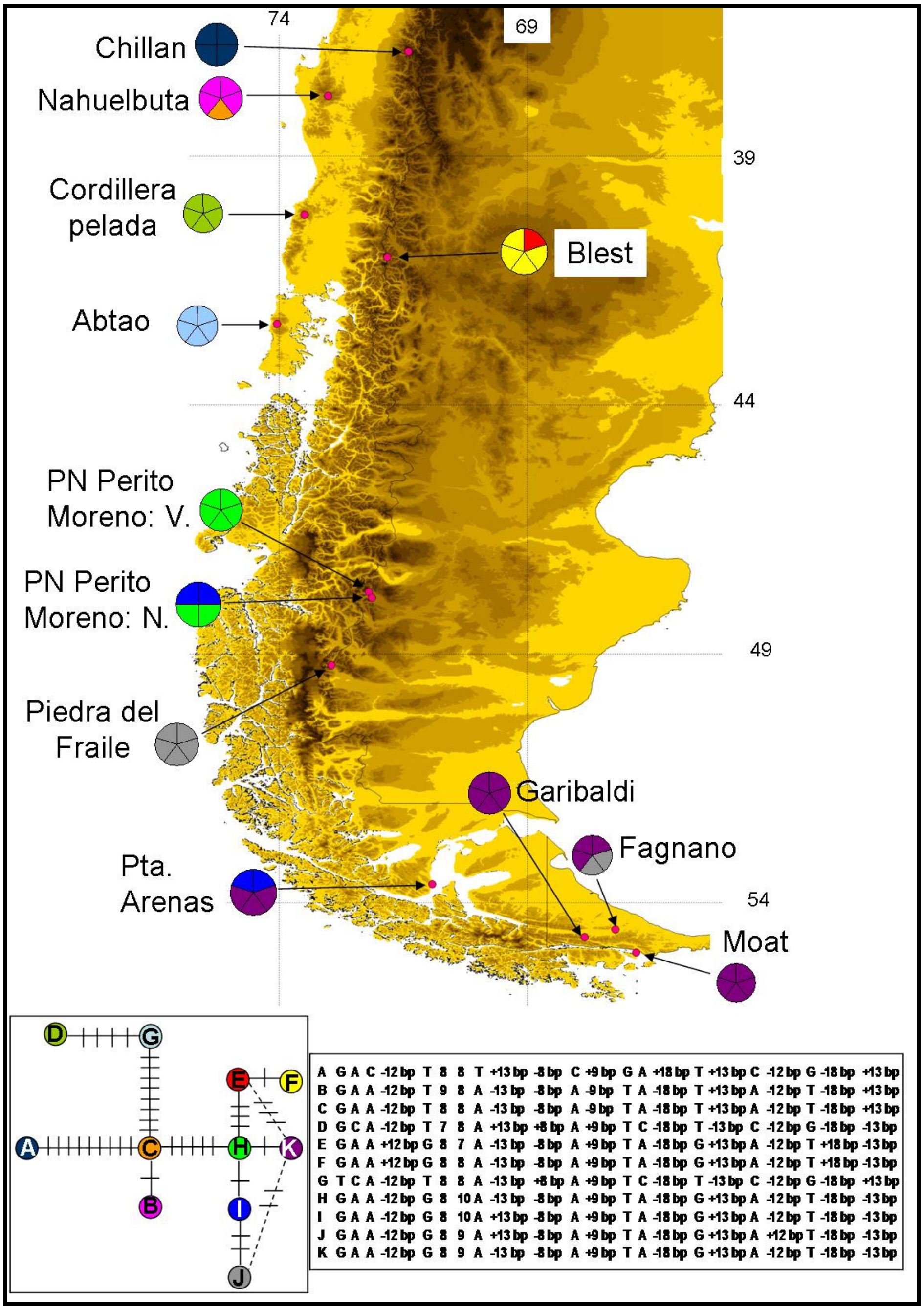
A minimum spanning network tree constructed using substitutions to establish the backbone of the tree is shown in Figure 3.8, and using substitutions, indels and SSRs together is shown in Figure 3.9. Constructing tress from all data, versus those excluding cpSSRs from the network backbone recovered the same major groupings of samples. This indicates that the SSR data does not show excessive homoplasy in this data set. Using the tree constructed with substitutions, populations in the north (north Andes "W" and north coastal) and central coastal are clustered separately from those further south, but with each population generally on a long branch from all other populations, indicative of extensive divergence (Figure 3.8). The one exception is the close similarity between Cordillera pelada and Abtao which are just one step apart. The haplotypes from Nahuelbuta are most closely related to the other major clade containing populations which occur from Blest at 41°S in the eastern Andes down to the far south (coloured light purple in the MSN). Within this group of populations, four are polymorphic, and in two of these (Blest and PN. Perito Moreno: N.) the different haplotypes differ by a single step (Figure 3.8). When indels and SSR's are used to contribute towards the interior structure of the tree the separation of all populations is more pronounced, especially between northern, central coastal populations and the rest (Figure 3.9). Populations from Central Andes "E", south Andes "E" and far south, maintain a similar separation between the two trees (Figure 3.8; Figure 3.9).



1	G	A	C	T	T	C	G	A	T	C	G
2	G	A	A	T	A	A	T	A	T	A	T
3	G	C	A	T	A	A	T	C	T	C	G
4	G	A	A	G	A	A	T	A	G	A	T
5	T	C	A	T	A	A	T	C	T	C	G

A	-12 bp	8	8	+13 bp	-8 bp	+9 bp	+18 bp	+13 bp	-12 bp	-18 bp	+13 bp
B	-12 bp	9	8	-13 bp	-8 bp	-9 bp	-18 bp	+13 bp	-12 bp	-18 bp	+13 bp
C	-12 bp	8	8	-13 bp	-8 bp	-9 bp	-18 bp	+13 bp	-12 bp	-18 bp	+13 bp
D	-12 bp	7	8	+13 bp	+8 bp	+9 bp	-18 bp	-13 bp	-12 bp	-18 bp	-13 bp
E	+12 bp	8	7	-13 bp	-8 bp	+9 bp	-18 bp	+13 bp	-12 bp	+18 bp	-13 bp
F	+12 bp	8	8	-13 bp	-8 bp	+9 bp	-18 bp	+13 bp	-12 bp	+18 bp	-13 bp
G	-12 bp	8	8	-13 bp	+8 bp	+9 bp	-18 bp	-13 bp	-12 bp	-18 bp	+13 bp
H	-12 bp	8	10	-13 bp	-8 bp	+9 bp	-18 bp	+13 bp	-12 bp	-18 bp	-13 bp
I	-12 bp	8	10	+13 bp	-8 bp	+9 bp	-18 bp	+13 bp	-12 bp	-18 bp	-13 bp
J	-12 bp	8	9	+13 bp	-8 bp	+9 bp	-18 bp	+13 bp	+12 bp	-18 bp	-13 bp
K	-12 bp	8	9	-13 bp	-8 bp	+9 bp	-18 bp	+13 bp	-12 bp	-18 bp	-13 bp

Figure 3.8. MSN and haplotype distribution map for *Gentianella magellanica*, using substitutions only to construct the backbone



A	G	A	C	-12 bp	T	8	8	T	+13 bp	-8 bp	C	+9 bp	G	A	+18 bp	T	+13 bp	C	-12 bp	G	-18 bp	+13 bp
B	G	A	A	-12 bp	T	9	8	A	-13 bp	-8 bp	A	-9 bp	T	A	-18 bp	T	+13 bp	A	-12 bp	T	-18 bp	+13 bp
C	G	A	A	-12 bp	T	8	8	A	-13 bp	-8 bp	A	-9 bp	T	A	-18 bp	T	+13 bp	A	-12 bp	T	-18 bp	+13 bp
D	G	C	A	-12 bp	T	7	8	A	+13 bp	+8 bp	A	+9 bp	T	C	-18 bp	T	-13 bp	C	-12 bp	G	-18 bp	-13 bp
E	G	A	A	+12 bp	G	8	7	A	-13 bp	-8 bp	A	+9 bp	T	A	-18 bp	G	+13 bp	A	-12 bp	T	+18 bp	-13 bp
F	G	A	A	+12 bp	G	8	8	A	-13 bp	-8 bp	A	+9 bp	T	A	-18 bp	G	+13 bp	A	-12 bp	T	+18 bp	-13 bp
G	T	C	A	-12 bp	T	8	8	A	-13 bp	+8 bp	A	+9 bp	T	C	-18 bp	T	-13 bp	C	-12 bp	G	-18 bp	+13 bp
H	G	A	A	-12 bp	G	8	10	A	-13 bp	-8 bp	A	+9 bp	T	A	-18 bp	G	+13 bp	A	-12 bp	T	-18 bp	-13 bp
I	G	A	A	-12 bp	G	8	10	A	+13 bp	-8 bp	A	+9 bp	T	A	-18 bp	G	+13 bp	A	-12 bp	T	-18 bp	-13 bp
J	G	A	A	-12 bp	G	8	9	A	+13 bp	-8 bp	A	+9 bp	T	A	-18 bp	G	+13 bp	A	+12 bp	T	-18 bp	-13 bp
K	G	A	A	-12 bp	G	8	9	A	-13 bp	-8 bp	A	+9 bp	T	A	-18 bp	G	+13 bp	A	-12 bp	T	-18 bp	-13 bp

Figure 3.9. MSN and haplotype distribution map for *Gentianella magellanica*, using all data to construct the backbone.

Table 3.9 Summary of phylogeographic patterns of *Gentianella magellanica*.

Distribution	**No. haplo-types**	**Phylogenetic divergence among haplotypes?**	**High diversity in part of range?**	**Unique northern haplotypes?**	**Unique central haplotypes?**	**Unique southern haplotypes?**	**Unique far southern haplo-types?**	**Comments**
North- Far South (Andes and coastal)	11	Yes	Yes	Yes (coast and Andes)	Yes (Andes and coast)	Yes	Yes	North and coastal populations are most divergent. All other populations contain a more closely related set of haplotypes.

3.4 Discussion

3.4.1 Is there evidence for geographically discrete genetic lineages?

If populations of *G. magellanica* had experienced long periods of historical isolation within parts of their ranges, this should lead to the presence of highly divergent lineages, such that chloroplast haplotypes in parts of the species range would be separated by many mutations from those in other parts of the range. The evidence for this is clear in the current data set. Populations in the north and coastal regions have unique and divergent chloroplast haplotypes. Another way of showing whether the species has a phylogeographical structure is by quantifying its spatial genetic variation. *Gentianella magellanica* shows significant among population differentiation (Gst = 0.76 and Nst = 0.951) (Table 3.8). The significant difference between Nst and Gst is consistent with ancient separation among populations. There is some geographical structure to the differentiation of populations, such that the average Fst per population decreases the more southerly the population (Table 3.7) indicating lower population differentiation in the south of the species range.

3.4.2 Do the observed patterns of genetic diversity and genetic differentiation correlate with previous hypotheses of historical suture zones or glacial refugia?

The clearest phylogeographic signal in the current data set is from the north of its distribution. Here we recovered divergent haplotypes between the sampled populations from the north Andes and the central coastal region. The rest of the populations located east of the Andes and southwards consisted entirely of a separate clade of relatively closely related haplotypes, including one haplotype which is widespread in the far south of Patagonia. These data suggest that many of these populations in the north and coastal parts of the range have been isolated from those east and further south for some time.

The presence of fixed unique haplotype in the northern and coastal populations suggests some level of isolation for these populations (Table 3.5). These populations of *G. magellanica* together with those from Blest are restricted to a high altitude location, and thus the populations can be thought of as occupying a habitat island surrounded by areas of unsuitable terrain (e.g. too warm and occupied by competitive evergreen rain forest). The genetically divergent populations in the north could be classified as currently being in a refugium, because suitable habitat conditions in this northern area are likely to have been more common in colder climates. One scenario which could have given rise to the observed distribution of haplotypes in the north /coastal regions is if the species was more widespread, but still patchily distributed during glacial maxima. The species is likely to have experienced altitudinal migrations in response to climate change, and the data are consistent with local populations persisting in the areas around these extant northern and coastal sites – expanding during glaciations, and contracting to high-elevation refugia during inter-glacials.

Considering the relationships between these divergent northern/coastal populations and the other populations, it is somewhat puzzling that the Nahuelbuta population is most closely related to the clade that dominates the southern area of distribution, rather than the population from Chillan (Figure 3.8). One might have predicted that Chillan in the Andes, would be most closely related to populations such as Blest (which is further down the Andean chain). However, although no perfectly satisfactory explanation exists for this, it is worth noting that the central depression is at a relatively high altitude in the area around Nahuelbuta, and may have acted as a bridge between the coastal cordillera and the Andes (e.g. see topographical relief on Figure 3.8; Figure 3.9).

Focusing on the more southern set of haplotypes (E-K, Figure 3.7), there is no simple single explanation to explain this pattern. Considering the geographical area over which these haplotypes are spread, they are more similar to each other, than those in the north and coastal regions. This result is consistent with this southerly region being covered by ice, and recolonisation, rather than a strong signal of *in situ* persistence, and marked divergence between all populations. However, the result is not as clear cut as a single haplotype dominating this previously glaciated area. Six haplotypes are present and the haplotypes from populations in the south and far-south are circumscribable in the haplotype network (Figure 3.8), separate from the haplotypes from Blest population. Thus although all of these haplotypes collectively share a common ancestor, there is subdivision within this group. What can be said is that in the far south, there is evidence for relatively widespread dispersal of individual haplotypes (J and K, Table 3.7; Figure 3.8). The present data (and density of sample points) does not allow us to go further than this and infer the location of refugia, or directionality to the recolonisation process. However, the presence of some phylogeogenetic/phylogeographic structure among this broad set of populations from Blest to Tierra del Fuego is consistent with origins from different (albeit not particularly divergent) refugia.

Among the South American species whose phylogeography has been studied, the most similar to *Gentianella magellanica* in terms of life history traits are two cold tolerant perennial herb species, *Hypochaeis incana* and *Hypochaeris palustris* (Tremetsberger *et al*., 2009, Muellner *et al*., 2005). It is difficult to draw direct comparisons with the first species because it has an eastern distribution only, lacking the coastal populations which are the most important in the studied specie. For *H. incana* it has been suggested that the three different clades detected could be related to glacial refugia. These are a northern clade distributed from 38°S to 43°S which correspond to the same area where Gentianella has higher levels of private haplotypes. The central clade is found from 44°S to 50°S and a southern clade from 50°S to 55°S. In *G. magellanica* this division into two southern clusters is not clear although populations south of 53°S all share the widespread haplotype (K) whereas none of the populations from further north has it. In the case of *H. palustris,* it has a somewhat similar phylogeographic pattern to *G. magellanica* in terms of the divergence of coastal and northern populations. Its coastal populations have high levels of diversity and are genetically isolated, as are those from the north. The furthest south the species was sampled was around 51°S. These populations are genetically differentiated from those further north, and the authors concluded (a) coastal populations have been isolated for long periods of time, but have contributed to recolonization of the Andes, (b) that multiple refugia were present in the Andean chain.

Cold tolerant Patagonian trees such as *Nothofagus pumilio, Nothofagus betuloides, Nothofagus dombeyi, Nothofagus nitida, Nothofagus antarctica* (Acosta and Premoli, 2010; Mathiasen and Premoli, 2010) and thermophilous trees such as *Nothofagus obliqua* (Azpilicueta *et al*., 2009) have a similar but not as clear pattern as *G. magellanica*. Populations are generally not so genetically isolated, but these studies have shown evidence for differentiation between populations north and south of a zone between ~40°S or 43°S, which matches the observation from *G. magellanica* of a widely distributed group of haplotypes from Blest (41°S) southwards, and the separation of this lineage from those further north and west.

3.5 Conclusions

The key points that emerge from this investigation of *G. magellanica*, an annual endemic to Patagonia are:

1. There is high density of private haplotypes in the north-central populations, suggesting multiple refugia, of which 2 correspond to northern refugia (north of 37°47'S) which may have been situated during the LGM at the central depression or on the lower slopes of the Andes cordillera for population Chillan and on the central depression and on the lower slopes of the coastal cordillera for Nahuelbuta population. In the central area, coastal populations may have constituted one refugium (we count this as one because there is only one difference between Cordillera pelada and Abtao) this could have

been distributed on the western side of the central depression or on the lower slopes of the Coastal cordillera. It is important to notice that no big difference appears between the populations separated by the Chacao channel (possibly due to gene flow from the mainland to the island due to a decrease of the sea level). The refugium for the central-south eastern populations is difficult to identify, although the Blest population has higher levels of private haplotypes than the rest of the south and far south populations, so a refugium in the central Andes could be suggested. However it is difficult to identify whether this population occupied a refugium on the eastern or western slopes of the Andes. South of Blest there is two private haplotypes, one in the area of PN. Perito Moreno and another private to Tierra del Fuego. So with these data we suggest that there were ~4 refugia; one north of 42°S on the coastal cordillera, a second in the central depression and Andes areas, and possibly two southern refugia at 47°S and south of 53°S.

2. Our sampling of the species on the west and east of the Andes cordillera is not comprehensive enough for us to provide a robust test of evidence for the main Andean cordillera as a barrier to gene flow. However the population from Nahuelbuta is genetically closer to the eastern southern populations than to the geographically closer populations from the coastal cordillera, so there is no evidence for a major east-west divide in this species.

3. Northern population from Chillan show higher differentiation than southern populations. This could be related to higher altitudes of the Andes cordillera north of 37°S, producing a geographical barrier for gene flow. There is also a decrease in the differentiation of the populations going south, being the most diverse populations from 36°S to 42°S.

Chapter 4: Phylogeography of Prumnopitys andina, a threatened conifer restricted to a few populations in the Coastal and Andean cordilleras

4.1 Introduction

Southern South America has high plant species richness and endemism in the area from 36°S to 40°S (Villagran *et al.*, 1998; Villagran and Le-Quesne, 1996). However, the biodiversity in this region is threatened due to habitat fragmentation caused by changes in land use to agriculture, forestry and urbanization (Echeverria *et al.*, 2006). There have only been a small number of studies on range-restricted endemic plants in this region. Studies to date include *Araucaria araucana* (Bekessy *et al.*, 2002), *Berberidopsis corallina* (Etisham-Ul-Haq *et al.*, 2001), *Legrandia concinna* (Martinez *et al.*, 2011) and *Pitavia punctata* (Stark, per.com.). These studies have indicated a high level of genetic differentiation between coastal and Andes populations (Bekessy *et al.*, 2002 and Stark pers. Com.), and within the Andean cordillera, some differentiation between northern and southern populations (Martinez *et al.*, 2011; Bekessy *et al.*, 2002; Etisham-Ul-Haq *et al.*, 2001).

The subject of the current chapter is *Prumnopitys andina* (Poepp. ex Endl.) de Laub. This species belong to the conifer family Podocarpaceae. Is a perennial tree up to 15 m high with thin leaves and a distinctive fleshy fruit, used to feed animals and to produce jam or alcoholic drinks (Hoffmann, 1997; Marticorena and Rodriguez, 1995). Trees are randomly monoecious or dioecious (Figure 4.1). It is endemic to Chile growing mainly in the Andes between Regions: VI, VII, VIII, IX, and X (35°53’-39°30’S); and in the coastal cordillera with only one known population, in the eastern slope of the Nahuelbuta Cordillera (Figure 4.2) (Gardner *et al.*, 2006; Hechenleitner *et al.*, 2006; Gosling *et al.*, 2005; Villagran and Armesto, 2005; Gardner and Lara, 2003). On both cordilleras it grows from 500 to 1,300 m (Figure 4.3), but over the restricted distribution of this species there is no clear correlation between altitude and latitude.

Several authors have cited a population in Argentina, although this information has been checked and suggests that this record is erroneous (Gardner *et al.*, 2006; Hechenleitner *et al.*, 2006). It occurs in moderate humid soils, mainly next to water courses on rocky slopes, growing with *Austrocedrus chilensis*, *Nothofagus glauca* and the esclerofilo forest type. It has been categorized by IUCN as a vulnerable species (VU B2ab(ii-v)) for the entire country and as rare and vulnerable for regions VI and VII respectively (Gardner *et al.*, 2006; Benoit, 1989).

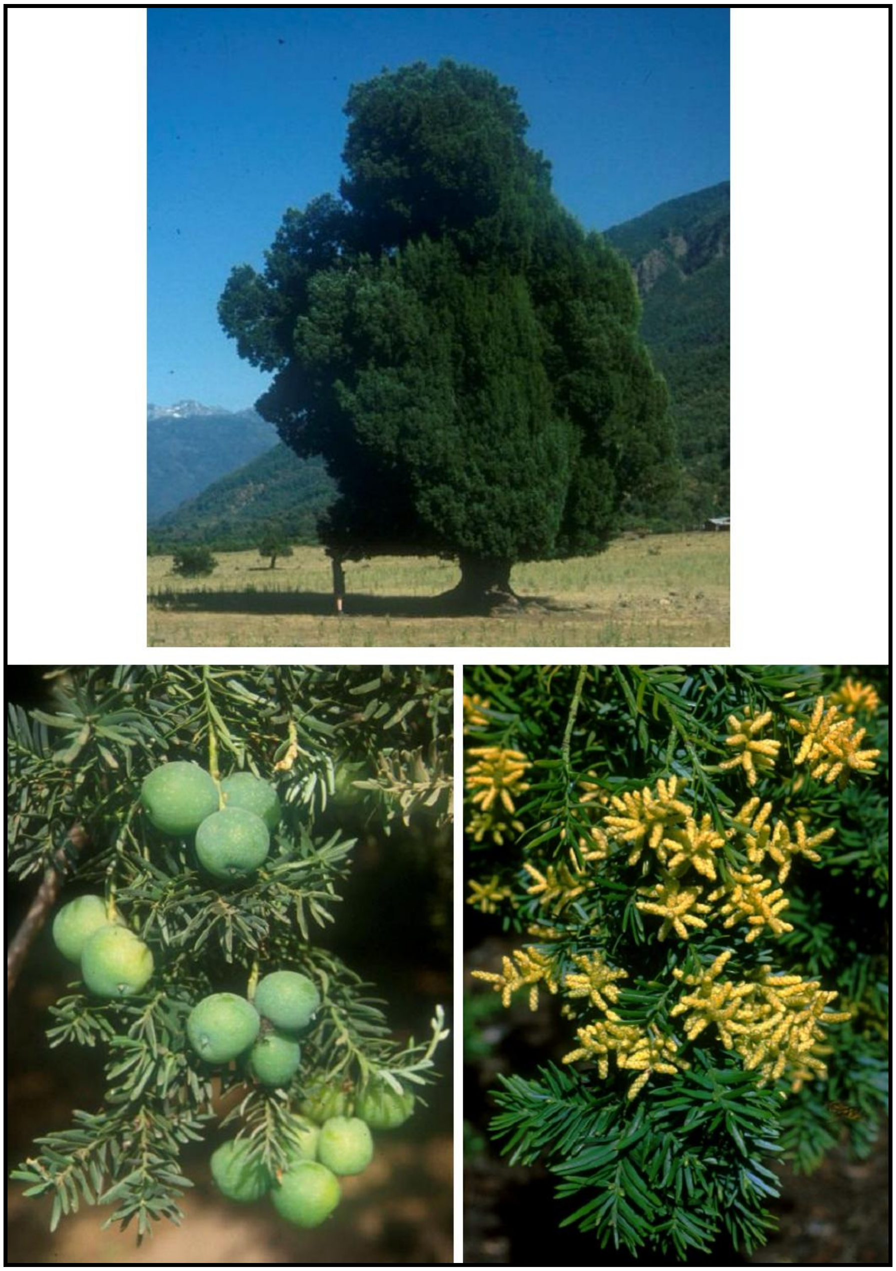


Figure 4.1. *Prumnopitys andina* clockwise from top: (a) mature tree, (b) close up of plant male flowers, (c) close up of plant fruits.

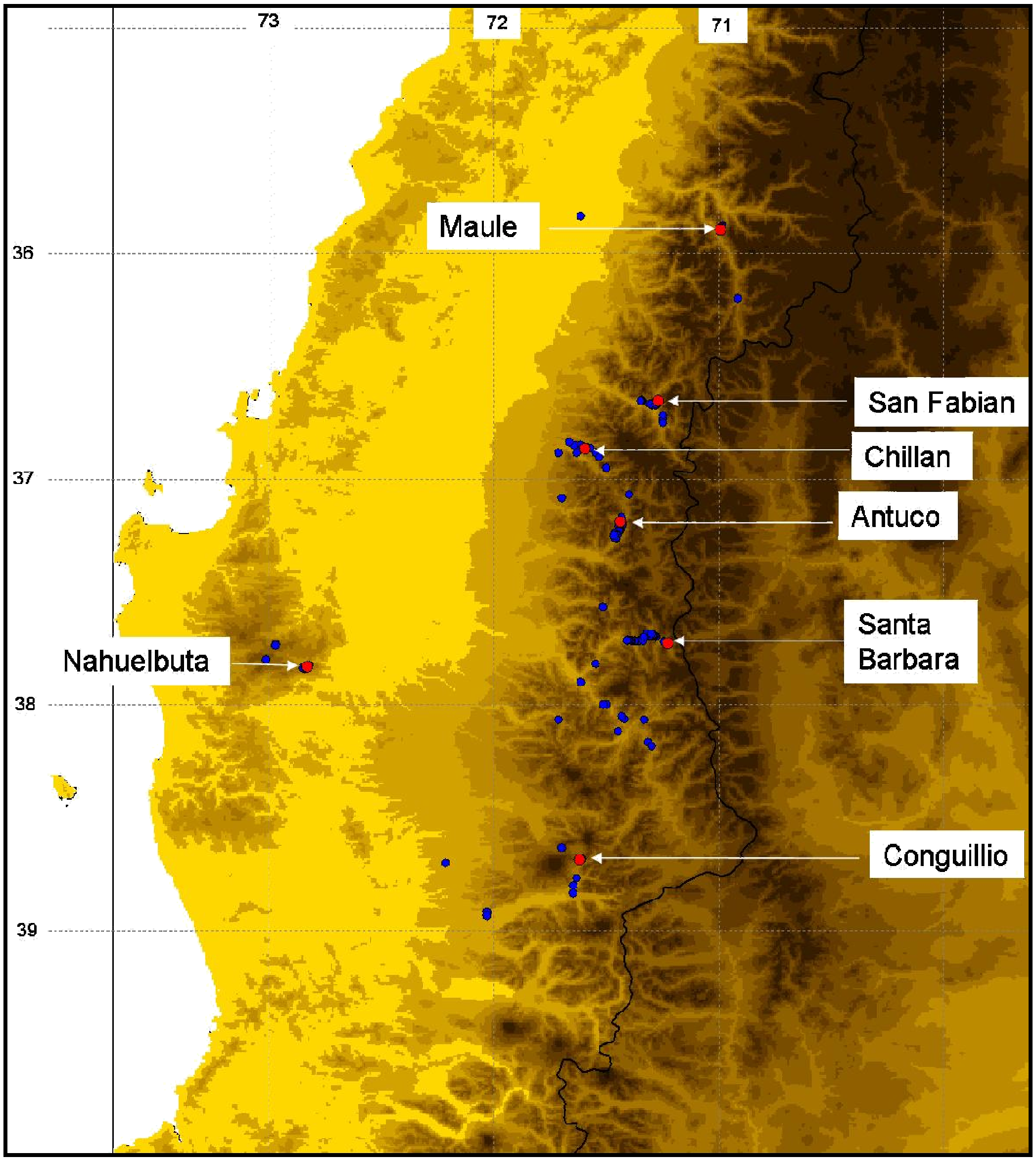


Figure 4.2. Distribution of *Prumnopitys andina.* The red dots indicate populations sampled for the current study and the blue dots are unsampled localities.

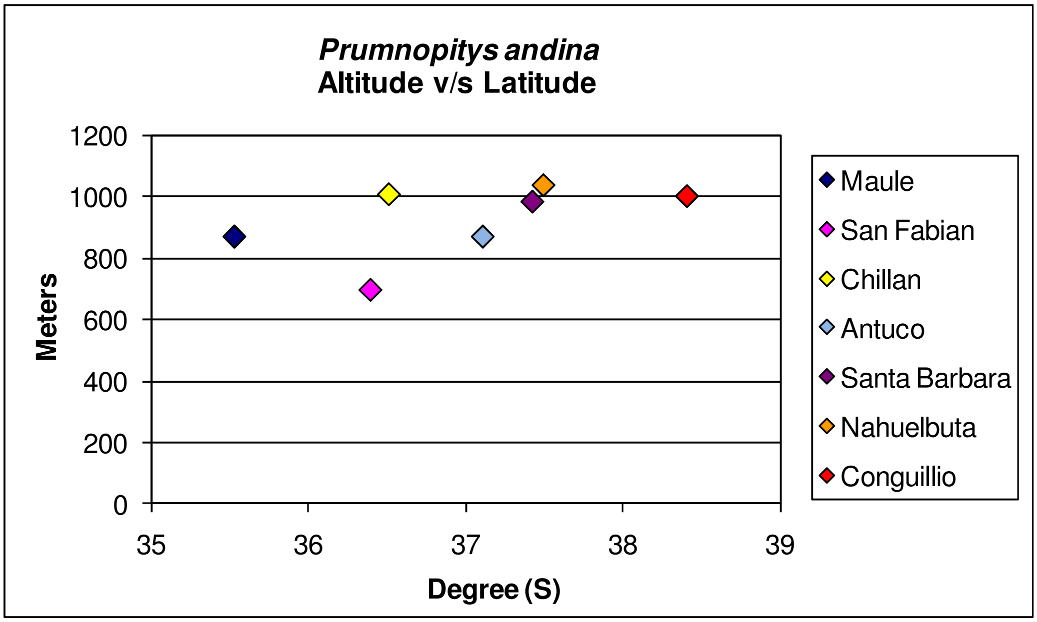


Figure 4.3. Plots of altitude and latitude from sample sites of *Prumnopitys andina*.

Evidence from fossil pollen suggests that this species was more widespread during glacial periods. There are pollen records for this species suggesting it was widespread in the central depression from 34°S to 42°S until 20,000 yr (Heusser *et al*., 2006a,b; Donoso, 1998) and also that it occurred on the eastern side of the Andes with high percentages from 17,000 yr to 14,900 yr disappearing until ca 9,000-10,000 yr (Markgraf *et al*., 2009; 1986).

The aims of this study are to examine whether there is any phylogeographic structure evident in this narrow endemic, that matches structure observed in more widespread species studied in Chapters 2 and 3. At the outset, there are two reasons to expect lower phylogeographic signal. Firstly – the smaller geographical distances involved are likely to limit the extent of differentiation. Secondly, as *Prumnopitys andina* is a conifer, the expectation is that its chloroplast DNA is paternally inherited (Wilson and Owens, 2003). The greater dispersal of pollen compared to seeds is likely to lead to lower levels of phylogeographic structure compared to that detected with maternally inherited markers (Ennos *et al.,* 1999).

4.2 Materials and Methods

4.2.1 Sampling Methodology

Species distribution data (Table 4.1) was obtained from the following herbaria: Royal Botanic Garden (E), Herbario Universidad de Concepcion (CONC). Seven populations of *Prumnopitys andina* were sampled throughout its entire range. This includes the only population at the Chilean coastal cordillera and the Andean cordillera populations, from 35°S until ~39°S (Table 4.1 and Figure 4.2). Populations were classified as being part of the Coastal range or the Andean mountains. To facilitate discussion of the data, these northern Andean populations have been classified in three groups: north, central and south (Table 4.1), although it should be stressed that all of these populations fall within the classification of 'northern' in Chapters 2 and 3.

For each population at least three individuals were collected as voucher herbarium specimens and deposited at the following herbaria: Royal Botanic Garden Edinburgh (E), Herbario de Concepcion (CONC) and Museo de Historia Natural de Chile (MNHN).

For genetic analyses, fresh plant tissue was collected from the wild, and up to 20 individuals per population were sampled, with a minimum inter-plant distance of at least 5 m, where possible, to avoid sampling related individuals. Plant samples, which consist of leaves from between 3 to 5 twigs of *P. andina* were placed in a silica-gel in zip-sealable plastic sample bags.

Table 4.1. Location of samples of *Prumnopitys andina*

Area	**Population**	**Location**	**Latitude (S)**	**Longitude (W)**	**Altitude (m)**	**Population size***
North North Andes	Maule	Valle del maule, road to corral de salas	35°53'32.9"	70°59'37.9"	871	300
Central North Andes	San Fabian	San Fabian de Alico, Sector el ingles, next to Rio Sauce	36°39'08.4"	71°16'12.4"	696	150
Central North Andes	Chillan	Termas de Chillan, road to Cerrillos	36°51'51.5"	71°35'55.1"	1240	>100
Central North Andes	Antuco	Fundo los ciervos, next to river Polcura	37°11'18.7"	71°26'29.9"	868	2000
Central North Andes	Santa Barbara	Alto Biobio, river Trapananda	37°43'33.8"	71°13'46.6"	859	2000
Coastal	Nahuelbuta	Cordillera de Nahuelbuta, Cerro pelado	37°49'52.0"	72°49'54.9"	1127	<100
South North Andes	Conguillio	PN. Conguillio, Laguna verde	38°41'10"	71°37'16.4"	980	>1000

* population size is based on an approximation of the number of mature individuals. This was possible for this species, due to extensive distributional surveys and data compilations undertaken by the International Conifer Conservation Project.

4.2.2 Molecular methods

DNA extraction

Approximately 50 mg of dried leaf material was ground using a porcelain mortar and pestle. All samples were extracted using a CTAB buffer. The CTAB method was modified from Doyle and Doyle (1987) with the inclusion of 0.1% insoluble polyvinylpoly-pyrrolidine (PVPP) to the 2x CTAB buffer. Two 24:1 chloroform/isoamyl alcohol washes were performed and the DNA precipitated overnight in freezer-cold isopropanol. Depending on the size of the resultant pellet, the DNA was dissolved in 50-70 µl of TE (10 mM TRIS-HCl, 1 mM EDTA, pH7.4). The quality and quantity of extracted DNA was checked under UV light on 1.0% agarose gels containing 400 mg of Ethidium bromide.

PCR amplification

The reaction mixture (50 *ul*) contained:

- 33 *ul* water
- 5 *ul* 10x NH_4 Reaction Buffer (160mM $(NH_4)_2SO_4$, 670 mM Tris-HCI (pH 8.8 at 25°C), 0.1% stabilizer (Bioline)
- 2.5 *ul* 50 mM $MgCl_2$ Solution (Bioline)
- 5 *ul* 10 mM dNTP Mix; dATP, dGTP, dCTP and dTTP as lithium salts (pH 7.5)
- 1.5 *ul* 10 mM Forward primers
- 1.5 *ul* 10 mM Reverse primers
- 0.25 *ul* BIOTAQ DNA polymerase (Bioline)
- 1.0 *ul* template DNA (for some individuals the amount of DNA used, varied between 0.5 to 2 *ul*)

Amplification of seven plastid regions was carried out using the following PCR profile (Table 4.2):

Table 4.2. PCR profile for seven analyzed chloroplast region for *Prumnopitys andina*. All primer pairs had 30 cycles.

Process	***atpB-rbcL***		***matK***		***trnD-trnT***		***trnH-trnK***		***trnL-trnF***		***trnS-trnFM***		***trnT-trnL***	
	Temp. (°C)	Time	Temp. (°C)	Time	Temp. (°C)	Time	Temp. (°C)	Time	Temp. (°C)	Time	Temp. (°C)	Time	Temp. (°C)	Time
Denaturation	94	4 m	94	3 m	94	4 s	94	4 m	94	4 m	94	4 m	94	5 m
Denaturation	92	45 s	94	45 s	95	45 s	92	45 s	92	45 s	92	45 s	94	1 m
Annealing	49	75 s	56	50 s	55	45 s	62	45 s	62	45 s	62	45 s	55	1 m
Extension	72	75 s	72	80 s	73	3 m	72	2 m	72	2 m	72	2 m	72	5 m
Final elongation	72	10 m	72	5 m	72	10 m	72	10 m	72	10 m	72	10 m	72	10 m

Temp.= temperature; m= minutes; s=seconds

All PCR's were performed using a PCT-200 DNA Engine cycler (BIO-RAD). PCR products quality and quantity was assessed by electrophoresis on 1.0% agarose gels containing 400 mg of Ethidium bromide and checked under UV light, comparing the products with 1kb+ standard markers.

Sequencing PCR

PCR products were purified by QIAquick PCR Purification Kit (Qiagen). QIAquick PCR purification was carried out following the manufacturer's recommendations. Sequencing was performed using DTCS Quickstart sequencing mix on a CEQ 8000 automated sequencer (Beckman & Coulter). The sequencing platform has a total reaction volume of 10 *ul*, using a fluorescent dye of DTCS Quickstart mix, the cycle sequencing profile is: 96°C 20 sec. and 50°C 20 sec. + 60°C 4 min. for 35 cycles. The resulting sequences were manually edited and analysed using Sequencher® 4.7 (Gene Codes Corporation).

Identification of chloroplast DNA variation

The seven chloroplast regions were initially tested for PCR amplification on one sample per population (Table 4.3). From this initial screen, only one region was polymorphic, five were easy to amplify and sequence but showed no variation, and one region did not amplify (Table 4.4). The variable region (*trnS-trnFm*) was then sequenced in 15 individuals per population.

Table 4.3. Primers, reaction conditions and usefulness of eleven chloroplast regions of *Prumnopitys andina.*

Chloroplast region	**Primer sequence 5'-3'** **F=Forward R=Reverse**	**Annealing temperature °C**	**Extension time (sec)**	**Amplification lengh**	**PCR success**	**Sequence polymorphism**
atpB-rbcL	F: ACATCKARTACKGGACCAATAA R: AACACCAGCTTTRAATCCAA	49	115	-	No	-
matK	F: GAACTCGTCGGATGGAGTG R: GAGAAATCTTTTTCATTACTACAGTG	56	120	500	Yes	No
trnD-trnT	F: ACCAATTGAACTACAATCCC R: CTACCACTGAGTTAAAAGGG	54.5	180	1650	Yes	No
trnH-trnK	F: ACGGGAATTGAACCCGCGCA R: CCGACTAGTTCCGGGTTCGA	62	120	3000	Yes	No
trnL-trnF	F: CGAAATCGGTAGACGCTACG R: GGGGATAGAGGGACTTGAAC	62	120	1650	Yes	No
trnS-trnFM	F: GAGAGAGAGGGATTCGAACC R: CATAACCTTGAGGTCACGGG	62	120	1300	Yes	Yes
trnT-trnL	F: CATTACAAATGCGATGCTCT R: TCTACCGATTTCGCCATATC	55	120	500	Yes	No

4.2.3 Molecular Data Analysis

To define chloroplast haplotypes and to format the molecular data for population genetic software packages, the sequence variation (substitutions and variation in chloroplast microsatellite length) was scored and analysed using a Chloroplast Excel macro (French pers.com.). Substitutions were quantified by coding the most common type as 0 and the rarer character state as 1. Microsatellites (SSR) variants were coded according to the length of the repeats.

The number of haplotypes per population was recorded, as was the distribution of private haplotyes (haplotypes found only in a given population or geographical regions). Quantification of genetic diversity and differentiation was undertaken using Nei's gene diversity estimators including total gene diversity (Ht), gene diversity present within populations (Hs) and gene diversity between populations (Gst) using Haplodiv (Pons and Petit, 1995). Pairwise population differentiation (Fst) was estimated using Arlequin ver. 2.000 (Schneider *et al*., 2000). To assess whether there is evidence for phylogeogenetic structure in the data set, Nst was estimated using Haplonst (Pons and Petit, 1996). To test whether Nst was significantly different from Gst, a randomisation test was undertaken using Permut (Pons and Petit, 1996). A minimum spanning network tree (MSN) was generated to visualise relationships and levels of mutational change between haplotypes using Arlequin ver. 2.000 (Schneider *et al*., 2000).

4.3 Results

4.3.1 General patterns of sequence and haplotype diversity

A total of eight haplotypes were detected in *P. andina*. These were based on 4 substitutions and one variable cpSSR (Table 4.4).

Table 4.4. Chloroplast haplotypes detected in *Prumnopitys andina* for the *TrnS-trnFM* region.

Haplotype name	***trnS-trnFM***				
	130 Bp	250 Bp	490 Bp	530 Bp	590 Bp
A	T	C	T	10	A
B	T	A	C	10	G
C	G	C	C	10	G
D	T	C	C	10	G
E	T	C	T	10	G
F	T	C	C	11	G
G	T	C	C	9	G
H	T	C	C	11	A

4.3.2 Diversity and distribution of genetic variation in Prumnopitys andina.

The number of haplotypes recovered from individual populations range from 1-4, with 6/7 (85.7%) of populations showing haplotype polymorphism (Figure 4.4; Table 4.5; Table 4.6), and levels of gene diversity range from 0-0.8 (mean Hs = 0.355) with a noticeable higher levels in the northern most population (Table 4.6; Figure 4.5). Of the 8 haplotypes, 3 are private to individual populations (haplotype C is private to Maule, haplotype E is private to San Fabian and haplotypes H is private to Conguillio). There is no clear cut pattern to the distribution of private haplotypes, only higher frequencies in Andean populations, instead of coastal population (Figure 4.6). The most widespread haplotype was D, occurring in all populations, and present in at least 10/15 individuals at all sites other than Maule. The mean Gst = 0.184, and Nst = 0.120, both significantly different from zero. There was no evidence for phylogeographic structure (Nst is not greater than Gst).

There is no clear cut difference in levels of population differentiation throughout the range, other than the most northerly and most southerly populations are the most differentiated from all others (Figure 4.7). The population from the coastal cordillera (Nahuelbuta) shows no evidence for isolation and has the lowest overall mean pairwise Fst with all other populations (Figure 4.7).

When the haplotypes are visualised on a minimum spaning tree, haplotype D occupies the interior node tree, with haplotypes B, C, G, E and F connected to this by a single step each (Figure 4.8). There is little structure to the tree.

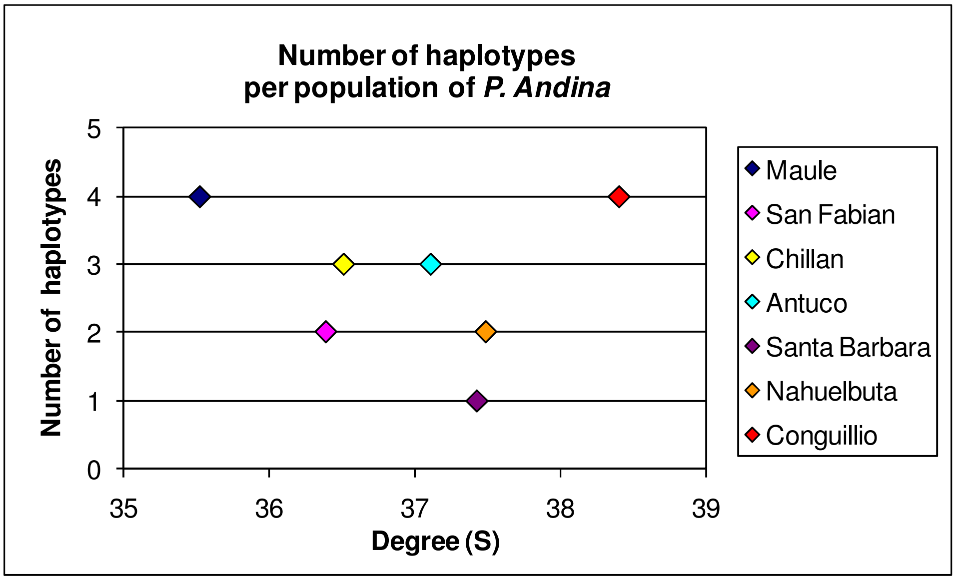


Figure 4.4. Number of haplotypes per population, plotted according to latitude for *Prumnopitys andina.*

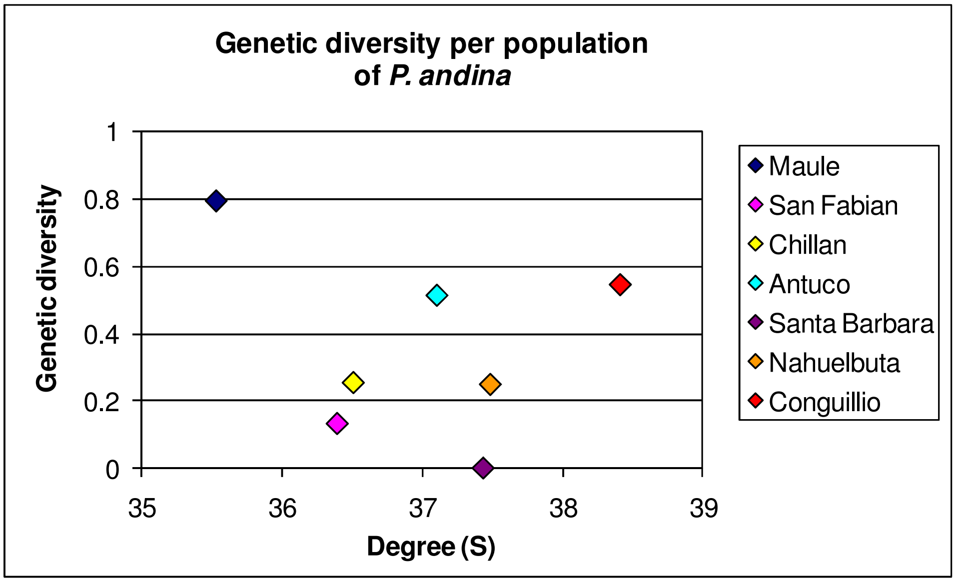


Figure 4.5. Gene diversity per population, plotted according to latitude for *Prumnopitys andina.*

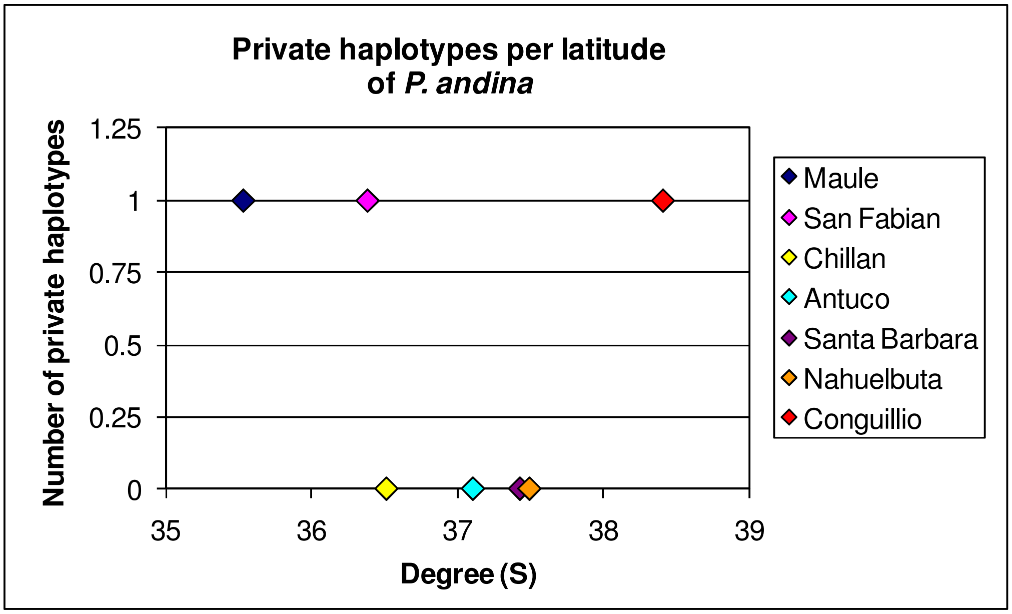


Figure 4.6. Number of private haplotypes plotted according to latitude of *Prumnopitys andina.*

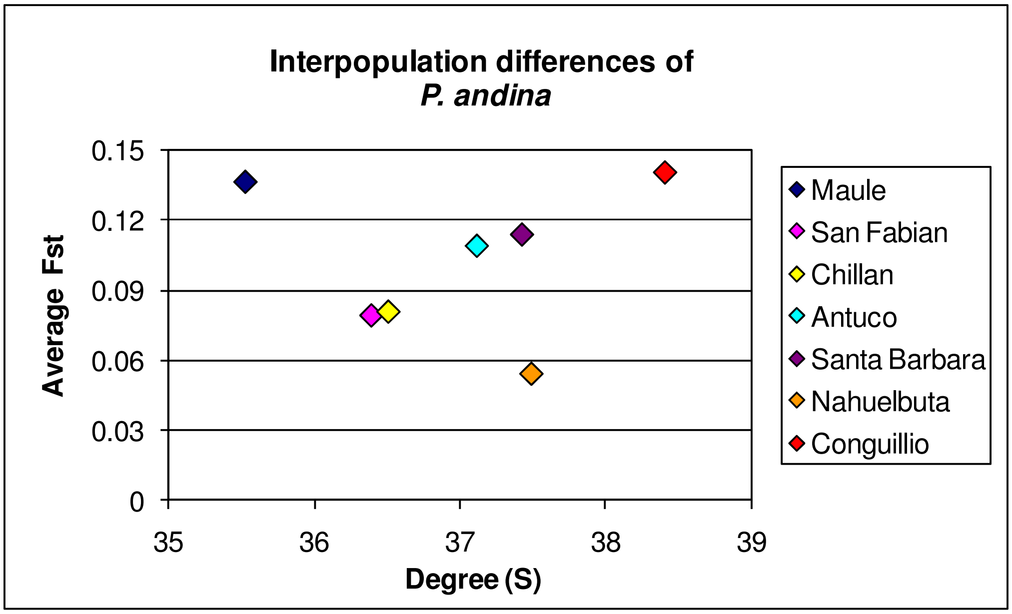
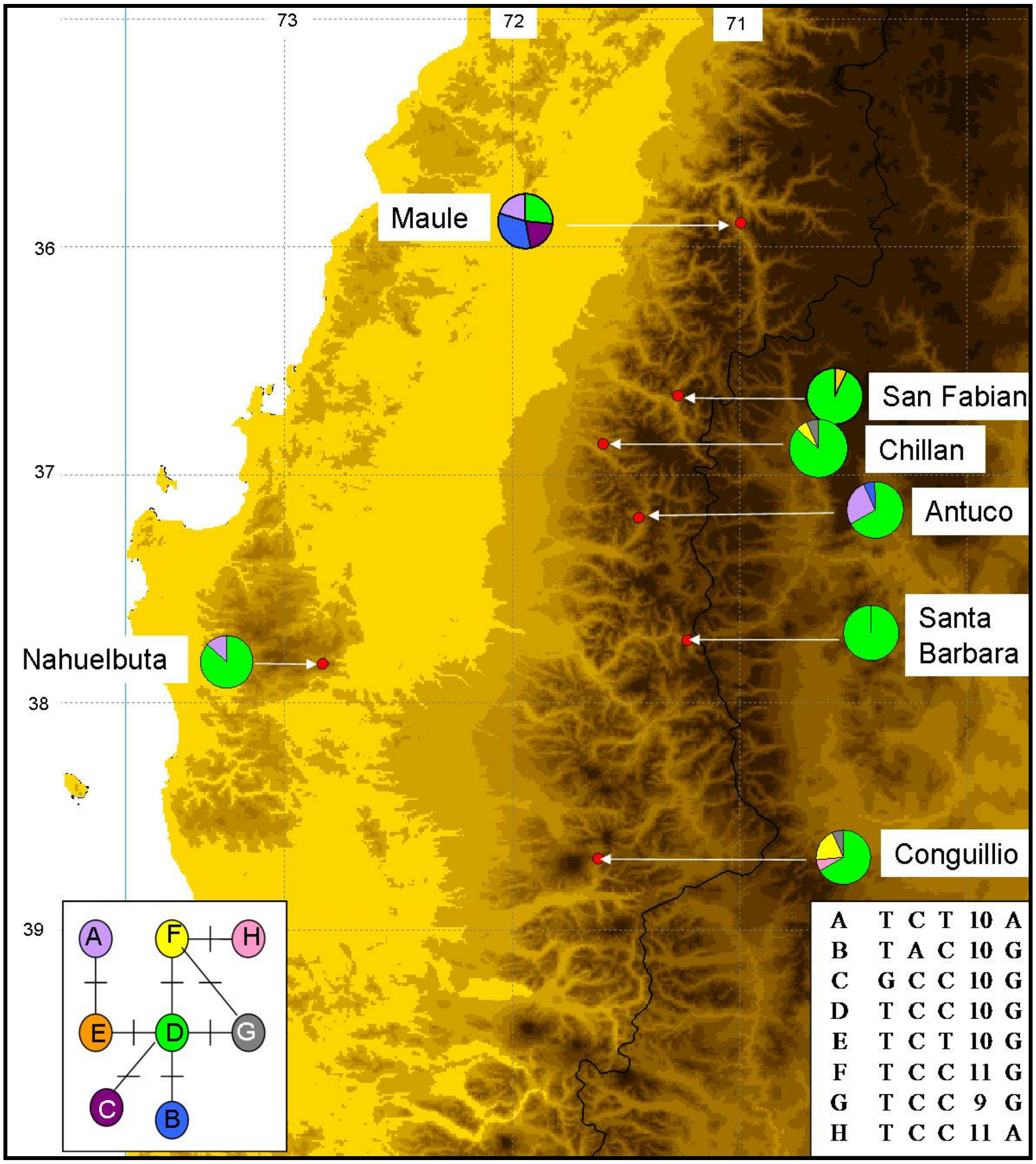


Figure 4.7. Comparison of the mean pairwise Fst for anyone population plotted against all others, according to latitude.



A	T	C	T	10	A
B	T	A	C	10	G
C	G	C	C	10	G
D	T	C	C	10	G
E	T	C	T	10	G
F	T	C	C	11	G
G	T	C	C	9	G
H	T	C	C	11	A

Figure 4.8. MSN and haplotype distribution map for *Prumnopitys andina.*

Table 4.5. Diversity and differentiation of chloroplast haplotypes in *Prumnopitys andina.*

Specie	No. Populations	No. Individuals	Number and types of mutations per region		No. haplotypes	Mean number of haplotypes/pop	H_S (s.e.)	V_S (s.e.)	H_T (s.e.)	V_T (s.e.)	G_{ST} (s.e.)	N_{ST} (s.e.)
			Subs	SSRs								
Prumnopitys andina	7	105	4	1	8	1.1	0.355 (0.103)	0.380 (0.132)	0.435 (0.131)	0.432 (0.143)	0.184	0.120

Pop = population; Hs = Population diversity; Ht = Total gene diversity; Gst = Coeficient of differentiation amog populations; Vs = Population phylogenetic diversity; Vt = Total phylogenetic diversity

Table 4.6. Distribution of haplotype variation among populations of *Prumnopitys andina.*

Population	Region	Latitude (S)	N	Number of individuals with each haplotype								Number of private haplotypes	He
				A	B	C	D	E	F	G	H		
Maule	North Andes	35°53'32.9"	15	3	5	3	4					1	0.791
San Fabian	Central Andes	36°39'08.4"	15				14	1				1	0.133
Chillan	Central Andes	36°51'51.5"	15				13		1	1		0	0.257
Antuco	Central Andes	37°11'18.7"	15	4	1		10					0	0.514
Santa Barbara	Central Andes	37°43'33.8"	15				15					0	0.000
Nahuelbuta	Coastal	37°49'52.0"	15	2			13					0	0.248
Conguillio	South Andes	38°41'10"	15				10		3	1	1	1	0.543

Table 4.7. Summary of phylogeographic patterns of *Prumnopitys andina* to form a comparative framework with Chapters 2 and 3.

Distribution	**No haplotypes**	**Phylogenetic divergence among haplotypes?**	**High diversity in part of the range?**	**Unique northern haplotypes?**	**Unique coastal haplotypes?**	**Unique central haplotypes?**	**Unique southern haplotypes?**	**Comments**
North Andes and coast	8	No	Yes (northern most population)	n/a	No	n/a	n/a	Northern population has highest genetic diversity. Relatively even distribution of haplotypes throughout the range. Coastal population not differentiated

n/a= non aplicable

4.4 Discussion

4.4.1 Is there evidence for geographically discrete genetic lineages?

There is little evidence for phylogeographic structure in this species. Overall, population differentiation is low (Gst = 0.184), and there are no long branches in the minimum spanning tree (Figure 4.8).

There is some geographical structure to the differentiation of populations, such that the average Fst per population is higher in the most northerly and most southerly populations (Maule and Conguillio). These matches with the landscape context of these two populations which are more geographically isolated compared to the intervening populations (see

Figure 4.2). The northern population at Maule also has high gene diversity. Interestingly, this high diversity is not correlated with population size (see Table 4.1). Thus some larger populations elsewhere (e.g. Antuco and Santa Barbara which contain ca. 2,000 individuals) contain less diversity than Maule (which has ca. 300 individuals). This is consistent with long term persistence in the Maule area.

Perhaps the most striking result is the similarity of the coastal population to all others. This is consistent with no long term separation of these populations and recent common ancestry. One possible explanation for this is that long distance pollen transfer has kept these populations genetically similar. However, the prevailing winds are westerly, which would imply an influx of genes from the coastal cordillera to the Andes rather than vice versa as a reason for the genetic similarities. It is noteworthy that pollen connectivity among the Andean populations is less likely as they are arranged on a N-S axis perpendicular to the prevailing winds, and with many mountain barriers between populations.

Coastal populations have a slight similarity with the northern and central populations Maule and Antuco, instead to the southernmost population Conguillio, this same pattern has been shown for the wide distributed *Nothofagus pumilio* (Mathiasen and

Premoli, 2010) and the range restrict *Berberidopsis corallina (*Etisham-Ul-Haq *et al*., 2001), suggesting similar refugia for coastal-northern populations.

4.4.2 Do the observed patterns of genetic diversity and genetic differentiation correlate with previous hypotheses?

The genetically atypical and diverse population in this study is from the Maule valley. This region has an unusual floristic composition and contains about 20 % of the endemic plant species in Chile (Martin Gardner pers. comm.). Another study which has shown genetic differentiation between populations north of ~36°S and those immediately to the south is on *Nothofagus pumilo*, although this difference was less pronounced than differences elsewhere in its range (Mathiasen and Premoli, 2010). No corresponding pattern is found at 36°S in *Nothofagus obliqua* (Azpilicueta *et al*., 2009) although again, the most northerly populations of this species are interesting in that they show the highest diversity of those in the Andes (in the case of *N. obliqua* this diversity is found at ~34°S).

In conclusion, placing these overall findings in the context of post-glacial history is difficult due to the low levels of differentiation. This is likely due to the small spatial scale of the study, and also the use of paternally inherited markers. The lack of detectable divergence of the coastal populations suggests that this region does not contain populations which have been isolated for long time periods. This is consistent with the observation that the species was widespread in the central depression until around 20,000 yr, although to facilitate comparisons with other studies it would be desirable to test this with maternally inherited markers. The highest gene diversity and lowest frequency of the most common haplotype at Maule point to this most northerly population as being a potentially important genetic refuge for this species and in contrast to the coastal population, suggest it has experienced some degree of isolation.

Chapter 5 Summary and Conclusions

5.1 Summary of Findings

This thesis has investigated phylogeographic structure in seven Patagonian plant species. In this chapter I bring together the results from the individual chapters to establish the extent of concordance among studies. To aid in the comparisons, the number of populations per degree of latitude for all seven species are shown in Figure 5.1. The geographic distribution of populations, individuals and private haplotypes are shown in Table 5.1. The distribution of haplotypes and their phylogenetic relationships for all seven species are shown in Figure 5.2. The number of haplotypes per population for all seven species are shown in Figure 5.3, and plotted together in Figure 5.4. The gene diversities of individual populations for all seven species are shown in Figure 5.5, and plotted together in Figure 5.6. The number of private haplotypes per population for all seven species are shown in Figure 5.7, and plotted together in Figure 5.8, and plotted as the standardised number of private haplotypes per degree of latitude in Figure 5.9. The mean pairwise Fst for each population with all other conspecific populations is shown for all seven species in Figure 5.10, and plotted together in Figure 5.11. A summary of haplotype diversity for all seven species is given in Table 5.2 and an overview of phylogeographic patterns in the seven species in Table 5.3.

The clearest signal that arises from this study is the north-south gradient in the number of private haplotypes. This is illustrated clearly in Figure 5.7 which shows that across multiple species, there is a greater tendency for northern populations to have private haplotypes compared with those from further south. If the data is expressed as the standardised number of private haplotypes per populations, averaged over all populations for a given degree of latitude, there is a clear correlation between the number of private haplotypes and latitude ($R^2 = 0.821$, $p<0.01$). This relationship is also robust to standardisation by numbers of individual samples and a similar relationship emerges when this is done (data not shown).

The same picture emerges namely a general decline from north to south (Figure 5.9). Populations in northern coastal regions such as Nahuelbuta, Ruiles, and Andean populations such as Chillan, Quillen and San Fabian show evidence for private haplotypes in multiple species. The absence of this level of divergence among populations in the south is striking. The most obvious conclusion from this, is that the genetic data, in general are supportive of their being long term persistence of these species in northern populations, which isolation among different parts of their ranges, but with southern populations, in general, sharing more recent common ancestry and colonisation. The key transition point between the populations showing the higher number of private haplotypes is around 42-43°S (see Figure 5.9). This is consistent with the presence of a suture zone around 43°S in *Nothofagus pumilio* (Mathiasen and Premoli, 2010) and *Podocarpus nubigena* (Quiroga and Premoli, 2010), and supports the notion that this may be an important biogeographical boundary for multiple species. Mathiasen and Premoli (2010) noted that this suture zone correlated with the presence of an ancient palaeo-basin (the Chubut basin). Using molecular dating, they concluded that *Nothofagus* species had experienced isolation north and south of this zone for something approaching 40 million years. The current study lacks calibration points for applying molecular dating, but there is no clear evidence for such ancient divergence here or indeed any real evidence for divergent northern and southern lineages (e.g. Figure 5.2).

There are some cases where haplotypes are detected in the south that are not found further north (Figure 5.2). There are two sites close together (Lago Desierto and Piedra del Fraile) in *Escallonia virgata* which share a unique haplotype, and two plants with a private haplotype from Lago Roca in *Discaria chacaye* (just to the south of the above sites). In the latter case, this could be a sampling artefact, but in the case of *Escallonia virgata,* the consistent pattern of uniform haplotypes in other populations does makes these two southern populations stand out, and these haplotypes could be indicative of a different history to these southern populations. Likewise, in *Gentianella magellanica* – one could argue that the southern cluster (‘clade’ 4 in Figure 5.2) is evidence for a southerly refugium and a northwards colonisation, but this is not clear cut, as within this 'clade', there are private haplotypes (E,F) in the north of the distribution.

My favoured summary explanation for the results in this thesis, is that north of 43°S there has been long term survival of populations of these species, and that in many cases, just subsets of the diversity in the northern populations migrated southwards. This fits with reconstructions of the maximum extent of the ice sheets during past glaciations, which inferred that the northernmost presence limit of continuous ice sheets was around this point (ca. 42-43°S, Glasser *et al.,* 2008). It also correlates with reconstructions of past weather conditions which predict a northerly shift of westerly winds, which led to the area south of 42°S being much drier during the last glacial maximum, than it is today (Manzini *et al.,* 2008; Moreno, 1997), and hence less suitable for plant growth. I conclude that the evidence for southern refugia is weaker, and although possible in species like *Gentianella magellanica* and *Escallonia virgata*, it appears to be the exception rather than the rule.

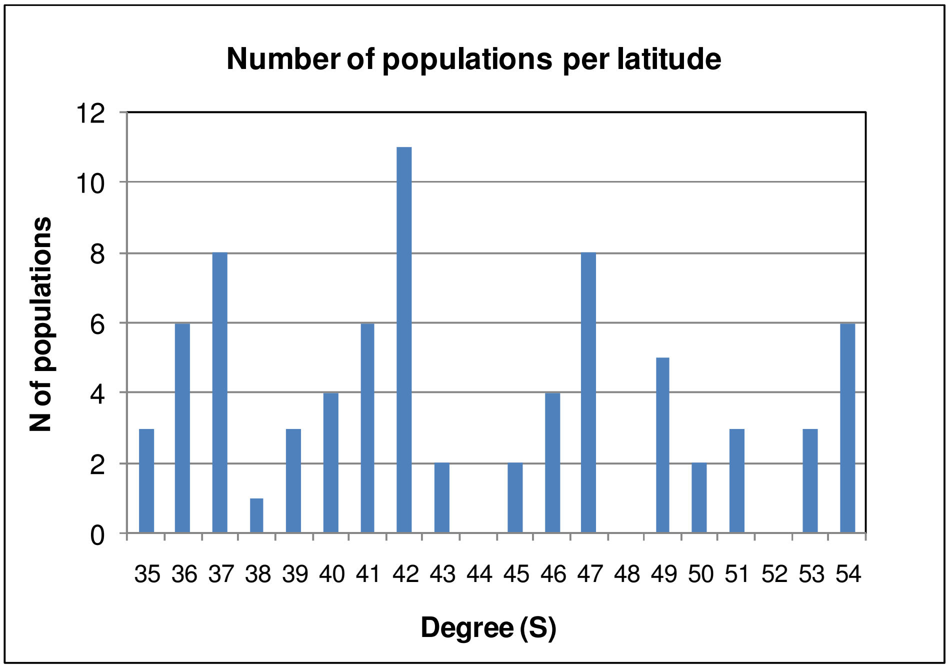


Figure 5.1. Latitudinal population distributions of seven Patagonian plant species.

Table 5.1. Geographic distribution of populations, individuals and private haplotypes in seven Patagonian plant species, including totals

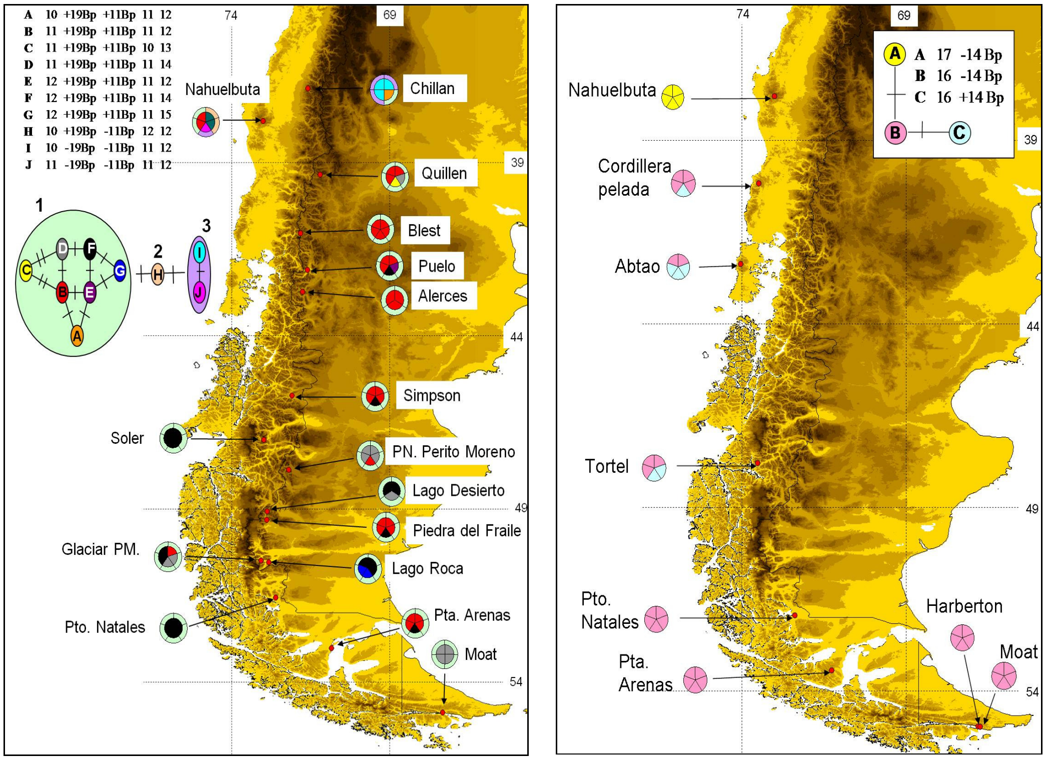
Latitude (S)	***Discaria chacaye***				***Donatia fascicularis***				***Escallonia virgata***				***Gentianella magellanica***			
	N pop	N priv	N priv/N pop	N ind	N pop	N priv	N priv/N pop	N ind	N pop	N priv	N priv/N pop	N ind	N pop	N priv	N priv/N pop	N ind
35																
36	1	2	2	4					1	1	1	5	1	1	1	4
37	1	2	2	5	1	1	1	5	1	0	0	5	1	2	2	5
38																
39	1	1	1	5					1	1	1	5				
40					1	0	0	5					1	1	1	5
41	1	0	0	5					1	0	0	5	1	2	2	5
42	2	1	0.5	8	1	0	0	5	1	0	0	5	1	1	1	5
43																
44																
45	1	0	0	5					1	0	0	5				
46									1	0	0	5				
47	2	0	0	10	1	0	0	5	2	0	0	8	2	0	0	9
48																
49	2	0	0	8					2	1	0.5	10	1	0	0	5
50	2	0	0	10												
51	1	0	0	5	1	0	0	5	1	0	0	5				
52																
53	1	0	0	5	1	0	0	5					1	0	0	4
54	1	0	0	4	2	0	0	10					3	0	0	15

N pop = number of populations; N priv = number of private haplotype

Table 5.1. Continued

Latitude (S)	***Prumnopitys andina***				***Tepualia stipularis***				***Weinmannia trichosperma***				**Totals**			
	N pop	N priv	N priv/N pop	N ind	N pop	N priv	N priv/N pop	N ind	N pop	N priv	N priv/N pop	N ind	**N pop**	**N priv**	**N priv/N pop**	**N ind**
35	1	1	1	15	1	1	1	5	1	1	1	1	**3**	**3**	**1**	**21**
36	2	1	0.5	30	1	2	2	5					**6**	**7**	**1.167**	**48**
37	3	0	0	45					1	1	1	5	**8**	**6**	**0.75**	**70**
38	1	1	1	15									**1**	**1**	**1**	**15**
39									1	1	1	5	**3**	**3**	**1**	**15**
40					1	1	1	5	1	1	1	5	**4**	**3**	**0.75**	**20**
41					1	0	0	5	2	0	0	9	**6**	**2**	**0.333**	**29**
42					3	0	0	14	3	2	0.67	13	**11**	**4**	**0.364**	**50**
43					1	1	1	5	1	0	0	5	**2**	**1**	**0.5**	**10**
44																
45													**2**	**0**	**0**	**10**
46					1	0	0	5	1	0	0	4	**3**	**0**	**0**	**14**
47					1	0	0	5	1	0	0	5	**9**	**0**	**0**	**42**
48																
49													**5**	**1**	**0.2**	**23**
50													**2**	**0**	**0**	**10**
51													**3**	**0**	**0**	**15**
52																
53													**3**	**0**	**0**	**14**
54													**6**	**0**	**0**	**29**

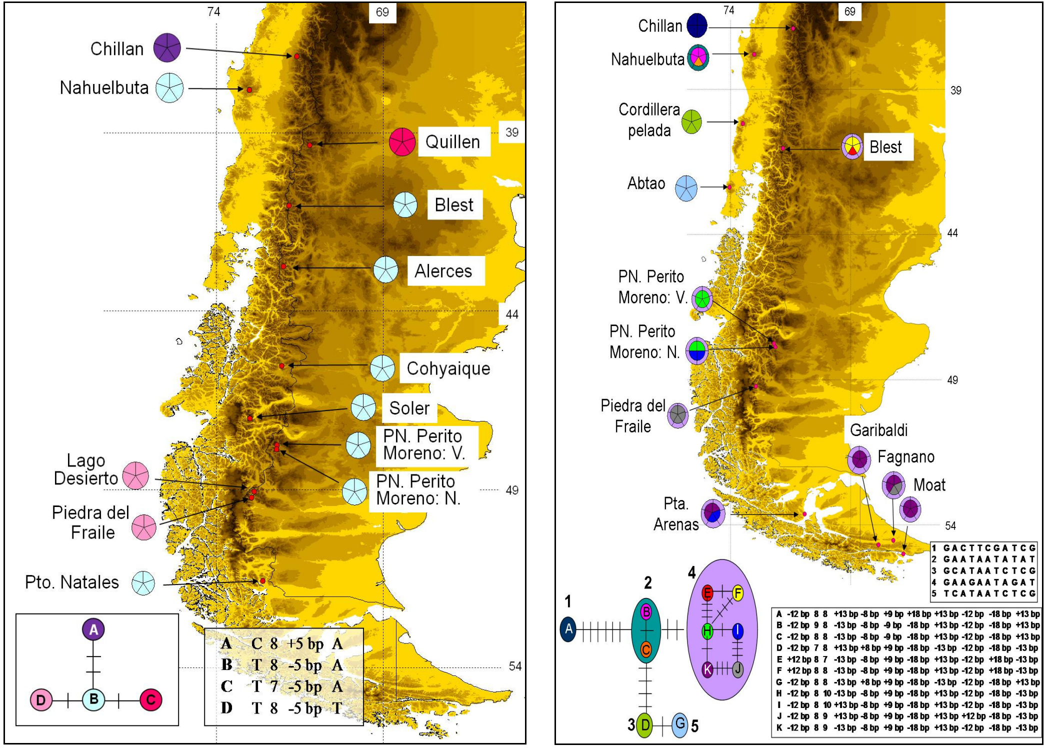
N pop = number of populations; N priv = number of private haplotype



A	10	+19Bp	+11Bp	11	12
B	11	+19Bp	+11Bp	11	12
C	11	+19Bp	+11Bp	10	13
D	11	+19Bp	+11Bp	11	14
E	12	+19Bp	+11Bp	11	12
F	12	+19Bp	+11Bp	11	14
G	12	+19Bp	+11Bp	11	15
H	10	+19Bp	-11Bp	12	12
I	10	-19Bp	-11Bp	11	12
J	11	-19Bp	-11Bp	11	12

A	17	-14 Bp
B	16	-14 Bp
C	16	+14 Bp

D. chacaye *D. fascicularis*



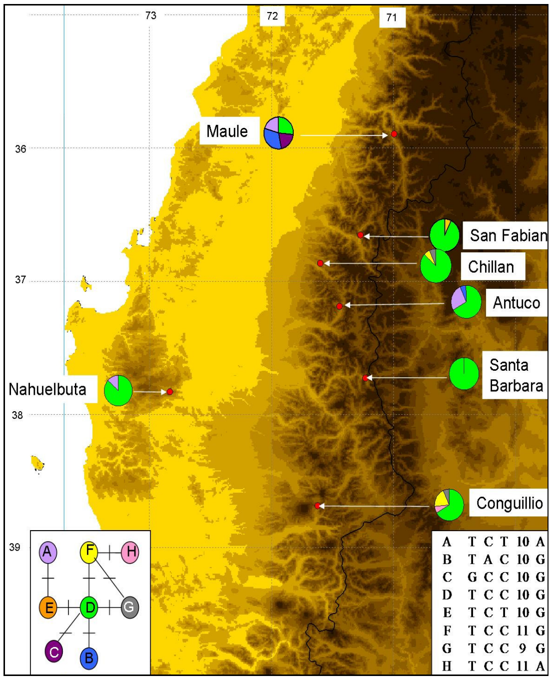
A	C	8	+5 bp	A
B	T	8	-5 bp	A
C	T	7	-5 bp	A
D	T	8	-5 bp	T

1	G	A	C	T	T	C	G	A	T	C	G
2	G	A	A	T	A	A	T	A	T	A	T
3	G	C	A	T	A	A	T	C	T	C	G
4	G	A	A	G	A	A	T	A	G	A	T
5	T	C	A	T	A	A	T	C	T	C	G

A	-12 bp	8	8	+13 bp	-8 bp	+9 bp	+18 bp	+13 bp	-12 bp	-18 bp	+13 bp
B	-12 bp	9	8	-13 bp	-8 bp	-9 bp	-18 bp	+13 bp	-12 bp	-18 bp	+13 bp
C	-12 bp	8	8	-13 bp	-8 bp	-9 bp	-18 bp	+13 bp	-12 bp	-18 bp	+13 bp
D	-12 bp	7	8	+13 bp	+8 bp	+9 bp	-18 bp	-13 bp	-12 bp	-18 bp	-13 bp
E	+12 bp	8	7	-13 bp	-8 bp	+9 bp	-18 bp	+13 bp	-12 bp	+18 bp	-13 bp
F	+12 bp	8	8	-13 bp	-8 bp	+9 bp	-18 bp	+13 bp	-12 bp	+18 bp	-13 bp
G	-12 bp	8	8	-13 bp	+8 bp	+9 bp	-18 bp	-13 bp	-12 bp	-18 bp	+13 bp
H	-12 bp	8	10	-13 bp	-8 bp	+9 bp	-18 bp	+13 bp	-12 bp	-18 bp	-13 bp
I	-12 bp	8	10	+13 bp	-8 bp	+9 bp	-18 bp	+13 bp	-12 bp	-18 bp	-13 bp
J	-12 bp	8	9	+13 bp	-8 bp	+9 bp	-18 bp	+13 bp	+12 bp	-18 bp	-13 bp
K	-12 bp	8	9	-13 bp	-8 bp	+9 bp	-18 bp	+13 bp	-12 bp	-18 bp	-13 bp

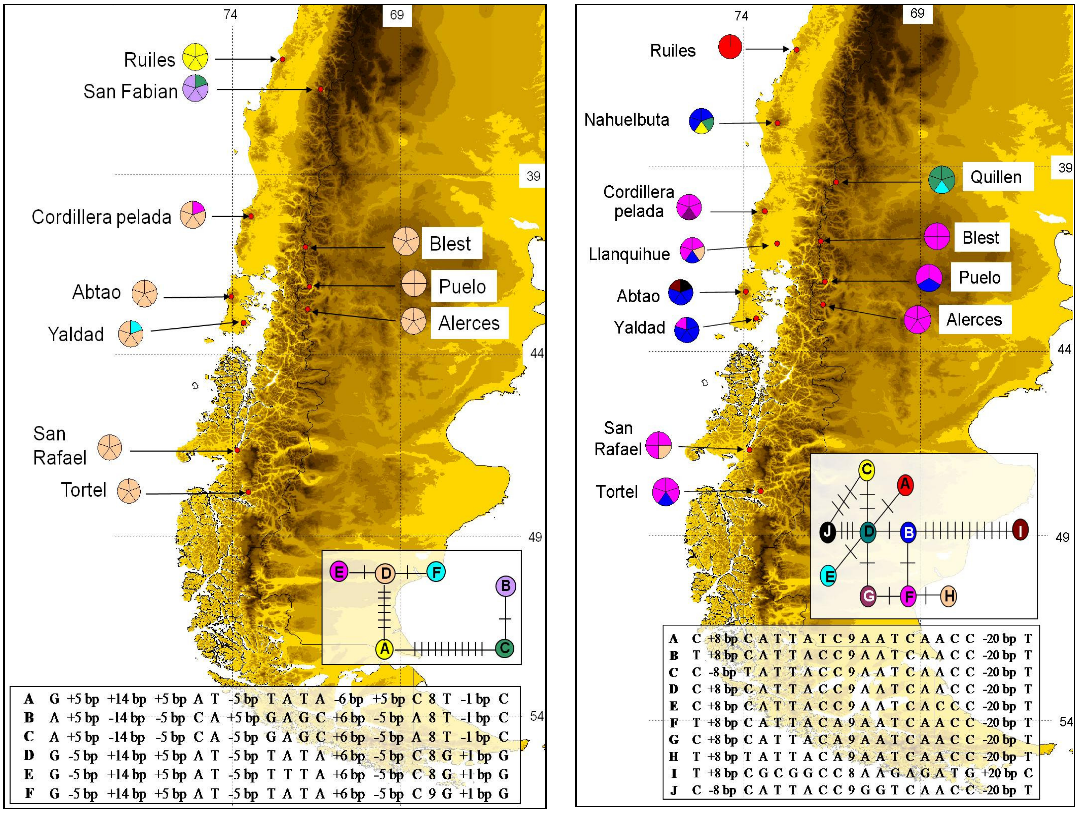
E. virgata G. magellanica

Figure 5.2. The distribution and relationships of chloroplast haplotypes in seven Patagonian plant species.



A	T	C	T	10	A
B	T	A	C	10	G
C	G	C	C	10	G
D	T	C	C	10	G
E	T	C	T	10	G
F	T	C	C	11	G
G	T	C	C	9	G
H	T	C	C	11	A

P. andina



A	G	+5 bp	+14 bp	+5 bp	A	T	-5 bp	T	A	T	A	-6 bp	+5 bp	C	8	T	-1 bp	C
B	A	+5 bp	-14 bp	-5 bp	C	A	+5 bp	G	A	G	C	+6 bp	-5 bp	A	8	T	-1 bp	C
C	A	+5 bp	-14 bp	-5 bp	C	A	-5 bp	G	A	G	C	+6 bp	-5 bp	A	8	T	-1 bp	C
D	G	-5 bp	+14 bp	+5 bp	A	T	-5 bp	T	A	T	A	+6 bp	-5 bp	C	8	G	+1 bp	G
E	G	-5 bp	+14 bp	+5 bp	A	T	-5 bp	T	T	T	A	+6 bp	-5 bp	C	8	G	+1 bp	G
F	G	-5 bp	+14 bp	+5 bp	A	T	-5 bp	T	A	T	A	+6 bp	-5 bp	C	9	G	+1 bp	G

A	C	+8 bp	C	A	T	T	A	T	C	9	A	A	T	C	A	A	C	C	-20 bp	T
B	T	+8 bp	C	A	T	T	A	C	C	9	A	A	T	C	A	A	C	C	-20 bp	T
C	C	-8 bp	T	A	T	T	A	C	C	9	A	A	T	C	A	A	C	C	-20 bp	T
D	C	+8 bp	C	A	T	T	A	C	C	9	A	A	T	C	A	A	C	C	-20 bp	T
E	C	+8 bp	C	A	T	T	A	C	C	9	A	A	T	C	A	C	C	C	-20 bp	T
F	T	+8 bp	C	A	T	T	A	C	A	9	A	A	T	C	A	A	C	C	-20 bp	T
G	C	+8 bp	C	A	T	T	A	C	A	9	A	A	T	C	A	A	C	C	-20 bp	T
H	T	+8 bp	T	A	T	T	A	C	A	9	A	A	T	C	A	A	C	C	-20 bp	T
I	T	+8 bp	C	G	C	G	G	C	C	8	A	A	G	A	G	A	T	G	+20 bp	C
J	C	-8 bp	C	A	T	T	A	C	C	9	G	G	T	C	A	A	C	C	-20 bp	T

T. stipularis

W. trichosperma

Figure 5.2. Continued.

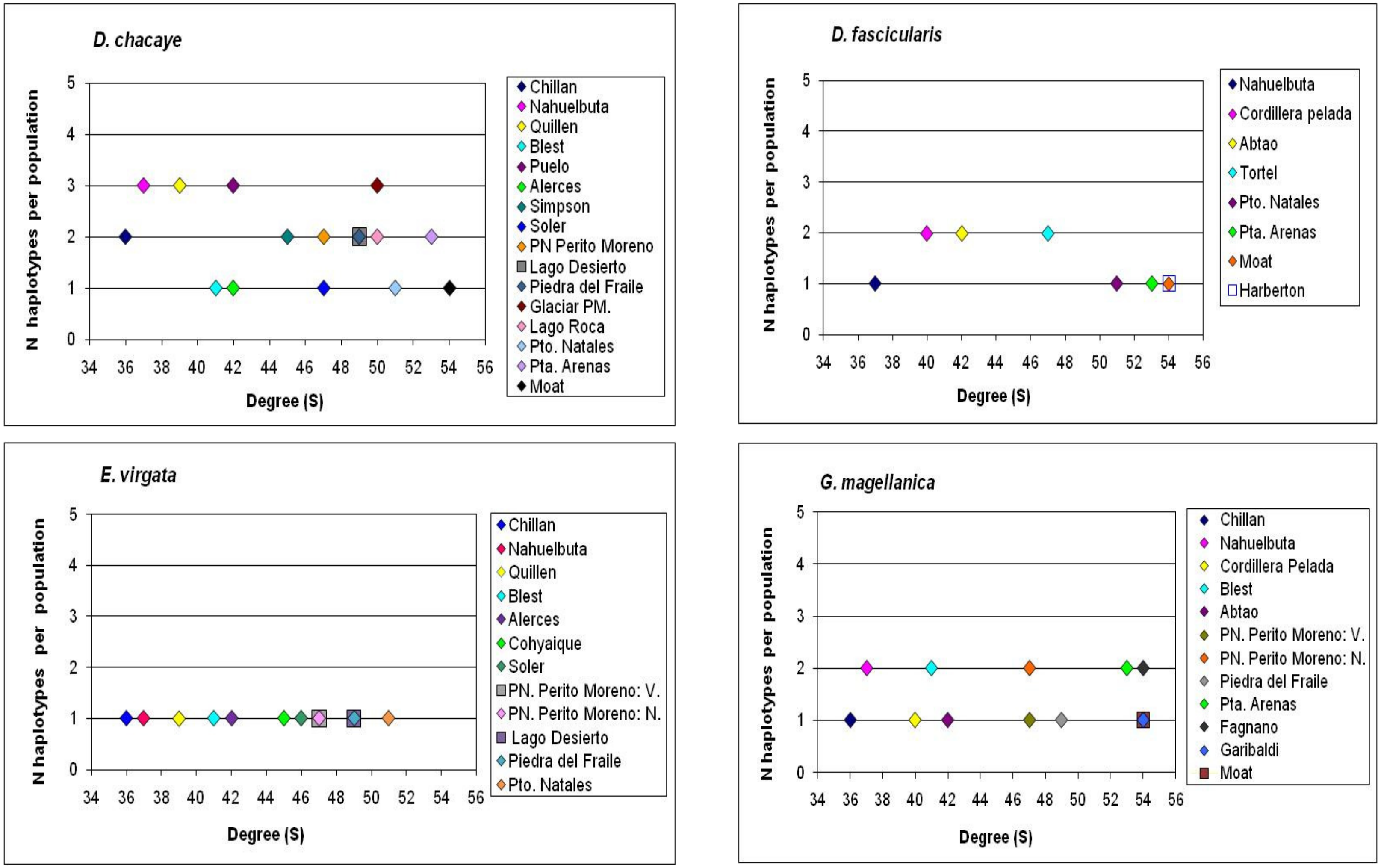


Figure 5.3. Number of haplotypes in each population of seven Patagonian plant species, plotted against latitude.

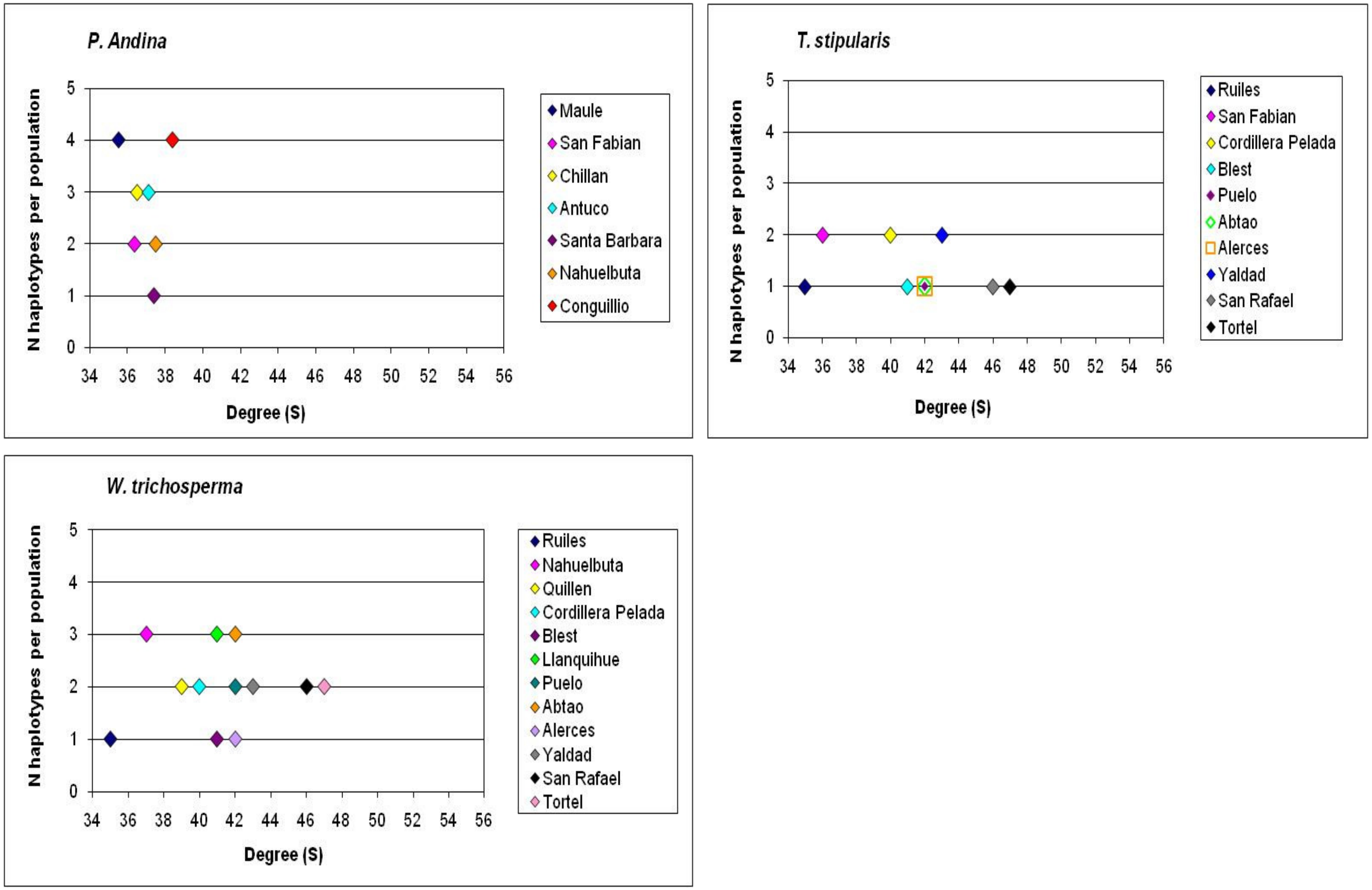


Figure 5.3. Continued

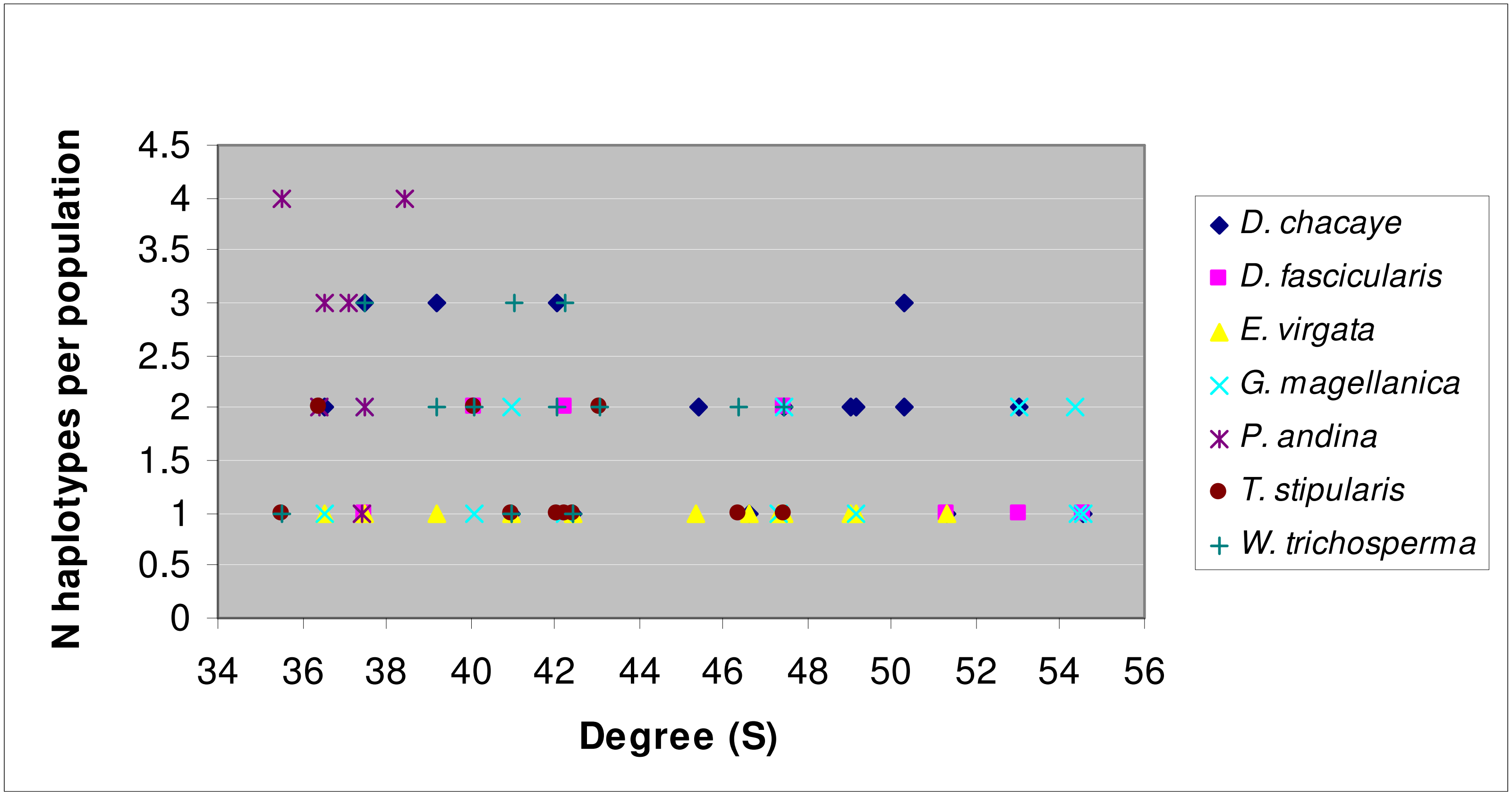


Figure 5.4. Number of haplotypes in each population of seven Patagonian plant species (plotted together) against latitude

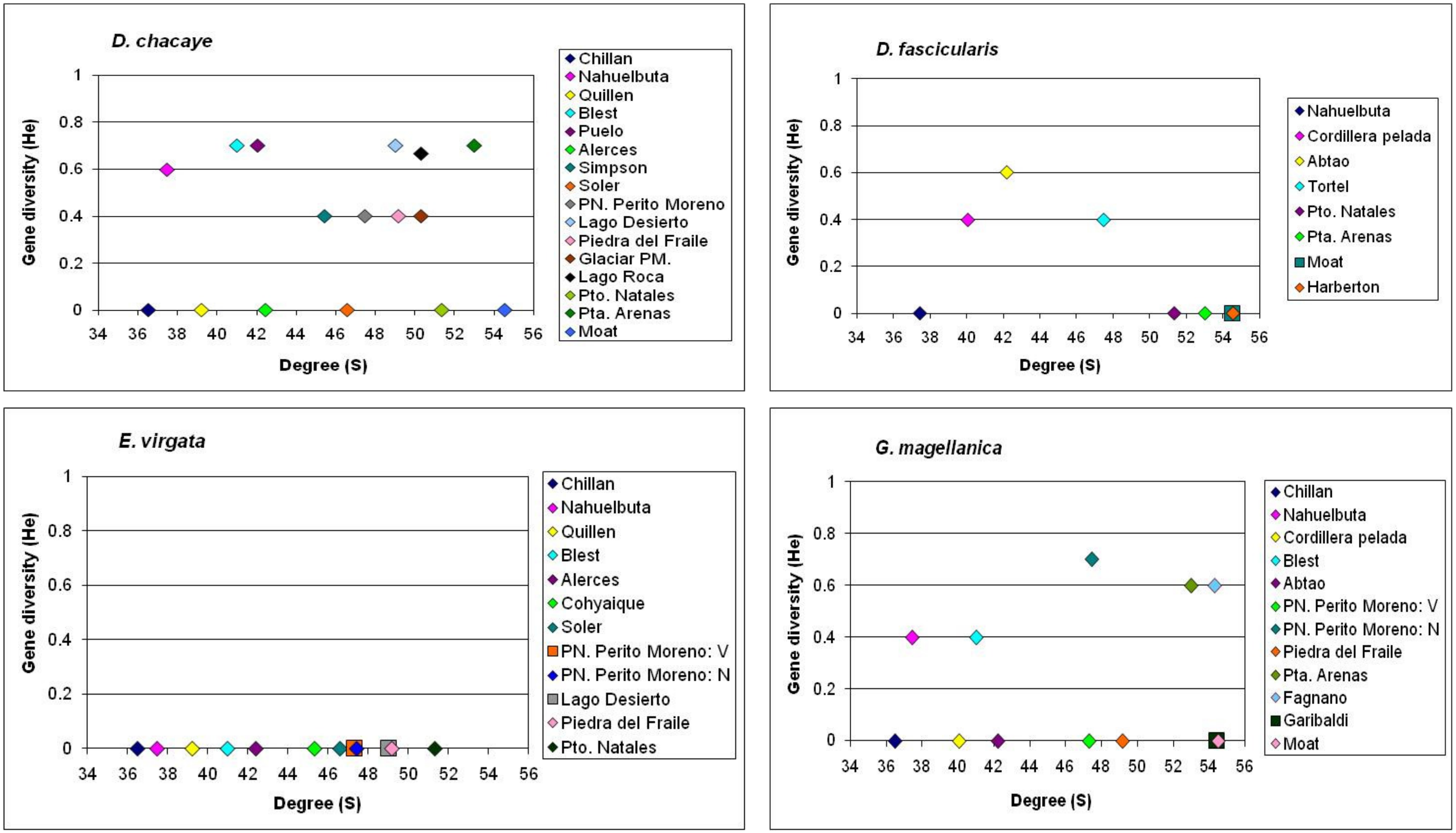


Figure 5.5. Gene diversity in each population of seven Patagonian plant species, plotted against latitude.

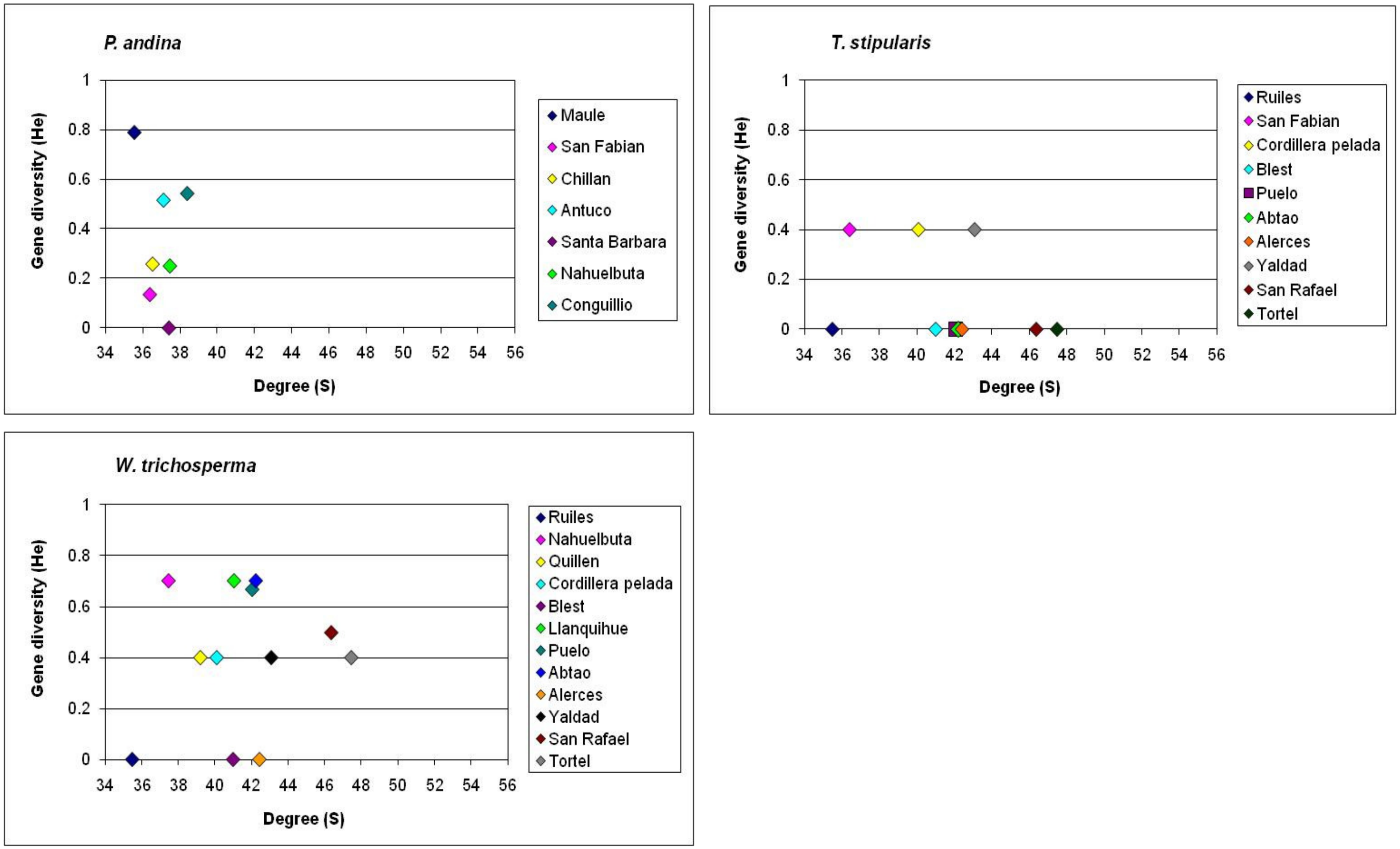


Figure 5.5. Continued.

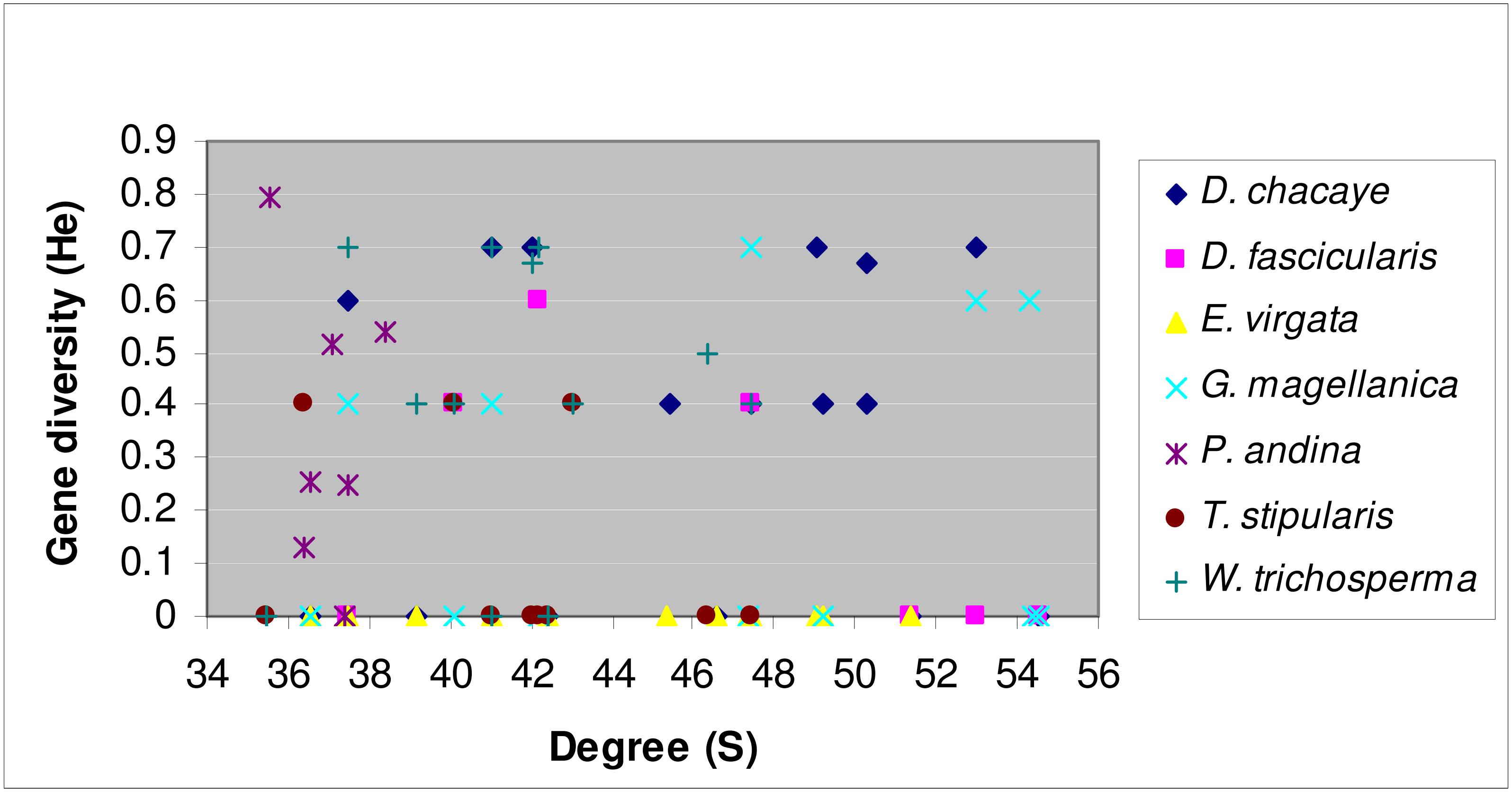


Figure 5.6. Gene diversity in each population of seven Patagonian plant species (plotted together) against latitude.

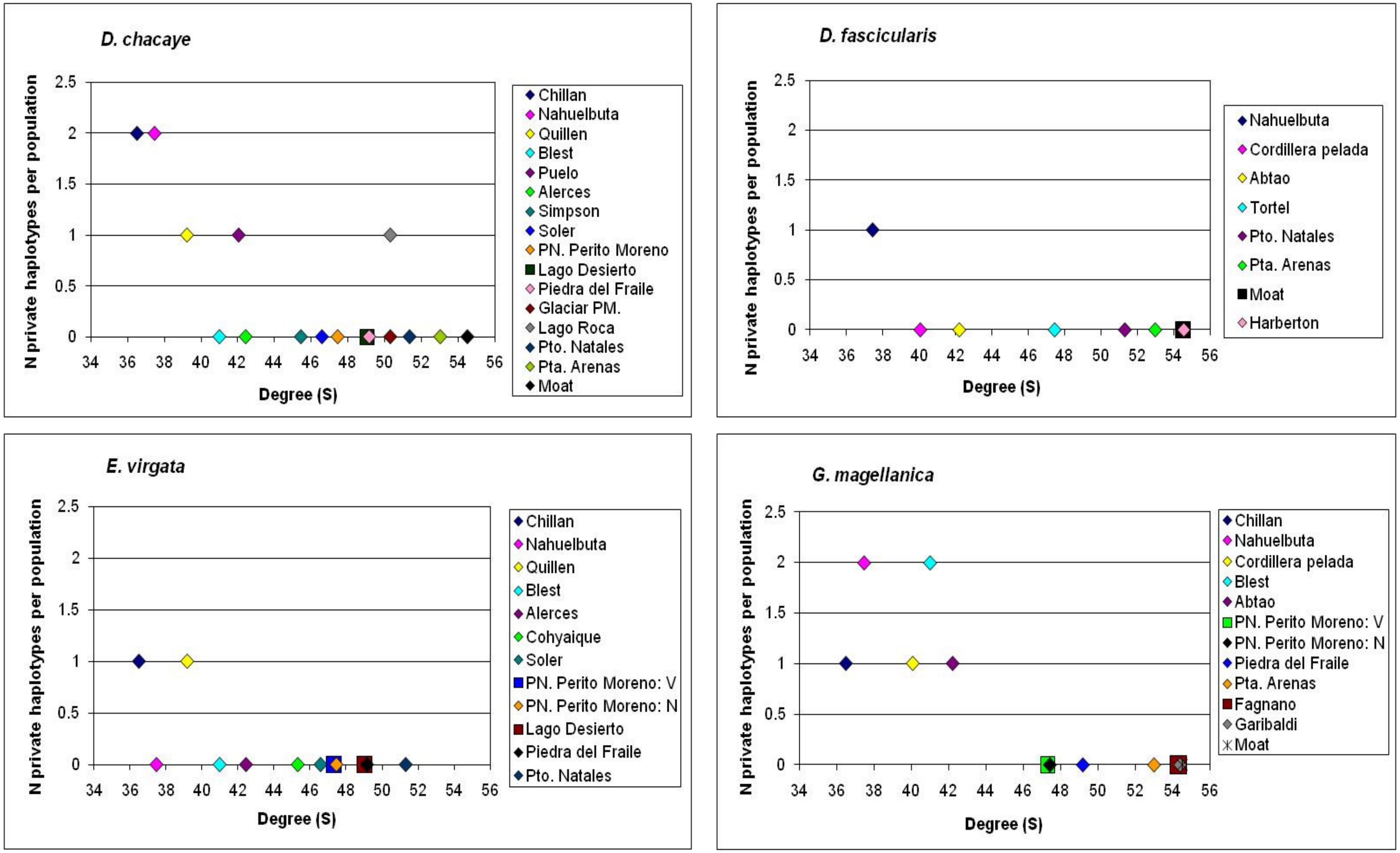
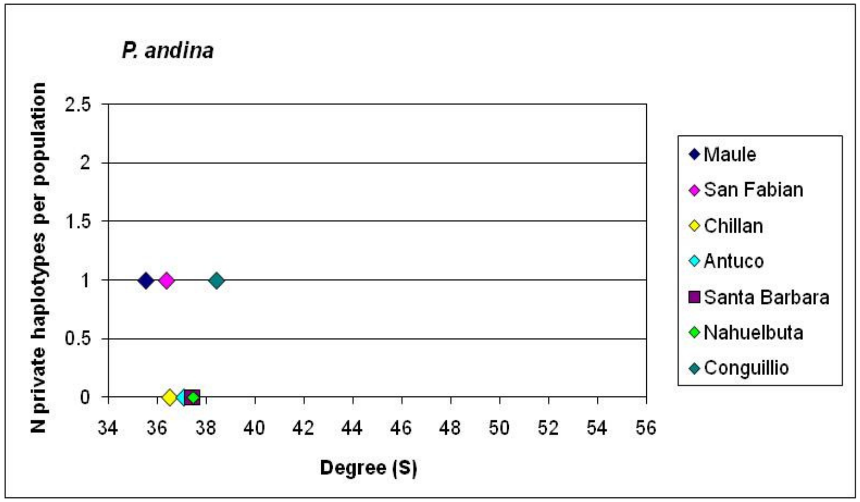
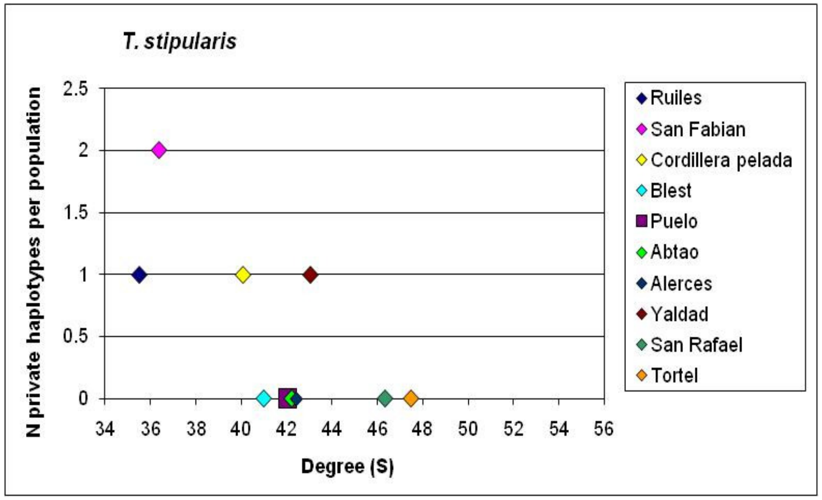


Figure 5.7. Number of private haplotypes in each population of seven Patagonian plant species, plotted against latitude.


P. andina
N private haplotypes per population
2.5
2
1.5
1
0.5
0
34
36
38
40
42
44
46
48
50
52
54
56
Degree (S)
Maule
San Fabian
Chillan
Antuco
Santa Barbara
Nahuelbuta
Conguillio


T. stipularis
N private haplotypes per population
2.5
2
1.5
1
0.5
0
34
36
38
40
42
44
46
48
50
52
54
56
Degree (S)
Ruiles
San Fabian
Cordillera pelada
Blest
Puelo
Abtao
Alerces
Yaldad
San Rafael
Tortel

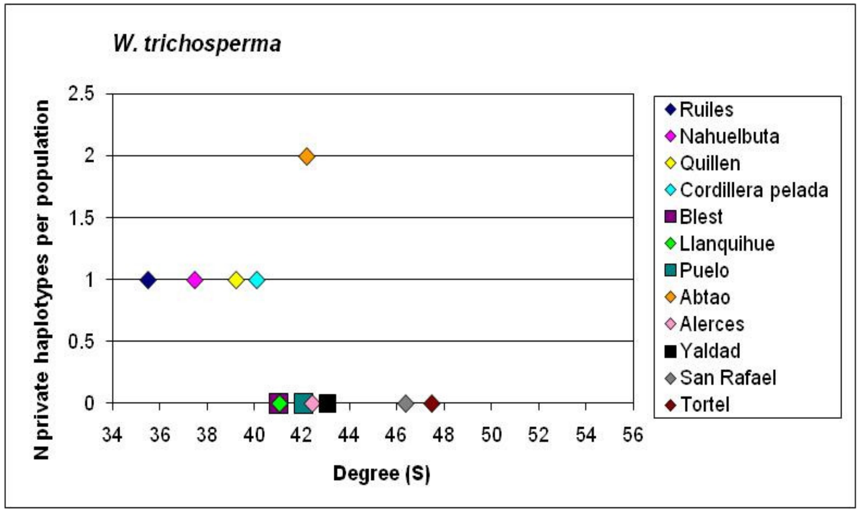

W. trichosperma
N private haplotypes per population
2.5
2
1.5
1
0.5
0
34
36
38
40
42
44
46
48
50
52
54
56
Degree (S)
Ruiles
Nahuelbuta
Quillen
Cordillera pelada
Blest
Llanquihue
Puelo
Abtao
Alerces
Yaldad
San Rafael
Tortel

Figure 5.7. Continued.

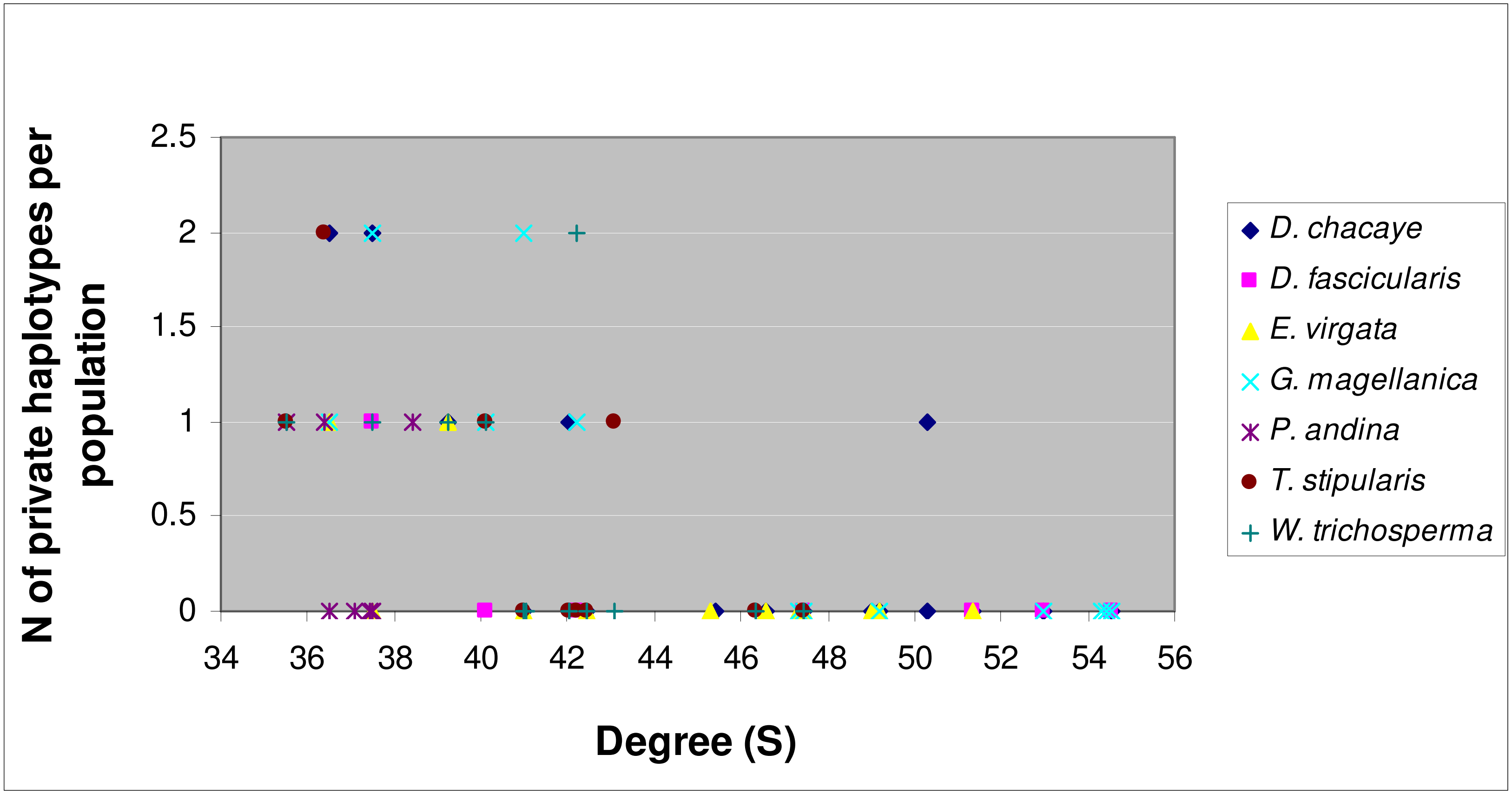


Figure 5.8. Number of private haplotypes in each population of seven Patagonian plant species (plotted together) against latitude.

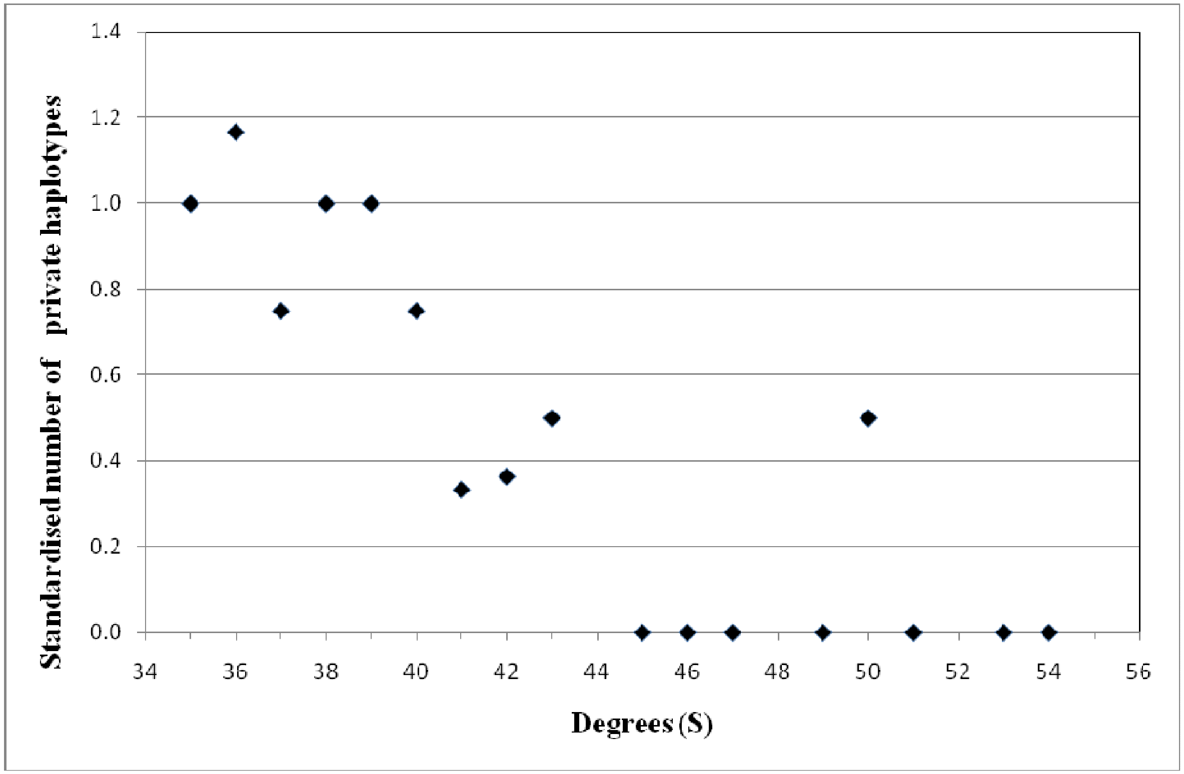


Figure 5.9. Number of private haplotypes per population, plotted by degree of latitude and standardised by the number of populations sampled at each degree of latitude (e.g. total number of private haplotypes summed across all populations for each latitudinal degree, divided by the total number of sampled populations for each latiduinal degree).

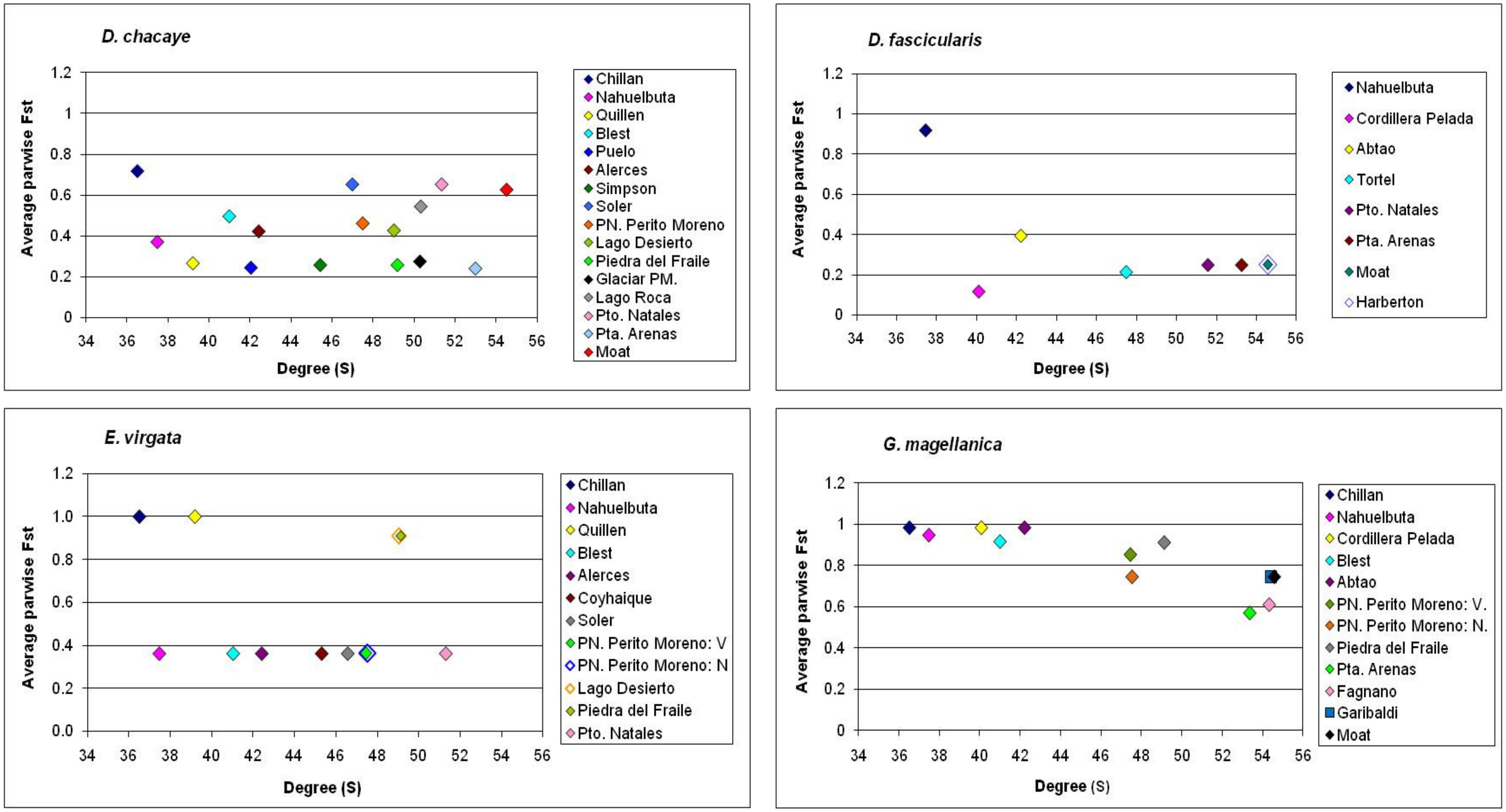


Figure 5.10. The average pairwise Fst value for each population (against all other conspecific populations) for seven Patagonian plant species plotted against latitude.

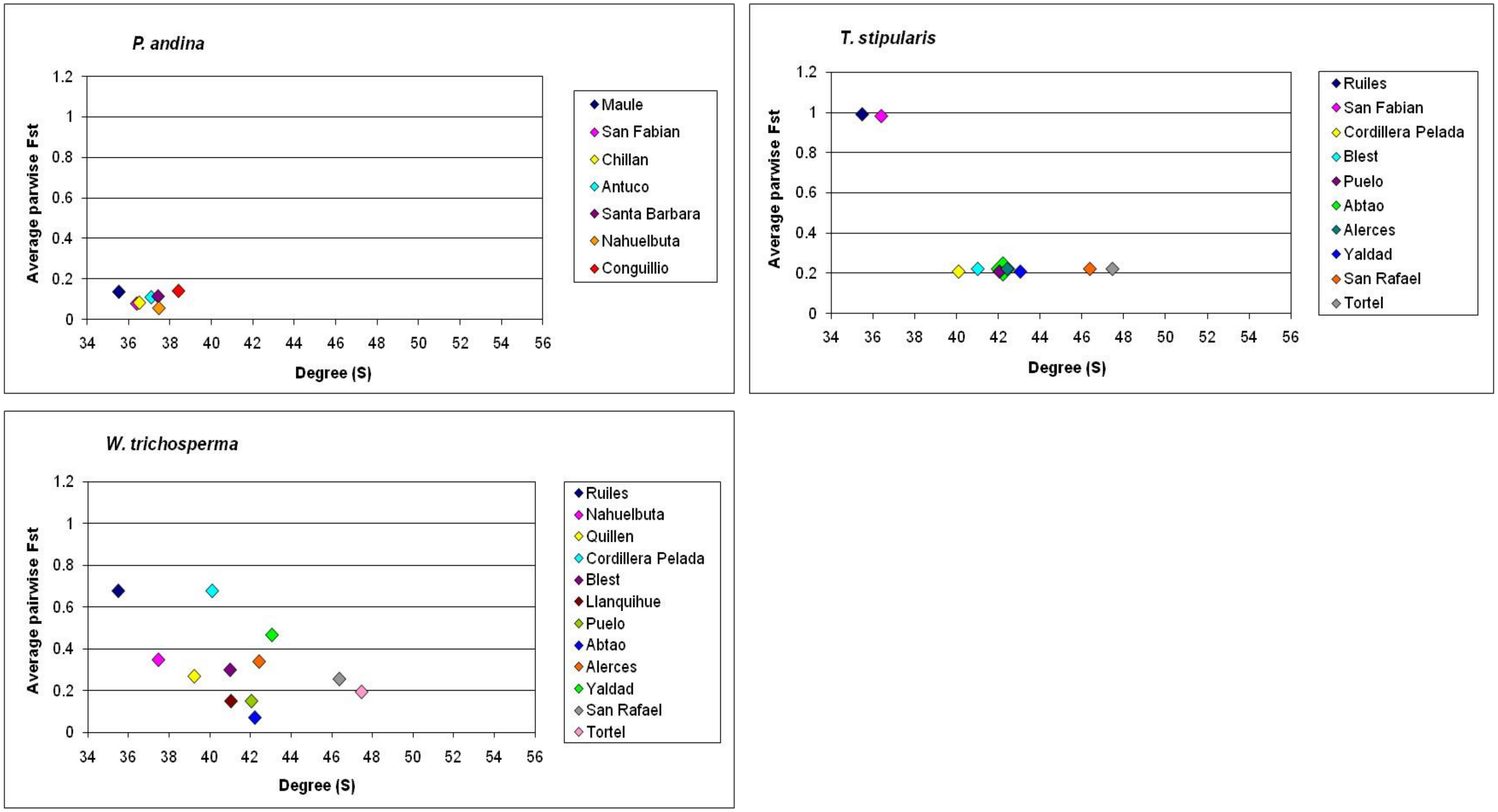


Figure 5.10. Continued.

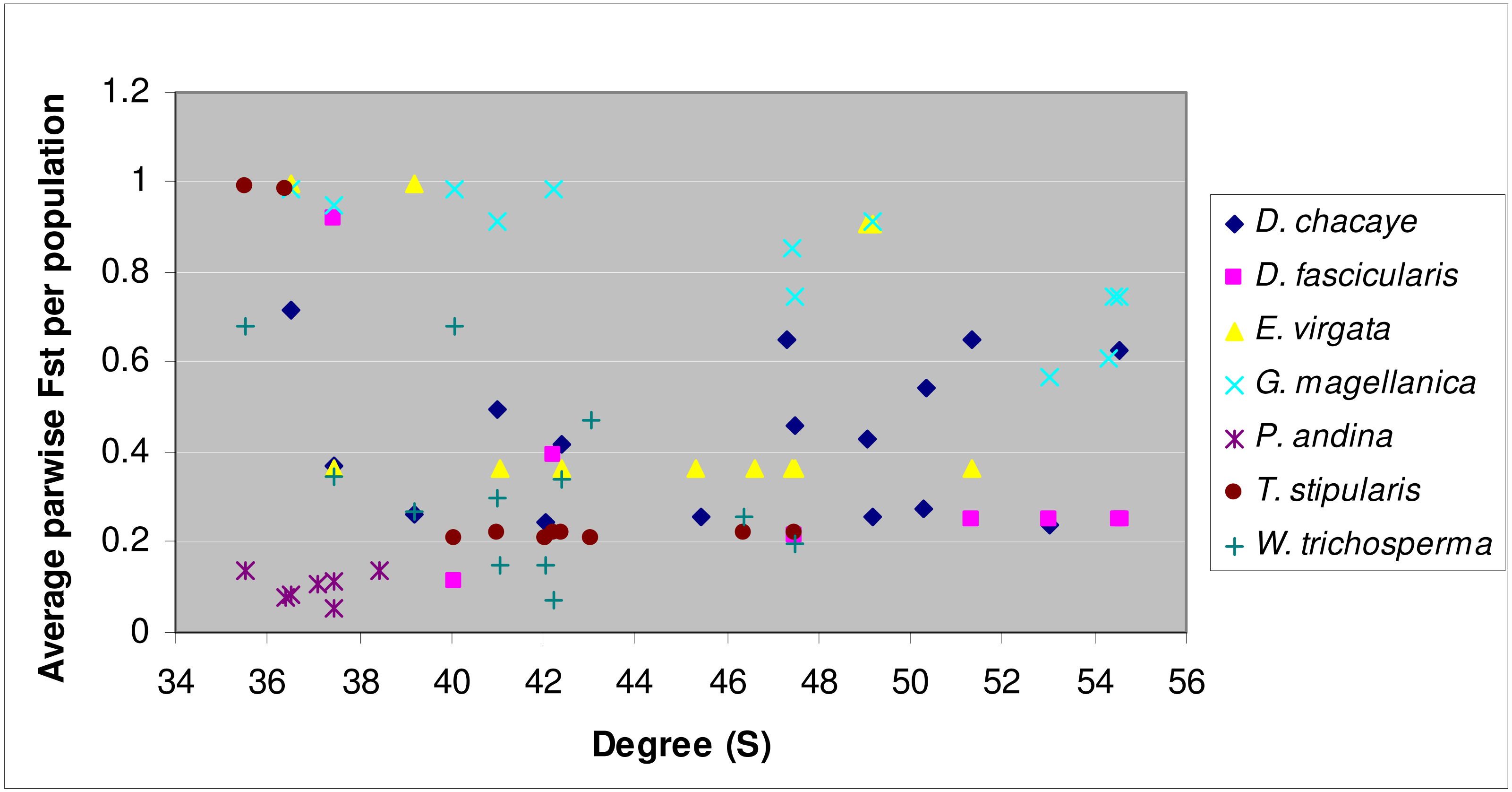


Figure 5.11. The average pairwise Fst value for each population (against all other conspecific populations) for seven Patagonian plant species (plotted together) against latitude.

Table 5.2. Summary of haplotype diversity in populations of seven Patagonian plant species. No. Species = number of species sampled from each site. N = sample size per species, H = number of haplotypes, P = number of private haplotypes.

Area	**Pop.**	**Lat. (S)**	**Long. (W)**	**No. species**	***D. chacaye***			***D. fascicularis***			***E. virgata***			***G. magellanica***			***P. andina***			***T. stipularis***			***W. trichosperma***			**All species**	
					N	H	P	N	H	P	N	H	P	N	H	P	N	H	P	N	H	P	N	H	P	Mean H	Mean P
North coastal	Ruiles	35°49'	72°30'	2																5	1	1	1	1	1	1.0	1.0
North Andes “W”	Maule	35°53'	70°59'	1													15	4	1							4.0	1.0
North Andes “W”	San Fabian	36°39'	71°22'	2													15	2	1	5	2	2				2.0	1.5
North Andes “W”	Chillan	36°51'	71°35'	4	4	2	2				5	1	1	4	1	1	15	3	0							1.8	1.0
North Andes “W”	Antuco	37°11'	71°26'	1													15	3	0							3.0	0.0
North Andes “W”	Santa Barbara	37°43'	71°13'	1													15	1	0							1.0	0.0
North coastal	Nahuelbuta	37°47'	73°00'	6	5	3	2	5	1	1	5	1	0	5	2	2	15	2	0				5	3	1	2.0	1.0

Table 5.2. Continued

Area	**Pop.**	**Lat. (S)**	**Long. (W)**	**No. species**	***D. chacaye***			***D. fascicularis***			***E. Virgata***			***G. magellanica***			***P. andina***			***T. stipularis***			***W. trichosperma***			**All species**	
					N	H	P	N	H	P	N	H	P	N	H	P	N	H	P	N	H	P	N	H	P	Mean H	Mean P
North Andes "E"	Conguillio	38°41'	71°37'	1													15	4	1							4.0	1.0
North Andes "E"	Quillen	39°21'	71°12'	3	5	3	1				5	1	1										5	2	1	2.0	1.0
Central coastal	Cordillera pelada	40°09'	73°25'	4				5	2	0				5	1	1				5	2	1	5	2	1	1.8	0.8
Central Andes "E"	Blest	41°00'	71°48'	5	5	1	0				5	1	0	5	2	2				5	1	0	4	1	0	1.2	0.4
Central valley	Llanquihue	41°04'	73°03'	1																			5	3	0	3.0	0.0
Central Andes "E"	Puelo	42°06'	71°36'	3	5	3	1													4	1	0	3	2	0	2.0	0.3
Central coastal	Abtao	42°22'	73°57'	4				5	2	0				5	1	1				5	1	0	5	3	2	1.8	0.8
Central Andes "E"	Alerces	42°43'	71°45'	4	3	1	0				5	1	0							5	1	0	5	1	0	1.0	0.0
Central coastal	Yaldad	43°06'	73°39'	2																5	2	1	5	2	0	2.0	0.5
South Andes "E"	Cohyaique	45°32'	72°02'	1							5	1	0													1.0	0.0

Central valley= Central depression.

Table 5.2. Continued

Area	**Pop.**	**Lat. (S)**	**Long. (W)**	**No. species**	***D. chacaye***			***D. fascicularis***			***E. virgata***			***G. magellanica***			***P. andina***			***T. stipularis***			***W. trichosperma***			**All species**	
					N	**H**	**P**	**N**	**H**	**P**	**N**	**H**	**P**	**N**	**H**	**P**	**N**	**H**	**P**	**N**	**H**	**P**	**N**	**H**	**P**	**Mean H**	**Mean P**
South Andes “E”	Simpson	45°43'	72°05'	1	5	2	0																			2.0	0.0
South Andes “W”	San Rafael	46°36'	73°51'	2																5	1	0	4	2	0	1.5	0.0
South Andes “E”	Soler	46°59'	72°59'	2	5	1	0				5	1	0													1.0	0.0
South Andes “E”	PN. Perito Moreno: V.	47°32'	72°10'	2							5	1	0	5	1	0										1.0	0.0
South Andes “E”	PN. Perito Moreno: N.	47°45'	72°12'	3	5	2	0				3	1	0	4	2	0										1.7	0.0
South Andes “W”	Tortel	47°47'	73°31'	3				5	2	0										5	1	0	5	2	0	1.7	0.0
South Andes “E”	Lago Desierto	49°03'	72°51'	2	3	2	0				5	1	0													1.5	0.0
South Andes “E	Piedra del Fraile	49°18'	72°54'	3	5	2	0				5	1	0	5	1	0										1.3	0.0

Table 5.2. Continued

Area	**Pop.**	**Lat. (S)**	**Long. (W)**	**No. species**	***D. chacaye***			***D. fascicularis***			***E. virgata***			***G. magellanica***			***P. andina***			***T. stipularis***			***W. trichosperma***			**All species**	
					N	**H**	**P**	**N**	**H**	**P**	**N**	**H**	**P**	**N**	**H**	**P**	**N**	**H**	**P**	**N**	**H**	**P**	**N**	**H**	**P**	**Mean H**	**Mean P**
South Andes "E"	Glaciar PM.	50°29'		1	5	3	0																			3.0	0.0
South Andes "E"	Lago Roca	50°32'	72°49'	1	5	2	1																			2.0	1.0
South Andes "E"	Pto. Natales	51°33'	72°33’	3	5	1	0	5	1	0	5	1	0													1.0	0.0
Far South	Pta. Arenas	53°00'	70°50'	3	5	2	0	5	1	0				4	2	0										1.7	0.0
Far South	Fagnano	54°32'	67°14'	1										5	2	0										2.0	0.0
Far South	Garibaldi	54°41'	67°50'	1										5	1	0										1.0	0.0
Far South	Moat	54°52'	67°44'	3	4	1	0	5	1	0				5	1	0										1.0	0.0
Far South	Harberton	54°57'		1				5	1	0																1.0	0.0

Table 5.3. Summary of the phylogeographic structure of seven Patagonian plant species.

Species	Distribution	No. haplotypes	Phylogenetic divergence among haplotypes?	High diversity in part of range?	Unique northern haplotypes?	Unique central haplotypes?	Unique southern haplotypes?	Unique far southern haplotypes?	Comments
Disacaria chacaye	North -Far South (Andes and coastal)	10	minimal	Yes	Yes (Andes and coastal)	Yes (Andes, but rare)	Yes (Andes but rare)	No	North and coastal populations encompass most diversity of haplotypes. Relatively even distribution of haplotypes throughout rest of the range
Donatia fascicularis	North -Far South (coastal and south Andes only)	3	No	Yes (ish)	Yes (in the coastal range)	No (but one haplotype restricted to central-southern regions)	No (but one haplotype restricted to central-southern regions)	No	Far south uniform, most northerly coastal population is unique, polymorphism in central/southern coastal populations.
Escallonia virgata	North - South (Andes and coastal)	4	No	No	Yes (in the Andes)	No	Yes	n/a	No intra-population variation, both northern Andes populations have unique haplotypes, two neighbouring populations in south share a unique haplotype

Table 5.3. Continued

Species	Distribution	No. haplotypes	Phylogenetic divergence among haplotypes?	High diversity in part of range?	Unique northern haplotypes?	Unique central haplotypes?	Unique southern haplotypes?	Unique far southern haplotypes?	Comments
Prumnopitys andina	North Andes and coast	8	No	Yes (northern most population)	n/a	No	n/a	n/a	Northern population has highest genetic diversity. Relatively even distribution of haplotypes throughout the range. Coastal population not differentiated
Gentianella magellanica	North- Far South (Andes and coastal)	11	Yes	Yes	Yes (coast and Andes)	Yes (Andes and coast)	Yes	Yes	North and coastal populations are most divergent. All other populations contain a more closely related set of haplotypes.
Tepualia stipularis	North-South (Andes and coastal)	6	Yes	Yes	Yes (Andes and coastal)	Rare variants in coastal range	No	n/a	Divergent haplotypes north, elsewhere dominated by one haplotype, coastal populations contain this and other low frequency variants
Weinmannia trichosperma	North-South (Andes and coastal)	10	Yes	Yes	Yes (Andes and coastal)	Yes (but rare coastal and central)	Yes (but rare)	n/a	Populations with private haplotypes are northern/coastal; One plant from Chiloe very divergent

n/a= non aplicable

In general, there is an increased topographical heterogeneity and isolation of populations in the landscape towards the north as the Andes become higher, so altitudinal gradients are steeper, and there are habitat gaps such as the central depression between the Andes and the Coastal cordillera. This matches the general trend for declining pairwise Fst values for each population along a north to south gradient. The pattern over all species is confounded by absolute differences among species, but Figure 5.11 shows the general trend that the highest population average pairwise Fst values for anyone species, is in the northernmost populations, and in some species (e.g. *Gentianella magellanica* and *Weinmannia trichosperma*) there is an almost linear correlation with latitude, with Fst declining southwards.

Of the seven species examined, there was divergence of the northern coastal populations with those from elsewhere in all species except *Prumnopitys andina* and *Escallonia virgata.* Where multiple coastal populations were sampled (e.g. *Donatia fasicularis, Gentianella magellanica* and *Weinmannia trichosperma*), these population were strongly differentiated from each other. This matches with previous studies showing divergence of coastal populations (e.g. Mathiasen and Premoli, 2010; Muellner *et al.,* 2005; Allnutt *et al.,* 1999). This body of evidence suggests that the coastal populations are important reservoirs of novel genetic variants. These charismatic sites (e.g. Figure 5.12) are surrounding by degraded landscapes. The recurrent detection of unique genetic variants across multiple species in these isolated sites in the coastal cordillera provides an added impetus to their conservation.

An interesting follow up to this, is that although the distinction between the northern coastal populations and those in the Andes is generally clear (albeit with exceptions), the distinction between coastal and Andean populations decreases as one heads southwards. Thus for *Tepualia stipularis,* coastal populations (e.g. Yaldad) share haplotypes with those east of the Andes (e.g. Blest; Figure 5.2) Likewise, there is haplotype sharing between southern coastal and eastern Andean populations of *Weinmannia trichosperma*. This suggests that at these latitudes, the Andes have not proved to be an insurmountable barrier to gene flow at some point in time. Also there was no clear effect of population size on the levels of genetic diversity within populations. In the main, this is because the differences in diversity levels within populations are in general small, and susceptible to sampling artifacts. However, in cases like *P. andina* which was sampled from 15 individuals per population it is noteworthy that the Maule population, which is the most genetically diverse population, has the smallest population size.

The North American and European species have gone through even more severe glacial periods than the South American species; with colder climates and thicker ice sheets and having higher levels of isolation. Although for North America plants there are several patterns that match with the Patagonian flora of this study, a southern west pacific refugia (Shafer *et al.*, 2010) could be similar to the northern coastal refugia found in the southern hemisphere. The special topography of Patagonia is very similar to the west of North America and it also shows a similar refugia pattern, with different haplotypes between both mountain ranges, showing an altitudinal migration in response to climate change (Grugger *et al.*, 2010; Nettel *et al.*, 2009). Another similarity is the Alexander Archipelago refugia that, due to the lower sea level during the LGM, some species survived there and colonized inland areas after the glacial retreat. This pattern could be similar to what we have found in *D. fascicularis* which shows possible refugia in the islands west of the glacial ice sheet. Finally, the North American arctic refugium or even refugia within ice sheets (nunataks), could be related to the southern refugia which the results of this research has suggested by *E.virgata.*

The European flora had its major refugia in the extreme south, in southern and central Italy, Corsica, Iberia and the Balkans (Petit *et al.*, 2003). This refugia composed mainly of thermophilous species, can be related to the northern refugia shared for all species of this study which was north of 43°S. Furthermore, it has been suggested that the northern cryptic refugia contained mainly cold-tolerant species with distributions in central and Eastern Europe including the Russian plain (Medail and Diedma, 2009), this pattern is similar to the southern refugia for *E. virgata* and possibly for *G. magellanica* and *D. chacaye*. The difference between Europe and North America, with southern South American phylogeographic patterns, could be related to the similarities found between North America and Patagonia, which includes a very similar topography and also a coastal, possible ice free, archipelago.

Figure 5.12. The top plate shows Araucaria araucana at Nahuelbuta, and the bottom plate shows the surrounding landscape (looking eastwards across the central depression to the Andean cordillera)

5.2 Future Work

The study of seven Patagonian species has provided evidence for some correlated patterns of genetic structure despite the different characteristics of individual species. There is a clear signal is of higher diversity of haplotypes towards the north, and evidence for long term persistence in northern and northern coastal sites, with only limited evidence for southern refugia.

Inevitably, in a study of this nature, there are many more areas one would have liked to have pursued, but which could not have been completed within the time frame of a PhD project and further questions are raised in light of the data. One area for further work would be to increase the sample density to gain additional resolution on the distribution of different haplotypes. The field component of this project was a major undertaking, sampling a total of 77 populations from 33 sites evenly distributed across Patagonia, often in remote and inaccessible areas. To build on this, an expedition down the western coast of Patagonia to obtain samples from the island archipelagos along the coast would be useful, and would provide insights into whether divergent coastal haplotypes are a feature of northern coastal populations, or whether coastal refugia persisted further south on these westerly island chains. An expedition of this nature would be costly (10s of thousands of pounds) as it would require ship hire for a lengthy period of time.

Another major target for sampling would be to increase the sample density in the northern part of the range, as populations in this area show evidence of high divergence. I searched for *T. stipularis* at Quillen and *G. magellanica* at Mt Chapelco (200 km south from Quillen) but did not find them, despite reliable records for these species at these sites.

The second area for further work would be to use nuclear markers on these samples. My field collections included up to 20 plants per population (so the sample sets are available), and the initial intention was to use nuclear markers for at least some of the species. However, I attempted AFLPs on *Gentianella magellanica,* but was not happy with the quality of the resulting data, and time constraints prevented me pursuing this further. As next generation sequencing technologies become ever cheaper, it should be (relatively) straightforward to develop sets of nuclear markers for these species and to use these to gain additional insights into their history and genetic structure. Obtaining data from multiple unlinked loci, could also allow more sophisticated methods of data analysis, to make inferences on the timing of divergence of populations and their historical populations sizes (Lowe *et al.,* 2004).

References

Abarzua AM, Villagran C, Moreno PI (2004) Deglacial and postglacial climate history in east-central isla grande de Chiloé, southern Chile (43°S). *Quaternary Research*, **62**, 49-59.

Abbott RJ, Comes HP (2003) Evolution in the Arctic: a phylogeographic analysis of the circumarctic plant, *Saxifraga oppositifolia* (Purple saxifrage). *New Phytologist*, **161**, 211-224.

Acosta MC, Premoli AC (2010) Evidence of chloroplast capture in south American *Nothofagus* (subgenus *Nothofagus*, Nothofagaceae). *Molecular Phylogenetics and Evolution*, **54**, 235-242.

Allnutt TR, Newton AC, Lara A, Premoli AC, Armesto JJ, Vergara R, Gardner M (1999) Genetic variation in *Fitzroya cupressoides* (alerce), a threatened south American conifer. *Molecular Ecology*, **8**, 975-987.

Allnutt TR, Courtis JR, Gardner M, Newton AC (2001) Genetic variation in wild Chilean and cultivated British populations of *Podocarpus salignus* D. Don (Podocarpaceae). *Edinburgh Journal of Botany*, **58**, 459-473.

Allnutt TR, Newton AC, Premoli AC, Lara A (2003) Genetic variation in the threatened south American conifer *Pilgerodendron uviferum* (Cupressaceae), detected using RAPD markers. *Biological Conservation*, **114**, 245-253.

Armesto JJ, Leon P, Arroyo MK (1995a) Los bosques templados del sur de Chile y Argentina: una isla biogeográfica. In: *Ecología de los Bosques Nativos de Chile* (eds Armesto JJ, Villagrán C, Arroyo MK), pp. 23-28, Editorial Universitaria, Santiago.

Armesto JJ, Smith-Ramirez C, Arroyo MK (1995b) Biodiversidad y conservacion del bosque templado en Chile. *Ambiente y Desarrollo*, **8**, 19-24.

Arroyo MK, Rozzi JR, Simonetti J, Marquet JA, Salaberry M (1999) Central Chile. In: *Hotspots. Earth's Biologically Richest and Most Endangered Terrestrial Ecoregions* (eds Mittermeier RA, Myers N, Robles-Gil P, Mittermeier CG), pp. 161-171, CEMEX-Agrupacion Sierra Madre, México.

Arroyo MK, Cavieres LA, Humaña AM (2004) Experimental evidence of potential for persistent seed bank formation at a subantartic alpine site in Tierra del Fuego, Chile. *Annals of the Missouri Botanical Garden*, **91**, 357-365.

Aubry KB, Statham MJ, Sacks BN, Perrine JD, Wisely SM (2009) Phylogeography of the north American red fox: vicariance in pleistocene forest refugia. *Molecular Ecology*, **18**, 2668-2686.

Avise JC (2000) Phylogeography: the history and formation of species. Harvard University Press, Cambridge Massachusetts.447pp.

Azpilicueta MM, Marchelli P, Gallo LA (2009) The effects of quaternary glaciations in Patagonia as evidenced by chloroplast DNA phylogeography of southern beech *Nothofagus obliqua. Tree Genetics & Genomes*, **5**, 561-571.

Barret S, Harder L, Worley A (1997) The comparative biology of pollination and mating in flowering plants. In: *Plant life histories: Ecology, phylogeny and evolution* (eds Silverton S, Franco M and Harper JL), pp. 57-76, Cambridge University press, Cambridge.

Barrett SCH (1998) The evolution of mating strategies in flowering plants. *Trends in Plant Science*, **3**, 335-341.

Beheregaray LB (2008) Twenty years of phylogeography: the state of the field and the challenges for the Southern Hemisphere. *Molecular Ecology*, **17**, 3754-3774.

Bekessy SA, Allnutt TR, Premoli AC, Lara A, Ennos RA, Burgman MA, Cortes M, Newton AC (2002) Genetic variation in the vulnerable and endemic monkey puzzle tree, detected using RAPDs. *Heredity*, **88**, 243-249.

Benoit I (1989) Libro rojo de la flora terrestre de Chile, Primera Parte. Ministerio de Agricultura, Corporación Nacional Forestal. Santiago Chile. 157 pp.

Bennett KD, Tzedakis PC, Willis KJ (1991) Quaternary refugia of north European trees. *Journal of Biogeography*, **18**, 103-115.

Bianchi MM, Massaferro J, Roman Ross G, Amos AJ, Lami A (1999) Late pleistocene and early holocene ecological response of lake El Trebol (Patagonia, Argentina) to environmental changes. *Journal of Paleolimnology*, **22**, 137-148.

Blisniuk P, Stern L, Chamberlain CP, Idleman B, Zeitler P (2006) Climatic and ecologic changes during miocene surface uplift in the southern Patagonian Andes. Backbone of the Americas, Patagonia to Alaska Symposium, Geological Society of America.

Brunsfeld SJ, Sullivan J, Soltis DE, Soltis PS (2000) Comparative phylogeography of north-western North America: a synthesis. In: *Integrating Ecology and evolution in a spatial context* (eds Silvertown J, Antonivics J), pp. 319-339, British Ecological Society, London.

Bull-Herenu K, Martínez EA, Squeo FA (2005) Structure and genetic diversity in *Colliguaja odorifera* Mol. (Euphorbiaceae), a shrub subjected to pleisto-holocenic natural perturbations in a mediterranean south American region. *Journal of Biogeography*, **32**, 1129-1138.

Caetano S, Prado D, Pennington RT, Beck S, Oliveira-Filho A, Spichiger R, Naciri Y (2008) The history of seasonally dry tropical forests in eastern south America: inferences from the genetic structure of the tree *Astronium urundeuva* (Anacardiaceae). *Molecular Ecology*, **17**, 3147-3159.

Caldenius CC (1932) Las Glaciaciones cuaternarias en la Patagonia y Tierra del Fuego. *Geografiska Annaler*, **14**, 1-164.

Carrasco B, Garnier M, Eaton L, Guevara R, Carú M (2009) Proximal causes of genetic variation between and within populations of raulí (*Nothofagus nervosa*). *Ciencia e Investigación Agraria*, **36**, 229-238.

Cavers S, Navarro C, Lowe AJ (2003) Chloroplast DNA phylogeography reveals colonization history of a Neotropical tree, *Cedrela odorata* L., in Mesoamerica. *Molecular Ecology*, **12**, 1451-1460.

Cavers S, Navarro C, Hopkins P, Ennos R, Lowe A.J (2005) Regional and population-scale influences on genetic diversity partitioning within Costa Rican populations of the pioneer tree *Vochysia ferruginea* Mart. *Silvae Genetica*, 54, 258-264.

Chen S, Wu G, Zhang D, Gao Q, Duan Y, Zhang F, Chen S (2008) Potential refugium on the Qinghai-Tibet plateau revealed by the chloroplast DNA phylogeography of the alpine species *Metagentiana striata* (Gentianaceae). *Botanical Journal of the Linnean Society*, **157**, 125-140.

Clapperton C (1993) Quaternary Geology and Geomorphology of South America. Elsevier Science Publishers BV, Amsterdam. 779 pp.

Corbella H and Lara LE (2008) Late cenozoic quaternary volcanism in Patagonia and Tierra del Fuego. In: *The Late Cenozoic of Patagonia and Tierra del Fuego* (eds Rabassa J), pp. 95-119, Elsevier, Amsterdam.

Coronato AMJ, Coronato F, Mazzoni E, Vasquez (2008) The physical geography of patagonia and Tierra del Fuego. In: *The Late Cenozoic of Patagonia and Tierra del Fuego* (eds Rabassa J), pp. 13-55, Elsevier, Amsterdam.

Correa MN (1984) Flora patagonica. Parte IV-b. Dicotiledoneas Dialipetalas (Droseraceae a Leguminosae). Instituto Nacional de Tecnologia Agropecuaria Argentina. Buenos Aires. 309 pp.

Correa MN (1988) Flora patagonica. Parte V. Dicotiledoneas Dialipetalas (Oxalidaceae a Cornaceae). Instituto Nacional de Tecnologia Agropecuaria Argentina. Buenos Aires. 381 pp.

Correa MN (1998) Flora patagonica. Parte I. Instituto Nacional de Tecnologia Agropecuaria Argentina. Buenos Aires. 391 pp.

D'Antoni HL (1983) Pollen analysis of Gruta del Indio. *Quaternary of South America and Antarctic Peninsula*, **1**, 83-104.

Denton GH, Lowell TV, Heusser CJ, Schluchter C, Andersen BG, Heusser LE, Moreno PI, Marchant DR (1999a) Geomorphology, stratigraphy and radiocarbon chronology of Llanquihue drift in the area of the southern Lake District, Seno de Reloncavi and isla grande de Chiloe, Chile. *Geografiska Annaler*, **81**, 167-229.

Denton GH, Lowell TV, Heusser CJ, Moreno PI, Andersen BG, Heusser LE, Schluchter C, Marchant DR (1999b) Interhemispheric linkage of paleoclimate during the last glaciation. *Geografiska Annaler*, **81**, 107-153.

Donoso C (1998) Bosques Templados de Chile y Argentina. Variación, Estructura y Dinámica, 4ta ed. Editorial Universitaria, Santiago. 484 pp.

Donoso C (2006) Especies arborescentes. In: *Las especies arbóreas de los bosques templados de Chile y Argentina. Autoecología* (eds Donoso C), pp. 586-591, Marisa Cuneo Ediciones, Valdivia, Chile.

Donoso C and Escobar B (2006) *Weinmannia trichosperma* Cav. Tineo, Teñio, Teñiu, Tinel, Palo Santo, Maden, Mëdehue, Tarco. Familia Cunoniaceae. In: *Las especies arbóreas de los bosques templados de Chile y Argentina. Autoecología* (eds Donoso C), pp. 586-591, Marisa Cuneo Ediciones, Valdivia, Chile.

Doyle JJ and Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin*, **19**, 11-15.

Dumolin-Lapegue S, Demesure B, Fineschi S, Le Corre V, Petit RJ (1997) Phylogeographic structure of white oaks throughout the European continent. *Genetics*, **146**, 1475-1487.

Dutech C, Maggia L, Joly HI (2000) Chloroplast diversity in *Vouacapoua americana* (Caesalpinaceae), a neotropical forest tree. *Molecular Ecology*, **9**, 1427-1432.

Echeverria C, Coomes D, Salas J, Rey-Benayas J, Lara A, Newton A (2006) Rapid deforestation and fragmentation of Chilean temperate forests. *Biological Conservation*, **130**, 481-494.

Ennos RA, Sinclair WT, Hu XS, Langdon A (1999) Using organelle markers to elucidate the history, ecology and evolution of plant populations. In: *Molecular systematics and plant evolution* (eds Hollingsworth PM, Bateman RM, Gornall RJ), pp. 1-19, Taylor & Francis, London.

Etisham-Ul-Haq M, Allnutt TR, Smith-Ramírez C, Gardner MF, Armesto JJ, Newton AC (2001) Patterns of genetic variation in *in* and *ex situ* populations of the threatened Chilean vine *Berberidopsis corallina*, detected using RAPD markers. *Annals of Botany*, **87**, 813-821.

Fabris HA (1953) Sinopsis preliminar de las Gencianaceas argentinas. *Boletín de la Sociedad Argentina de Botánica*, **4**, 233-259.

Ferris C, King RA, Hewitt GM (1999) Isolation within species and the history of glacial refugia. In: *Molecular systematics and plant evolution* (eds Hollingsworth PM, Bateman RM, Gornall RJ), pp. 1-19, Taylor & Francis, London.

Fesq-Martin M, Friedmann A, Peters M, Behrmann J, Kilian R (2004) Late-glacial and holocene vegetation history of the Magellanic rain forest in southwestern Patagonia, Chile. *Vegetation History and Archaeobotany*, **13**, 249-255.

Filippa EM and Barboza GE (2006) Novedades en Gentianaceae para América del Sur. *SIDA*, **22**, 129-143.

Galbreath KE, Hafner DJ, Zamudio KR, Agnew K (2010) Isolation and introgression in the intermountain west: contrasting gene genealogies reveal the complex biogeographic history of the American pika (*Ochotona princeps*). *Journal of Biogeography*, **37**, 344-362.

Gardner M and Lara A (2003) The Conifers of Chile: an overview of their distribution and ecology. *Acta Horticulturae*, **615**, 165-170.

Gardner MF, Hechenleitner V, Thomas P, Echeverria C, Escobar B, Brownless P, Martínez C (2006) Threatened Plants of Central and South Chile: Distribution, Conservation and Propagation. Universidad Austral de Chile & Royal Botanic Garden Edinburgh. 188 pp.

Gillies ACM, Navarro C, Lowe AJ (1999) Genetic diversity in mesoamerican populations of mahogany (*Swietenia macrophylla*), assessed using RAPDs. *Heredity*, **83**, 722-732.

Glasser NF, Jansson KN, Harrison S, Kleman J (2008) The glacial geomorphology and pleistocene history of south America between 38ºS and 56ºS. *Quaternary Science Reviews*, **27**, 365-390.

Godbout J, Fazekas A, Newton CH, Yeh FC, Bousquet J (2008) Glacial vicariance in the Pacific northwest: evidence from a lodgepole pine mitochondrial DNA minisatellite for multiple genetically distinct and widely separated refugia. *Molecular Ecology*, **17**, 2463-2475.

Gosling PG, Ives LM, Cunningham VJ, Hechenleitner P, Brownless P, Thomas P, Martinez C (2005) Preliminary advice on fruit handling, seed pretreatment and germination of embryos of *Prumnopitys andina*. *Sibbaldia*, **3**, 41-50.

Groot JJ and Groot CR (1966) Pollen spectra from deep-sea sediments as indicators of climatic changes in southern south America. *Marine Geology*, **4**, 467-524.

Gugger PF, Sugita S, Cavender-Bares J (2010) Phylogeography of Douglas-fir based on mitochondrial and chloroplast DNA sequences: testing hypotheses from the fossil record. *Molecular Ecology*, **19**, 1877-1897.

Haberle SG, Bennett KD (2004) Postglacial formation and dynamics of north Patagonian rainforest in the Chonos archipelago, southern Chile. *Quaternary Science Reviews*, **23**, 2433-2452.

Hamrick JL and Godt MJW (1996) Effects of life history traits on genetic diversity in plant species. *Philosophical transactions of the Royal Society of London*, **351**, 1291-1298.

Hechenleitner P, Gardner MF, Escobar B, Cabello A (2006) *Prumnopitys andina* (Poepp. ex Endl.) de Laub. Lleuque, Lleuqui, Uva de cordillera. In: *Las especies arbóreas de los bosques templados de Chile y Argentina. Autoecología* (eds Donoso C), pp. 109-115, Marisa Cuneo Ediciones. Valdivia.

Heuertz M, Fineschi S, Anzidei M, Pastorelli R, Salvini D, Paule L, Frascaria-Lacoste N, Hardy OJ, Vekemans X, Vendramin GG (2004) Chloroplast DNA variation and postglacial recolonization of common ash (*Fraxinus excelsior* L.) in Europe. *Molecular Ecology*, **13**, 3437-3452.

Heusser CJ (1983) Quaternary pollen record from laguna de Tagua Tagua, Chile. *Science*, **219**, 1429-1432.

Heusser CJ (1984) Late glacial-holocene climate of the lake District of Chile. *Quaternary Research*, **22**, 77-90.

Heusser CJ (1995a) Palaeoecology of a *Donatia-Astelia* cushion bog, magellanic moorland-subantarctic evergreen forest transition, southern Tierra del Fuego, Argentina. *Review of Palaeobotany and Palynology*, **89**, 429-440.

Heusser CJ (1995b) Three late quaternary pollen diagrams from southern Patagonia and their palaeoecological implications. *Palaeogeography, Palaeoclimatology, Palaeoecology*, **118**, 1-24.

Heusser CJ, Lowell TV, Heusser LE, Hauser A, Andersen BG, Denton GH (1996) Full-glacial-late-glacial palaeoclimate of the Southern Andes: evidence from pollen, beetle and glacial records. *Journal of Quaternary Science*, **11**, 173-184

Heusser CJ, Heusser LE, Lowell TV (1999) Paleoecology of the southern Chilean lake district-isla grande de Chiloe during middle-late Llanquihue glaciation and deglaciation. *Geografiska Annaler*, **81**, 231-284.

Heusser CJ, Lowell TV, Heusser LE, Moreira A, Moreira S (2000) Pollen sequence from the Chilean lake district during the Llanquihue glaciation in marine oxygen isotope stages 4-2. *Journal of Quaternary Science*, **15**, 115-125.

Heusser LE , Heusser CJ, Kleczkowski A, Crowhurst S (1999) A 50,000-yr pollen record from Chile of south American millennial-scale climate instability during the last glaciation. *Quaternary Research*, **52**, 154-158.

Heusser LE, Heusser CJ, Mix A, McManus J (2006a) Chilean and southeast pacific paleoclimate variations during the last glacial cycle: directly correlated pollen and $\delta^{18}O$ records from ODP Site 1234. *Quaternary Science Reviews*, **25**, 3404-3415.

Heusser LE, Heusser CJ, Pisias N (2006b) Vegetation and climate dynamics of southern Chile during the past 50,000 years: results of ODP Site 1233 pollen analysis. *Quaternary Science Reviews*, **25**, 474-485.

Hewitt GM (1999) Post-glacial re-colonization of European biota. *Biological Journal of the Linnean Society*, **68**, 87-112.

Hewitt GM (2000) The genetic legacy of the quaternary ice ages. *Nature*, **405**, 907-913.

Hewitt GM (2001) Speciation, hybrid zones and phylogeography – or seeing genes in space and time. *Molecular Ecology*, **10**, 537-549.

Hewitt GM, Ibrahim KM (2001) Inferring glacial refugia and historical migrations with molecular phylogenies. In: *Integrating Ecology and evolution in a spatial context* (eds Silvertown J, Antonivics J), pp. 271-294, British Ecological Society. London.

Ho TN and Liu SW (1993) New combinations, names and taxonomic notes on *Gentianella* (Gentianaceae) from South America and New Zealand. *Bulletin of the British Museum*, **23**, 61-65.

Hoffmann AE (1997) Flora Silvestre de Chile, zona Araucana. Fundación Claudio Gay. Santiago Chile. 257 pp.

Hoffmann AE, Arroyo MK, Liberona F, Munoz M, Watson J (1998) Plantas Altoandinas en la Flora Silvestre de Chile. Fundacion Claudio Gay. Santiago Chile. 281 pp.

Hubbard A, Hein AS, Kaplan MR, Hulton NRJ, Glasser N (2005) A modelling reconstruction of the last glacial maximum ice sheet and its deglaciation in the vicinity of the northern patagonian icefield, south America. *Geografiska Annaler*, **87**, 375-391.

Hulton NRJ, Sugden D, Payne A, Clapperton C (1994) Glacier modeling and the climate of Patagonia during the last glacial maximum. *Quaternary Research*, **42**, 1-19.

Hulton NRJ, Purves RS, McCulloch RD, Sugden DE, Bentley MJ (2002) The last glacial maximum and deglaciation in southern south America. *Quaternary Science Reviews*, **21**, 233-241.

Inzunza J (2003) Climas de Chile. In:
http://www2.udec.cl/~jinzunza/meteo/meteo.htm. Accessed August 2006

Kausel E (1953) El genero Escallonia en Chile. *Darwiniana*, **2**, 169-255.

King MG, Horning ME, Roalson EH (2009) Range persistence during the last glacial maximum: *Carex macrocephala* was not restricted to glacial refugia. *Molecular Ecology*, **18**, 4256-4269.

Lamy F, Hebbeln D, Wefe G (1999) High-resolution marine record of climatic change in mid-latitude Chile during the last 28,000 years based on terrigenous sediment Parameters, **51**, 83-93

Landrum L (1988) The myrtle family (Myrtaceae) in Chile. *Proceedings of the California Academy of Sciences*, **45**, 277-317.

Lowe A, Harris S, Ashton P (2004) Ecological Genetics: Design, Analysis, and Application. Backwell Science. Oxford, UK. 326 pp.

Lumley SH and Switsur R (1993) Late quaternary chronology of the Taitao peninsula, southern Chile. *Journal of Quaternary Science*, **8**, 161-165.

Mancini MV (1998) Vegetational changes during the holocene in Extra-Andean Patagonia, Santa Cruz Province, Argentina. *Palaeogeography, Palaeoclimatology, Palaeoecology*, **138**, 207-219.

Manzini MV, Prieto AR, Paez MM, Schäbitz F (2008) Late quaternary vegetation and climate of Patagonia. In: *The Late Cenozoic of Patagonia and Tierra del Fuego* (eds Rabassa J), pp. 351-367, Elsevier, Amsterdam.

Marchelli P and Gallo LA (2001) Genetic diversity and differentiation in a southern beech subjected to introgressive hybridization. *Heredity*, **87**, 284-293.

Marchelli P, Gallo LA (2004) The combined role of glaciation and hybridization in shaping the distribution of genetic variation in a Patagonian southern beech. *Journal of Biogeography*, **31**, 451-460.

Marchelli P, Gallo LA (2006) Multiple ice-age refugia in a southern beech of South America as evidenced by chloroplast DNA markers. *Conservation Genetics*, **7**, 591-603.

Marchelli P, Gallo LA, Scholz F, Ziegenhagen B (1998) Chloroplast DNA markers reveal a geographical divide across Argentinean southern beech *Nothofagus nervosa* (Phil.) Dim. et Mil. distribution area. *Theorical Application Genetics*, **97**, 642-646.

Marchelli P, Baier C, Mengel C, Ziegenhagen B, Gallo LA (2009) Biogeographic history of the threatened species *Araucaria araucana* (Molina) K. Koch and implications for conservation: a case study with organelle DNA markers. *Conservation Genetics*, **11**, 951-963.

Markgraf V, Bradbury JP, Fernandez J (1986) Bajada de Rahue, province of Neuquen, Argentina: An interstadial deposit in northern Patagonia. *Palaeogeography, Palaeoclimatology, Palaeoecology*, **56**, 251-258.

Markgraf V, McGlone M, Hope G (1995) Neogene paleoenvironmental and paleoclimatic change in southern temperate ecosystems-a southern perspective. *Trends in Ecology and Evolution*, **10**, 143-147.

Markgraf V, Whitlock C, Anderson RS, García A (2009) Late quaternary vegetation and fire history in the northernmost *Nothofagus* forest region: Mallín Vaca Lauquen, Neuquén province, Argentina. *Journal of Quaternary Science*, **24**, 248-258.

Marr KL, Allen GA, Hebda RJ (2008) Refugia in the cordilleran ice sheet of western north America: chloroplast DNA diversity in the arctic-alpine plant *Oxyria digyna*. *Journal of Biogeography*, **35**, 1323-1334.

Marticorena C and Rodriguez R (1995) Flora de Chile. Vol I Pteridophyta – Gymnospermae. Universidad de Concepción. Concepción Chile. 351 pp.

Martinez C, Premoli AC, Echeverria C, Thomas P, Hechenleitner P (2011) Genetic diversity of luma del norte, a range restricted and threatened Mirtaceae endemic to central Chile. *Bosque*, **32**, 30-38.

Mathiasen P, Premoli AC (2010) Out in the cold: genetic variation of *Nothofagus pumilio* (Nothofagaceae) provides evidence for latitudinally distinct evolutionary histories in austral south America. *Molecular Ecology*, **19**, 371-385.

McCulloch RD, Fogwill CJ, Sugden DE, Bentley MJ, Kubik PW (2005a) Chronology of the last glaciation in central strait of Magellan and Bahía Inútil, southernmost south America. *Geografiska Annaler*, **87**, 289-312.

McCulloch RD, Bentley MJ, Tipping RM, Clapperton CM (2005b) Evidence for late-glacial ice dammed lakes in the central strait of Magellan and Bahía Inútil, southernmost South America. *Geografiska Annaler*, **87**, 335-362.

Medail F, Diadema K (2009) Glacial refugia influence plant diversity patterns in the Mediterranean Basin. *Journal of Biogeography*, **36**, 1333-1345.

Moore DM (1983) Flora of Tierra del Fuego. Missouri Botanical Garden, Missouri, USA. 396 pp.

Moreno PI (1997) Vegetation and climate near lago Llanquihue in the Chilean lake district between 20 200 and 9500^{14}C yr BP. *Journal of Quaternary Science*, **12**, 485-500.

Moreno PI (2000) Climate, fire, and vegetation between about 13,000 and 9200 ^{14}C yr B.P. in the Chilean lake district. *Quaternary Research*, **54**, 81-89.

Moreno PI (2004) Millennial-scale climate variability in nothwest Patagonia over the last 15000yr. *Journal of Quaternary Science*, **19**, 35-47.

Moreno PI, Leon AL (2003) Abrupt vegetation changes during the last glacial to holocene transition in mid-latitude south America. *Journal of Quaternary Science*, **18**, 787-800.

Moreno PI, Lowell TV, Jacobson GL, Denton GH (1999) Abrupt vegetation and climate changes during the last glacial maximum and last termination in the Chilean lake district: A case study from canal de la Puntilla (41°S). *Geografiska Annaler*, **81**, 285-311.

Muellner AN, Tremetsberger K, Stuessy T, Baeza CM (2005) Pleistocene refugia and recolonization routes in the southern Andes: insights from *Hypochaeris palustris* (Asteraceae, Lactuceae). *Molecular Ecology*, **14**, 203-212.

Nettel A, Dodd RS, Afzal-Rafii Z (2009) Genetic diversity, structure, and demographic change in Tanoak, *Lithocarpus densiflorus* (Fagaceae), the most susceptible species to sudden oak death in California. *American Journal of Botany*, **96**, 2224-2233.

Nuñez-Ávila MC, Armesto JJ (2006) Relict islands of the temperate rainforest tree *Aextoxicon punctatum* (Aextoxicaceae) in semi-arid Chile: genetic diversity and biogeographic history. *Australian Journal of Botany*, **54**, 733-743.

Paez MM, Prieto AR, Mancini MV (1999) Fossil pollen from los Toldos locality: A record of the late-glacial transition in the Extra-Andean Patagonia. *Quaternary International*, **53**, 69-75.

Palma RE, Rivera-Milla E, Salazar-Bravo J, Torres-Pérez F, Pardiñas UFJ, Marquet PA, Spotorno AE, Meynard AP, Yates TL (2005) Phylogeography of *Oligoryzomys longicaudatus* (Rodentia: Sigmodontinae) in temperate south America. *Journal of Mammalogy*, **86**, 191-200.

Pastorino MJ and Gallo LA (2002) Quaternary evolutionary history of *Austrocedrus chilensis,* a cypress native to the Andean-Patagonian forest. *Journal of Biogeography*, **29**, 1167-1178.

Pastorino MJ, Marchelli P, Milleron M, Soliani C, Gallo, LA (2009) The effect of different glaciation patterns over the current genetic structure of the southern beech *Nothofagus antarctica*. *Genetica*, **136**, 79-88.

Petit RJ, Csaikl UM, Bordács S, Burg K, Coart E, Cottrell J, van Dam B, Deans JD, Dumolin-Lapègue S, Fineschi S, Finkeldey R, Gillies A, Glaz I, Goicoechea PG, Jensen JS, König AO, Lowe AJ, Madsen SF, Mátyás G, Munro RC, Olalde M, Pemonge M, Popescu F, Slade D, Tabbener H, Taurchini D, de Vries SGM, Ziegenhagen B, Kremer A (2002a) Chloroplast DNA variation in European white oaks. Phylogeography and patterns of diversity based on data from over 2600 populations. *Forest Ecology and Management*, **156**, 5-26.

Petit RJ, Brewer S, Bordacs S, Burg K, Cheddadi R, Coart E, Cottrell J, Csaikl UM, van Dam B, Deans JD, Espinel S, Fineschi S, Finkeldey R, Glaz I, Goicoechea PG, Jensen JS, Konig AO, Lowe AJ, Madsen SF, Matyas G, Munro RC, Popescu F, Slade D, Tabbener H, de Vries SGM, Ziegenhagen B, de Beaulieu J, Kremer A (2002b) Identification of refugia and post-glacial colonisation routes of European white oaks based on chloroplast DNA and fossil pollen evidence. *Forest Ecology and Management*, **156**, 49-74.

Petit RJ, Aguinagalde I, de Beaulieu J, Bittkau C, Brewer S, Cheddadi R, Ennos R, Fineschi S, Grivet D, Lascoux M, Mohanty A, Müller-Starck G, Demesure-Musch B, Palmé A, Martín JP, Rendell S, Vendramin GG (2003) Glacial refugia: Hotspots but not melting pots of genetic diversity. *Science*, **300**, 1563-1565.

Phillipsen IC and Metcalf AE (2009) Phylogeography of a stream-dwelling frog (*Pseudacris cadaverina*) in southern California. *Molecular Phylogenetics and Evolution*, **53**, 152-170.

Pons O and Petit RJ (1995) Estimation, variance and optimal sampling of gene diversity 1. Haploid locus. *Theoretical and applied genetics*, **90**, 462-470.

Pons O and Petit RJ (1996) Measuring and testing genetic differentiation with ordered and anordered alleles. *Genetics*, **144**, 1237-1245.

Premoli AC (1997) Genetic variation in a geographically restricted and two widespread species of south American *Nothofagus*. *Journal of Biogeography*, **24**, 883-892.

Premoli AC, Kitzberger T, Veblen TT (2000) Conservation genetics of the endangered conifer *Fitzroya cupressoides* in Chile and Argentina. *Conservation Genetics*, **1**, 57-66.

Premoli AC, Souto CP, Rovere AE, Allnut TR, Newton AC (2002) Patterns of isozyme variation as indicators of biogeographic history in *Pilgerodendron uviferum* (D.Don) Florín. *Diversity and Distributions*, **8**, 57-66.

Puscas M, Choler P, Tribsch A, Gielly L, Rioux D, Gaudeul M, Taberlet P (2008) Post-glacial history of the dominant alpine sedge *Carex curvula* in the European alpine system inferred from nuclear and chloroplast markers. *Molecular Ecology*, **17**, 2417-2429.

Quiroga MP and Premoli AC (2010) Genetic structure of *Podocarpus nubigena* (Podocarpaceae) provides evidence of quaternary and ancient historical events. *Palaeogeography, Palaeoclimatology, Palaeoecology*, **285**, 186-193.

Rabassa J (2008) Introduction. In: *The late cenozoic of Patagonia and Tierra del Fuego* (eds Rabassa J), pp. 1-5, Elsevier. Amsterdam.

Ramos VA and Ghiglione MC (2008) Tectonic evolution of the Patagonian Andes. In: *The late cenozoic of Patagonia and Tierra del Fuego* (eds Rabassa J), pp. 57-71, Elsevier. Amsterdam.

Reisch C (2008) Glacial history of *Saxifraga paniculata* (Saxifragaceae): molecular biogeography of a disjunct arctic-alpine species from Europe and north America. *Biological Journal of the Linnean Society*, **93**, 385-398.

Riedemann P, Aldunate G (2003) Flora nativa de valor ornamental. Identificación y Propagación, Chile Zona Sur. Editorial Andres Bello. 516 pp.

Rodriguez R, Matthei O, Quezada M (1983) Flora Arborea de Chile. Editorial de la Universidad de Concepcion Chile. 408 pp.

Rodriguez-Serrano E, Cancino RA, Palma RE (2006) Molecular phylogeography of *Abrothrix olivaceus* (Rodentia: Sigmodontinae) in Chile. *Journal of Mammalogy*, **87**, 971-980.

Ruzzante DE, Walde SJ, Cussac VE, Dalebout ML, Seibert J, Ortubay S, Habits E (2006) Phylogeography of the Percichthyidae (Pisces) in Patagonia: roles of orogeny, glaciation, and volcanism. *Molecular Ecology*, **15**, 2949-2968.

Ruzzante DE, Walde SJ, Gosse JC, Cussac VE, Habits E, Zemlak SS, Adams EDM (2008) Climate control on ancestral population dynamics: insight from Patagonian fish phylogeography. *Molecular Ecology*, **17**, 2234-2244.

Schneider S, Roessli D, Excoffier L (2000) Arlequin a software for population genetics data analysis. User manual ver. 2.000. Genetics and Biometry Lab. Dept. of Anthropology. University of Geneva, Geneva.

Sewell MM, Parks CR, Chase MW (1996) Intraspecific Chloroplast DNA Variation and Biogeography of North American Liriodendron L. (magnoliaceae). *Evolution*, **50**, 1147-1154.

Shafer ABA, Cullingham CI, Cote SD, Coltman DW (2010) Of glaciers and refugia: a decade of study sheds new light on the phylogeography of northwestern North America. *Molecular Ecology*, **19**, 4589-4621.

Simoes AC, Lemos-Filho JP, Ribeiro RA, Rodrigues F, Lovato MB (2007) Phylogeography of the Tree *Hymenaea stigonocarpa* (Fabaceae: Caesalpinioideae) and the influence of quaternary climate changes in the Brazilian cerrado. *Annals of Botany*, **100**, 1219-1228.

Soltis DE, Gitzendanner MA, Strenge DD, Soltis PS (1997) Chloroplast DNA intraspecific phylogeography of plants from the Pacific northwest of north America. *Plant Systematics and Evolution*, **206**, 353-373.

Souto CP and Premoli AC (2007) Genetic variation in the widespread *Embothrium coccineum* (Proteaceae) endemic to Patagonia: effects of phylogeny and historical events. *Australian Journal of Botany*, **55**, 809-817.

Spellman GM, Klicka J (2006) Testing hypotheses of pleistocene population history using coalescent simulations: phylogeography of the pygmy nuthatch (*Sitta pygmaea*). *Proceedings of the Royal Society B*, **273**, 3057-3063.

Sudgen DE, Bentley MJ, Fogwill CJ, Hulton NRJ, McCulloch RD, Purves RS (2005) Late-glacial glacier events in southernmost south America: A blend of “northern” and “southern” hemispheric climatic signals?. *Geografiska Annaler*, **87**, 273-288.

Tomimatsu H, Kephart SR, Vellend M (2009) Phylogeography of *Camassia quamash* in western north America: postglacial colonization and transport by indigenous peoples. *Molecular Ecology*, **18**, 3918-3928.

Tremetsberger K, Urtubey E, Terrab A, Baeza CM, Ortiz MA, Talavera M, König C, Temsch EM, Kohl G, Talavera S, Stuessy TF (2009) Pleistocene refugia and polytopic replacement of diploids by tetraploids in the Patagonian and subantarctic plant *Hypochaeris incana* (Asteraceae, Cichorieae). *Molecular Ecology*, **18**, 3668-3682.

Veit H, Garleff K (1995) Evolución del paisaje cuaternario y los suelos en Chile central-sur. In: *Ecología de los bosques nativos de Chile* (eds. Armesto JJ, Villagrán C, Arroyo MK), pp. 29-49, Editorial Universitaria, Santiago.

Villagran C (1991) Historia de los bosques templados del sur de Chile durante el tardiglacial y Postglacial. *Revista Chilena de Historia Natural*, **64**, 447-460.

Villagran C (1995) Quaternary history of the mediterranean vegetation of Chile. In: *Ecology and Biogeography of Mediterranean Ecosystems in Chile, California and Australia* (eds. Arroyo MK, Zedler PH and Fox MD), pp. 3-20, Springer-Verlag.

Villagran C (2001) Un modelo de historia de la vegetación de la cordillera de la costa de Chile central-sur: la hipótesis glacial de Darwin. *Revista Chilena de Historia Natural*, **74**, 793-803.

Villagran C, Armesto JJ (2005) Fitogeografía histórica de la Cordillera de la Costa de Chile. In: *Historia, biodiversidad y ecología de los bosques costeros de Chile* (eds. Smith-Ramírez C, Armesto JJ, Valdovinos C), pp. 99-119, Editorial Universitaria, Santiago.

Villagran C, Hinojosa LF (1997) Historia de los bosques del sur de Sudamérica, II: Análisis fitogeográfico. *Revista Chilena de Historia Natural*, **70**, 241-267.

Villagrán C and Le-Quesne C (1996) El interés biogeográfico-histórico de Chile Central-Sur: ¿Por qué debemos conservar su biota?. In: *Libro rojo de los sitios prioritarios para la conservación de la diversidad biológica de Chile* (eds. Muñoz M, Núñez H, Yañez J), pp. 160-172, Ministerio de Agricultura, Corporación Nacional Forestal, Santiago.

Villagran C, Moreno P, Villa R (1995) Antecedentes palinologicos acerca de la historia cuaternaria de los bosques chilenos. In: *Ecologia de los bosque nativos de chile* (eds. Armesto JJ., Villagran C. and. Arroyo M.K.), pp. 51-70, Editorial Universitaria, Santiago.

Villagran C, Le-Quesne C, Aravena JC, Jimenez H, Hinojosa F (1998) El rol de los cambios de clima del cuaternario en la distribución actual de la vegetación de Chile central-sur. *Bamberger Geographische Schriften Bd*, **15**, 227-242.

Vuilleumier F (1991) A quantitative survey of speciation phenomena in Patagonian birds. *Ornitologia Neotropical*, **2**, 5-28.

Wilson VR, Owens JN (2003) Cytoplasmatic inheritance in *Podocarpus totara* (Podocarpaceae). *Acta Horticulturae*, **615**, 171-172.

Whitlock C, Bianchi MM, Bartlein PJ, Markgraf V, Marlon J, Walsh M, McVoy N (2006) Postglacial vegetation, climate, and fire history along the east side of the Andes (lat 41-42.5°S), Argentina. *Quaternary Research*, **66**, 187-201.

Xu J, Pérez-Losada M, Jara CG, Crandall KA (2009) Pleistocene glaciation leaves deep signature on the freshwater crab *Aegla alacalufi* in Chilean Patagonia. *Molecular Ecology*, **18**, 904-918.

Zemlak TS, Habit EM, Walde SJ, Battini MA, Adams EDM, Ruzzante DE (2008) Across the southern Andes on fin: glacial refugia, drainage reversals and a secondary contact zone revealed by the phylogeographical signal of *Galaxias platei* in Patagonia. *Molecular Ecology*, **17**, 5049-5061.

Zuloaga F, Morrone O, Belgrano M (2009) Catalogo de las Plantas Vasculares del Cono Sur (Argentina, Sur de Brazil, Chile, Paraguay y Uruguay).