A Phylogenetic Investigation of the New Zealand Pteridaceae Ferns

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A Thesis Presented for the Degree Master of Science in Ecology and Evolution at
Victoria University of Wellington, New Zealand.
February, 2008

In search of *Pellaea* – Mt. Wellington, Auckland

September 2007

Abstract

The fern family Pteridaceae is among the largest fern families in New Zealand. It comprises 17 native species among five genera. Traditionally the classification of Pteridaceae was based on morphological characters. The advent of molecular technology, now makes it possible to test these morphology-based classifications. The Pteridaceae has previously been subjected to phylogenetic analyses; however representatives from New Zealand and the South Pacific have never been well represented in these studies. This thesis research aimed to investigate the phylogenetic relationships of the New Zealand Pteridaceae, as well as, the phylogenetic relationships of the New Zealand species to their overseas relatives. The DNA sequences of several Chloroplast loci (e.g. *trnL-trnF* locus, *rps4* and *rps4-trnS* IGS, *atpB*, and *rbcl*) were determined and the phylogenetic relationships of the New Zealand Pteridaceae and several species-specific question within the genus *Pellaea* and *Adiantum* were investigated. Results presented in this thesis confirm previously published phylogenetics of the Pteridaceae, which show the resolution of five major clades, i.e., cryptogrammoides, ceratopteridoids, pteridoids, cheilanthoids, and the adiantoids. The addition of the New Zealand species revealed a possible South West Pacific groups formed by the respective genera, where New Zealand species were generally more related to one another than to overseas relatives. Within the New Zealand *Pellaea*, the analysis of the *trnL-trnF* locus sequence data showed that the morphologically-intermediate plants *P. aff. falcata*, responsible for taxonomic confusion, were more closely related to *P. rotundifolia* than to *P. falcata*. Furthermore, the species collected on the Kermadec Islands, previously thought to be *P. falcata*, are genetically distinct from the Australian *P. falcata* and they could constitute a new species. *Adiantum hispidulum*, which is polymorphic for two different hair types being used to distinguish them as different species, was also reinvestigated morphologically and molecularly. Morphological inspection of hairs revealed three hair types as opposed to the previous thought two, and furthermore, they correspond to three different *trnL-trnF* sequences haplotypes.

Acknowledgements

I would first like to thank my supervisors, Dr. Leon Perrie and Dr. Peter Ritchie, for their help and support throughout my project.

This project was possible by a scholarship through New Zealand Museum Te Papa Tongarewa for work on plant molecular systematics.

The following people kindly provided fern specimens and offered insights for my study: Leon Perrie, Pat Brownsey, Phil Garnock-Jones, Ewen Cameron, Alex Buchanan, Peter de Lange, and David Glenny.

I would lastly like to thanks my family, friends, and most importantly my husband Skip. He has been the best support I could ask for and also an amazing field partner. I am sure he has a new found love for ferns!

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Chapter 1

1.1 General Introduction

1.1.1 New Zealand flora

New Zealand has been geographically isolated in the southern Pacific Ocean for nearly 80 million years. It is believed that through tectonic uprifting 115 mya, New Zealand began to separate from the supercontinent Gondwanaland and reached its current position in the Pacific around 55 mya (McLoughlin 2001). It was during this time, throughout the Cretaceous and early Tertiary era, that New Zealand was thought to have evolved its unique and diverse native flora (Mildenhall 1980). This New Zealand flora has attracted many botanists to focus studies on the biogeography, phylogenetics, and biodiversity of New Zealand plant species.

New Zealand shared a common origin with many other South Pacific landmasses (i.e., Australia, Tasmania, New Caledonia). With increased

taxonomic sampling it has become apparent that there are many similarities with respect to species of ferns and genera in angiosperms in the flora between New Zealand and these surrounding landmasses (Winkworth et al. 2002). These floral similarities have been a subject of debate among scientists and many hypotheses for the origins of the native New Zealand flora have been suggested. One is that much of New Zealand's flora has been isolated since its separation from Gondwana over 80 million years ago. However, evidence from molecular dating and phylogenetic analyses support another hypothesis, being that much of the New Zealand flora is a result of recent arrival by long distance dispersal where New Zealand species are found to be closely related to other overseas taxa and most often Australian taxa (Brownsey 2001b, Winkworth et al. 2002, Perrie and Brownsey 2007).

Long distance dispersal as the mechanism for explaining the recent origins of New Zealand's extant biota is becoming increasingly supported (Brownsey 2001b, Perrie and Brownsey 2007). It is also becoming increasingly important to understand these origins in order to determine the relationships among the species in the New Zealand flora, as well as to their overseas relatives. Investigations of the flora are an important part of an ongoing effort to accurately document plant species diversity in New Zealand. Currently, close to 1,900 species of angiosperms, 200 species of pteridophytes, and 20 species of gymnosperms make up the native New Zealand vascular flora (Brownsey and Smith-Dodsworth 2000, Wilton and Breitwieser 2000).

Accurate records of native, endemic, and introduced plant species and their evolutionary relationships provide necessary information for defining, understanding, and ultimately protecting the indigenous flora. It is also important to document the biota as it changes in response to new introductions. Long distance dispersal most likely played an important role

in the colonization of New Zealand's flora and it may continue to be an important process that shapes the composition of the flora in the future (de Lange and Crowcroft 1997, Heenan and de Lange 2004).

1.1.2 New Zealand fern flora

Pteridophytes occurring in the New Zealand Botanical Region, extending from the Kermadec Islands to Macquarie Island, have been useful plants for phylogenetic and biogeographical studies (Brownsey 2001b, Perrie and Brownsey 2005b, a, 2007, Shepherd et al. 2007a). They are part of an old lineage of vascular plants, having first evolved around 364 million years ago. Most of the leptosporangiate ferns, or true ferns, seen today are believed to have evolved around 200 million years ago and it is believed that many of the extant fern families in New Zealand evolved prior to the separation of New Zealand from Gondwanaland (Pryer et al. 2004). This ancient origin makes them ideal candidates for testing the competing hypotheses of the origins of New Zealand flora.

The first attempt to systematically catalogue the New Zealand ferns was by the Frenchman Achille Richard in 1832, although he had never visited New Zealand (Breitwieser et al. in press). In later years, as botanists began to visit New Zealand more frequently, more accurate records began to be developed. The initial volume of the Flora of New Zealand series was published in 1961, and described 164 pteridophyte species (Allan 1961). This work was followed by numerous descriptions and cataloging of new fern species. The number of fern species described in New Zealand grew from 164 to 196 by the mid 1980's (Breitwieser et al. in press).

Today, New Zealand is home to nearly 200 recognized indigenous pteridophyte species, from approximately 60 genera. Among the ferns, 25 of the 37 fern families worldwide recognized worldwide by Smith et al. (2006) are represented (Brownsey and Smith-Dodsworth 2000, Smith et al. 2006). Less than half of the native New Zealand fern flora is endemic and many of the species are found elsewhere in the South Pacific. Approximately 90 percent of those species found elsewhere occur in temperate Australia (Brownsey 2001b). Although the diversity of ferns in New Zealand is low compared to most tropical regions, the sheer abundance of ferns and their dominance in the flora for a temperate region make them an important component of the New Zealand biota (Brownsey 2001a).

1.1.3 *Phylogenetics of New Zealand flora*

Molecular phylogenetic studies play an important role in revealing the relationships between and among fern species and these techniques are becoming increasingly vital to the classification of species in conjunction with traditional morphological characters. The Aspleniaceae, Gleicheniaceae, *Polystichum*, and *Blechnum* are among the few of the 60 genera and 25 fern families represented in New Zealand that have undergone investigations of a molecular nature (Perrie et al. 2003, Perrie and Brownsey 2005b, a, Perrie et al. 2007, Perrie and Brownsey 2007, Shepherd et al. 2007a, Shepherd et al. 2007b). Most of the studies to date have focused on the genus *Asplenium*. Among representatives of *A. hookerianum*, it was found that its morphological variation of pinnules was not concordant with its genetic variation (Perrie and Brownsey 2005a). Further investigations of *Asplenium* gave insights into its complex evolutionary history and found that New Zealand *Asplenium* are not monophyletic (Perrie and Brownsey 2005b). Molecular studies on the fern family Gleicheniaceae suggest that the previous taxonomy was insufficient to

explain the relationships that are present among the members and is in need of revision (Perrie et al. 2007). All these investigations, with the addition of DNA sequence data, have improved the understanding of both the origins of these ferns and their relationships to one another and their overseas relatives.

1.1.4 Research objectives and contributions

The fern family Pteridaceae is among the largest of the fern families present in New Zealand. It contains 17 native species among five genera in New Zealand, with five of the species being endemic (Brownsey and Smith-Dodsworth 2000). The majority of the species in this family are distributed throughout New Zealand, occurring on both the North and South Islands, while a few species are restricted only to the North Island. The distributions of the species of New Zealand Pteridaceae are mapped in Appendix A. Furthermore, previous classifications of the New Zealand Pteridaceae were based primarily on traditional morphological characters. Advances in molecular technology have allowed for the use of DNA sequence data to test morphology-based classifications.

The Pteridaceae ferns on a global level are a large and very diverse family and are found throughout the world (Tryon et al. 1990). Recently, several studies have focused on resolving the relationships of this family with DNA sequence data (Kirkpatrick 2007, Prado et al. 2007, Schuettpelz et al. 2007, Zhang et al. 2007). While the taxonomic sampling efforts for these previous studies has greatly improved, the New Zealand and South Pacific species were not well represented in world-wide studies of the Pteridaceae.

The aim of this thesis research was to investigate the relationships of the Pteridaceae fern family by carrying out the first phylogenetic analysis for this

group of ferns as it is represented in New Zealand. Chloroplast DNA sequence data was obtained to infer the relationships between the five genera within New Zealand and also their relationship to the other members of the Pteridaceae on a world-wide scale (Chapter 2).

Several other species-specific questions were investigated dealing with particular members within the genera *Pellaea* (Chapter 3) and *Adiantum* (Chapter 4). Within the genus *Pellaea*, three species are recognized in New Zealand (Brownsey and Smith-Dodsworth 2000). However, morphologically-intermediate plants have caused confusion regarding the taxonomy of *P. rotundifolia* and *P. falcata*. Chloroplast DNA from the *trnL-trnF* locus was sequenced to determine the relationships of the morphologically-intermediate plants. Also, DNA sequence data from overseas *Pellaea* sect. *Platyloma* was obtained to determine the relationships of the New Zealand *Pellaea* species to their overseas relatives.

Within *Adiantum*, seven native species are recognized in New Zealand. However, the polymorphic *A. hispidulum* has been subject to debate as to whether those plants containing long lax hairs should be a distinct species from those plants with short rigid hairs. The *trnL-trnF* locus was selected to investigate molecular variation among the plants of *A. hispidulum*. In addition, several chloroplast loci were sequenced to determine the relationships between all of the native *Adiantum* species in New Zealand.

Collection efforts for this thesis project obtained 54 native plant samples from around New Zealand representing many wild populations, and will be deposited in the WELT herbarium. These samples were used along with previously collected Pteridaceae specimens for molecular and morphological analyses needed for this study. Newly generated DNA sequences were

obtained from several chloroplast loci (57 *trnL-trnF* locus, 21 *rbcL*, 21 *atpB*, and 16 *rps4* gene and *rps4-trnS* IGS) for the samples collected and will be deposited into GenBank for future use. The distributions of the species of New Zealand Pteridaceae have not been previously mapped. Comprehensive distribution maps were generated for each of the 17 native species by combining collection details from each of the three major herbaria; CHR, WELT, and AK. These maps are presented in Appendix A.

Molecular phylogenies were generated for the New Zealand genera, *Pellaea* and *Adiantum*. The largest to date data set of chloroplast *rbcL* sequence data for the Pteridaceae was compiled to determine the relationships of the New Zealand species to the Pteridaceae world-wide. The results of this study will not only clarify the diversity and relationships among the New Zealand Pteridaceae, but also the relationships among the Pteridaceae on a global scale.

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Chapter 2

Relationships of the New Zealand Pteridaceae

2.1 Introduction

The Pteridaceae fern family has been the subject of extensive taxonomic and phylogenetic research on the basis of non-molecular (Ching 1940, Copeland 1947, Tryon and Tryon 1982, Tyron et al. 1990) and molecular data (Sánchez-Baracaldo 2004, Smith et al. 2006, Kirkpatrick 2007, Prado et al. 2007, Schuettpelz et al. 2007, Zhang et al. 2007). This family is morphologically characterized by creeping rhizomes, marginal sori or sori borne along the veins, and a chromosome number that is typically $n=29$ or 30 (Tyron et al. 1990, Smith et al. 2006). It is distributed worldwide, though the majority of these ferns are centered in the tropics. The classification of this family is complicated by its sheer size, extensive distribution, morphological variability, and diverse ecological habitats; and despite the array of studies

focusing on this family, many relationships between the members of this family remain unclear.

Past classifications for the Pteridaceae have fluctuated greatly with respect to the number of genera recognized in this family. Numbers have been reported ranging from only a few (Ching 1940, Pichi Sermolli 1977) to 63 (Copeland 1947) and most recently 50 (Smith et al. 2006). A clearer picture of the relationships among the genera is being developed as more sophisticated phylogenetic methods are being employed. There has been agreement over the subfamily classifications of the Pteridaceae across recent studies (Hasebe et al. 1995, Pryer et al. 2004, Smith et al. 2006, Schuettpelz et al. 2007). However, the generic classifications within those subfamilies are still widely debated.

Using morphological characters alone as a basis for classifying generic relationships in the Pteridaceae has been shown by analysis of DNA sequences to be misleading, most notably in circumscription of the cheilanthoid ferns (Gastony and Rollo 1995, Kirkpatrick 2007). Because of convergence as a result of ecological habitats (Gastony and Rollo 1995), certain morphological characters fail to reflect the evolutionary lines determined by DNA sequence data. A study by Smith et al. (2006) revised the classifications of extant ferns to ordinal and familial rank, and also provided a synopsis of included genera. They combined both morphological and molecular data to derive a consensus phylogeny of fern relationships. In the Pteridaceae, Smith et al. (2006) recognized 50 genera, with over 950 species. Five putatively major monophyletic groupings were recognized within this family (see below). They pointed out that several of the genera they accepted in the Pteridaceae are likely to be paraphyletic or polyphyletic and in need of redefinition.

Utilizing molecular data, several recent studies on Pteridaceae ferns have addressed the generic relationships within this family. Prado et al. (2007) used *rbcL* sequence data to investigate relationships within the Pteridaceae, with an expanded taxon sampling of native Brazilian species. A study by Schuettpelz et al. (2007) provided a much more extensive investigation of the family by incorporating seven genera previously unsampled and expanding the data set to a multi-gene analysis with chloroplast loci *atpB* and *atpA* along with the commonly used *rbcL* gene.

The phylogenetic relationships within the Pteridaceae reported by both Prado et al. (2007) and Schuettpelz et al. (2007) were in general agreement (Figure 2.1) The Pteridaceae formed five major clades corresponding to those reported by Smith et al. (2006). However, the relationship of these clades to one another was further resolved. The major clades were as follows as named by Schuettpelz et al. (2007): 1) cryptogrammoids, 2) ceratopteridoids (Parkeraceae), 3) pteridoids, 4), adiantoids, and 5) cheilanthoids. The cryptogrammoids, containing approximately 23 species among the genera *Coniogramme*, *Cryptogramma*, and *Llavea*, were sister to rest of the Pteridaceae. The ceratopteridoids (6 species among *Acrostichum* and *Ceratopteris*) are sister to pteridoids (app. 400 species among *Pteris* and ferns belonging to the monotypic Platyzomatotoideae; and Taenitidoideae, 14 genera, as described by Tryon et al, 1990). The genus *Adiantum* (app. 150 species) and the vittaroid ferns (12 genera, 300 species) are collectively called the adiantoids and are sister to cheilanthoids (20 genera, 400 species). Both studies showed support with *rbcL* sequence data for including the vittaroids as nested within *Adiantum*, and also paraphyly of the genus *Pteris*. Despite the increased resolution found from these studies, questions still remain regarding the

monophyletic status of several genera including those found in New Zealand, i.e., *Adiantum*, *Cheilanthes*, *Pellaea*, *Pteris*, and *Anogramma*.

Figure 2.1. Summary topologies of most recent Pteridaceae studies from Prado et al, 2007 and Schuettpelz et al, 2007. Thickened branches indicate those clades that received good posterior probabilities and bootstrap values according their respective studies (Prado et al. 2007, Schuettpelz et al. 2007).

The *rbcL* gene has been a popular choice for inferring plant phylogenies and is still a common chloroplast marker used for constructing single gene phylogenies (Gastony and Rollo 1995, Hasebe et al. 1995, Prado et al. 2007). Despite the popularity of *rbcL*, multiple gene phylogenies are becoming increasingly favored for providing better resolution of evolutionary relationships. This present study utilizes two chloroplast genes, *rbcL* and *atpB*, both of which have been used in previous phylogenetic investigations of the Pteridaceae. These genes have proven to provide sufficiently variable DNA sequence data necessary for reconstructing relationships among the members of the Pteridaceae. This, along with large numbers of *rbcL* sequences available on GenBank, makes them ideal markers for phylogenetic investigations.

This study adds to the examination of the overall relationships of the fern family Pteridaceae by including all of the 17 native species in the five genera recognized in the New Zealand. In addition, estimated divergence dates for the genera within the New Zealand Pteridaceae are inferred from results obtained by previous studies to hypothesize whether the species of Pteridaceae arrived in New Zealand by long-distance dispersal or are of a vicariant origin (Pryer et al. 2004, Perrie and Brownsey 2007). Because this study constructs the most comprehensive *rbcL* data set to date by combining all of the Pteridaceae DNA sequences available from the different data sets of previous studies of Pteridaceae phylogenies (Prado et al. 2007, Schuettpelz et al. 2007, Zhang et al. 2007) with the data of the present study; it is possible to make the best inferences yet, regarding the relationships of the New Zealand Pteridaceae species to their overseas relatives.

New Zealand and other areas of the South Pacific are under-represented in previous DNA sequence sampling, as the present study is the first molecular investigation to be conducted on New Zealand Pteridaceae phylogenetics.

Previous studies of New Zealand *Asplenium* ferns suggest that regional biodiversity exists in areas of the South Pacific and Australasia (Perrie and Brownsey 2005) where hybridizing New Zealand species formed a closely related “Austral” group. The five genera of Pteridaceae native to New Zealand are *Anogramma* (one species), *Pteris* (four), *Adiantum* (eight), *Cheilanthes* (two), and *Pellaea* (three). Within New Zealand, most of these ferns are distributed throughout the country, although a few are quite rare and confined to certain areas (e.g., *Pellaea falcata* and *Adiantum formosum*). While most of New Zealand’s native Pteridaceae species are also found in Australia and elsewhere, three *Adiantum*, two *Pteris* and, one *Pellaea* are endemic to New Zealand (Brownsey and Smith-Dodsworth 2000).

Increased taxonomic sampling with individual and combined analyses of multiple genes in this study offered insight into the following questions: 1) What are the relationships and affinities of the New Zealand Pteridaceae to the overseas members of this family? 2) Does the addition of the New Zealand species change the Pteridaceae phylogeny as it has been reported in previous studies? 3) In the Pteridaceae, is there conflict between single gene phylogenies and combined gene phylogenies and is resolution gained with multiple gene analyses? 4) Do estimated divergence times support long-distance dispersal for the origins of the New Zealand Pteridaceae?

2.2 Material & Methods

2.2.1 Sample collection

A sample from each of the 17 Pteridaceae species native to New Zealand was included in the present study (Figure 2.2). A specimen referable to *Adiantum*

Figure 2.2. Distribution map of native New Zealand species collected for this study. The species collected from the Kermadec Islands are shown, in relation to New Zealand, in the top left. Samples beginning with P are in the WELT herbarium. WB and LRP are collection numbers for Whitney Bouma and Leon Perrie respectively.

hispidulum Swartz var. *pubescens* (Schkuhr) Large et Braggins, sometimes treated as a distinct species (*A. pubescens* Schkuhr; Paris, 1980), was also sampled, as were two species adventive to New Zealand, *Adiantum capillus-veneris* and *A. raddianum*. Samples were identified using Brownsey and Smith-Dodsworth (2000), and confirmed by sequencing the *trnL-trnF* locus for duplicate specimens for each native species (unpublished data; Chapters 3 and 4). The *trnL-trnF* locus is too variable for comparisons amongst the genera, but it supported the identification of the samples presented here.

2.2.2 DNA isolation/PCR/sequencing

Total DNA was extracted from dried silica gel tissue or fresh tissue, where available, according to a modified CTAB protocol (Doyle and Doyle 1990). Primers 1F (5'-ATGTCACCACAAACGGAGACTAAAGC-3') and 1361R (5'-TCAGGACTCCACTTACTAGCTTCACG-3') were used for amplifying the *rbcl* chloroplast gene (Schuettpelz and Pryer 2007). PCR amplifications, in a total volume of 20 μ l, were made with the following reagents: 1M betaine, 10x NH_4 reaction buffer (160mM $(\text{NH}_4)_2\text{SO}_4$, 670mM Tris-HCl (pH8.8 at 25°C), 0.1% Tween-20, BioLine), 1.5mM MgCl_2 (BioLine), 250 μ M dNTPs (BioLine), 10pmols of each forward and reverse primer, 1U of Taq DNA Polymerase (BioLine), and approximately 50ng of DNA template. Thermocycler conditions consisted of an initial denaturing of 95°C for 2 minutes, 34 cycles of 95°C for 1 minute, 50°C for 1 minute, 72°C for 1 minute, and a final 72°C for 5 minutes. Primers 672F (5'-TTGATACGGGAGCYCCTCTWAGTGT-3') and 384R (5'-GAATCCCAAACCTATTCGATTAGG-3') were used to amplify the *atpB* gene (Schuettpelz and Pryer 2007). PCR amplification mix and thermocycler settings were the same as for *rbcl*.

PCR products were visualized with agarose gel electrophoresis. Successfully amplified PCR products were purified with 0.2U of SAP (shrimp alkaline phosphatase, USB Corp., Cleveland, USA) and 1U EXO (exonuclease I, Exo I, USB Corp., Cleveland, USA) to remove residual reagents. Thermocycler conditions consisted of 37°C for 30 minutes followed by deactivation of enzymes at 80°C for 15 minutes. Cleaned products were sequenced at the Allan Wilson Center Genome Service with an ABI3730 Genetic Analyzers (Allan Wilson Centre Genome Service, Palmerston North, New Zealand). Newly obtained DNA sequences for the New Zealand Pteridaceae will be submitted to GenBank but sequence data and files can be found in the CD appendix.

2.2.3 Phylogenetic analyses

Three single-gene data sets (88-taxon *atpB*, 88-taxon *rbcL*, and 220-taxon *rbcL* with expanded sampling) and a combined two-gene (88-taxon *atpB+rbcL*) data set were constructed. The single-gene data sets (*atpB* and *rbcL*) and the combined data set contained 88 taxa, including the 17 species of Pteridaceae native to New Zealand, and 12 being assigned as outgroups. The same outgroups as Schuettpelz et al. (2007) were used because both *atpB* and *rbcL* sequences were available on GenBank. The remaining 55 DNA sequences for other members of the Pteridaceae were sourced from GenBank, again because they had both *atpB* and *rbcL* DNA sequences available to allow for a concatenated data set. The expanded *rbcL* data set contained 220 taxa, again including the New Zealand species with the remainder being Pteridaceae samples from recent studies (Prado et al. 2007, Schuettpelz et al. 2007, Zhang et al. 2007) obtained from GenBank (Table 2.1).

Newly generated DNA sequences for both *rbcL* and *atpB* were edited by eye using BioEdit version 5.0.9 (Hall 1999). Alignments were constructed using Clustal X version 1.83 (Thompson et al. 1997). These alignments were imported into MEGA version 4.0 (Tamura et al. 2007) and combined with imported sequences from GenBank. Summary statistics on alignments were calculated using MEGA v.4.0 (Table 2.2). Pairwise comparisons determined the number of nucleotide differences, transitions, and transversions both within and between the major groups of the Pteridaceae (Table 2.3). DAMBE (Xia and Xie 2001) software was used to plot the number of transitions and transversions versus the number of nucleotide differences (Appendix B) to investigate whether the data sets had become saturated with transitions and thus were inappropriate for phylogenetic analyses (Page and Holmes 1998). Before combining *rbcL* and *atpB* sequence data, a partition homogeneity test was run on PAUP* version 4.b10 (Swofford 2002) to test for conflict between the two data sets.

PAUP* v. 4.b10 was also used for implementing maximum parsimony and maximum likelihood analyses on all of the 88-taxon data sets. The heuristic searches under the parsimony criterion employed 10,000 replicates of random sequence addition and tree bisection-reconnection branch swapping. Support for the topology was assessed using bootstrapping, with 200 pseudoreplicates each using 100 replicates of random sequence addition. Maximum likelihood analyses were implemented using the appropriate model of evolution as assessed by ModelTest version 3.6 (Posada and Buckley 2004) according to the Akaike Information criteria. Maximum likelihood settings ran for 100 replicates of random sequence addition. Bootstrapping was again performed to assess the confidence with settings consisting of 100 pseudoreplicates, one random sequence addition, and, because of time constraints, branch swapping of nearest neighbor interchange (NNI). The 220-taxon *rbcL* data set

was subjected to maximum parsimony analysis using the same criteria as mentioned above. Maximum likelihood analyses were not run on this expanded data set because the time required would have been prohibitive.

MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001) was used to carry out Bayesian inference (nst=6, rates=invgamma, and default priors) on all four of the data sets in this study. Analyses were run for 5,000,000 generations with sampling every 1000 generations and the initial 50% discarded as burn-in. Standard deviations of split frequencies were checked at 2,500,000 generations to ensure a value of <0.01 (suggesting a convergence to stationary distribution had been achieved).

2.3 Results

2.3.1 Data

The alignment of the *rbcL* and *atpB* gene sequences was straightforward, with no insertions or deletions. Summary statistics (Table 2.2; found at the end of the chapter) for the *rbcL* data set showed that of the 1143 sites used, 496 were variable, with 413 being parsimony informative. The *atpB* data set comprised an alignment length of 1111 sites, of which 469 were variable and 384 were parsimony informative sites. Nucleotide composition for the *rbcL* alignment averaged: T= 26.3% C=22.0% A= 26.0% G= 25.8%. Average nucleotide composition for the *atpB* alignment showed: T= 27.5% C=19.9% A= 28.2% and G= 24.4%. The observed differences between transitions and transversions are given in Table 2.3. Both *atpB* and *rbcL* data sets had a similar number of observed transitions. However, the *rbcL* data set had a much higher proportion of transversions

Table 2.2.
Summary statistics for the four data sets analyzed in this study

Data Set	Alignment Length (bp)	Variable sites	Parsimony Informative sites	Best fitting Model (AIC)	Number MP Trees (score)	Score ML tree
88-taxon <i>rbcL</i>	1143	496	413	GTR+I+G	270 (2908)	15202.05189
88-taxon <i>atpB</i>	1111	469	384	TVM+I+G	319 (2284)	12283.04732
88-taxon <i>rbcL+atpB</i>	2254	956	797	GTR+I+G	39 (5228)	27717.61937
Expanded <i>rbcL</i>	1143	594	483	NA	10,000 (4617)	NA

AIC: Akaike Information Criteria MP: Maximum parsimony ML: Maximum likelihood

when compared with transition type changes suggesting a faster rate of evolution compared to *atpB* sequence data. Figures B1 and B2 in Appendix B show the rates of transitions and transversions versus the observed distance for each of the single gene 88-taxon *atpB* and 88-taxon *rbcL* data sets, respectively, and showed no sign of saturation.

The partition homogeneity test reported a P value greater than 0.05 indicating that the two data sets could be combined for analyses (P=0.09). Summary statistics for the 88-taxon *atpB+rbcL* data set showed that of the 2254 characters used 956 were variable and 797 were parsimony informative. The 220-taxon *rbcL* data set had the same amount of characters as the 88-taxon *rbcL* data set, but, with the additional taxa, had 594 variable sites of which 483 were parsimony informative.

2.3.2 Phylogenetic analyses

Five major monophyletic groups or clades were resolved within the Pteridaceae in this study. They will be referred to by the Pteridaceae clade names as presented in Schuettpelz et al. (2007); i.e., the cryptogrammooids, ceratopteridoids, pteridoids,

Table 2.3a Sequence difference matrix between major clades in single gene *atpB* data set.

Upper matrix corresponds to the percent difference and the lower is the total number of transitions over the number of transversions

Groups	cerapteridoids	pteridoids	cheilanthoids	<i>Adiantum</i> I	<i>Adiantum</i> II	vittaroids	outgroups	cryptogramoids
cerapteridoids		10.8	10.7	11.0	10.6	13.7	12.9	10.5
pteridoids	63/13		8.7	8.2	7.7	12.7	12.0	8.9
cheilanthoids	63/12	53/9		7.9	7.8	12.0	12.3	7.6
<i>Adiantum</i> I	62/14	47/11	48/7		6.3	10.9	12.1	9.1
<i>Adiantum</i> II	59/12	47/8	48/4	33/7		10.9	12.0	8.5
vittaroids	69/26	65/23	61/21	54/22	53/20		15.4	13.2
outgroups	72/17	70/13	74/11	71/13	71/11	82/24		12.2
cryptogramoids	60/13	53/9	47/6	54/9	51/6	69/22	73/12	

Table 2.3b Sequence difference matrix between major clades in single gene *rbcL* data set

Upper matrix corresponds to the percent difference and the lower is the total number of transitions over the number of transversions.

Groups	cerapteridoids	pteridoids	cheilanthoids	<i>Adiantum</i> I	<i>Adiantum</i> II	vittaroids	outgroups	cryptogramoids
cerapteridoids		11.9	12.9	13.6	13.1	16.1	14.9	12.4
pteridoids	58/42		9.3	10.6	10.6	13.8	12.9	9.3
cheilanthoids	71/37	56/22		9.9	10.1	13.6	12.7	9.9
<i>Adiantum</i> I	76/38	64/25	68/15		9.0	13.2	13.6	10.4
<i>Adiantum</i> II	72/37	68/24	70/14	58/15		13.5	13.1	10.4
vittaroids	82/53	74/41	78/36	76/34	78/35		16.5	15.2
outgroups	83/42	79/29	83/23	87/27	86/23	94/44		11.9
cryptogramoids	69/35	58/20	69/14	70/18	72/15	88/39	79/20	

cheilanthoids, and adiantoids. Within the adiantoids, three groups (*Adiantum* I, *Adiantum* II, and the vittaroids) were consistently recovered in the 88-taxon *atpB*, 88-taxon *rbcL*, and 88-taxon *atpB+rbcL* data sets. The group *Adiantum* I contained species with affinity to *A. capillus-veneris* (*A. malesianum*, *A. pedatum*, *A. peruvianum*, *A. tetraphyllum*, and sometimes *A. tenerum*). *Adiantum* II contained all the New Zealand species with the addition of the non-native *A. raddianum*. Core phylogenies from all three tree-building analyses of each of the three 88-taxon data sets are presented in Figures 2.3a-i. Many of the inferred relationships amongst the major clades differed between tree-building analyses and between which sequence data set was used.

Maximum Parsimony

Figure 2.3a represents the 50% majority rule consensus tree of 319 equally most parsimonious trees with a tree length of 2284 steps, for the 88-taxon *atpB* data set. There were 270 most parsimonious trees for the 88-taxon *rbcL* data set, of 2908 steps in length (Table 2.2). Figure 2.3d represents the 50% majority rule consensus tree of the 270 trees recovered from this analysis. Figure 2.3g represents the 50% majority rule consensus tree for the 36 recovered trees of the 88-taxon *atpB+rbcL* data set with a tree length of 5228 steps.

The consensus parsimony trees for each of the 88-taxon data sets recovered the major Pteridaceae clades as recorded by Schuettpelz et al. (2007). However, incongruence was found in respect to the relationships between these clades. In all three of the 88-taxon maximum parsimony analyses, the most parsimonious tree recovered the ceratopteridoids as sister to the rest of the Pteridaceae, which was not in agreement with the previous studies (Prado et al. 2007, Schuettpelz et al. 2007). The bootstrap analyses for the present study also recovered the ceratopteridoids as sister to the Pteridaceae but not supported with high bootstrap (BS) with the 88-taxon *atpB* (BS=60%), and only moderately supported for the 88-taxon *atpB+rbcL* analyses

Figure 2.3a – 3i.

Phylogenies for each of the three analyses; maximum parsimony (MP), Bayesian Inference (BI), and maximum likelihood (ML), for single gene *atpB*, *rbcL*, and combined *atpB+rbcL* data sets. Thickened branches indicate well supported bootstrap values (> 70) and good posterior probabilities (> 0.95).

(BS=72%). In contrast the bootstrap analysis of the 88-taxon *rbcL* data set recovered the ceratopteridoids as sister to the pteridoids (bootstrap trees not shown).

Other differences with past studies were produced in the maximum parsimony analyses. The 88-taxon *atpB* data set produced a clade containing cryptogrammoideae and pteridoideae, which was then sister to *Adiantum*. The vittaroideae, rather than being related to *Adiantum*, were placed sister to the remainder of Pteridaceae with the exception of the ceratopteridoideae. None of these relationships were supported with high bootstrap values.

Also worth noting was the relationships recovered between *Adiantum* and vittaroideae. Neither the 88-taxon *rbcL* nor the 88-taxon *rbcL+atpB* data sets resolved *Adiantum* as a well supported monophyletic group. *Adiantum* was recovered as either paraphyletic with respect to the vittaroideae with low support (BS= <50%) in the 88-taxon *rbcL*, or a poorly supported monophyletic group (BS= 55%) in the 88-taxon *rbcL+atpB* data set.

The maximum parsimony analysis for the 220-taxon *rbcL* dataset (Figure 2.4a-c) also recovered the five major putative groups in the Pteridaceae. In contrast to the three 88-taxon parsimony analyses, the 220-taxon *rbcL* data set produced a topology of the five major clades that was in agreement with previous studies with the cryptogrammoideae as sister to the Pteridaceae. This expanded data set also recovered a monophyletic *Adiantum* (Figure 2.4c), though it received low bootstrap support (BS =54%).

Bayesian Inference

Bayesian Inference (BI) of the 88-taxon *rbcL* data set (Figure 2.3e) produced a resolved tree with cryptogrammoideae being sister to the rest of the Pteridaceae although receiving low posterior probability (PP) support (PP=0.86). The remainder of the clades producing a topology in agreement with previous studies (Prado et al. 2007, Schuettpelz et al. 2007). The ceratopteridoideae (PP=1.0) were sister to the pteridoideae (PP <0.50) but the relationship did not receive good support (PP=0.82).

The fully resolved but poorly supported cheilanthoid clade (PP=0.55) was sister to the fully resolved adiantoids (PP=1.0) and the relationship received high support (PP=1.0). The genus *Adiantum* was paraphyletic, with the vittaroids being more closely related to *Adiantum* I than to *Adiantum* II. Although grouping the vittaroids with *Adiantum* was well supported (PP=1.0), the paraphyletic relationship of *Adiantum* was not (PP=0.67).

The 88-taxon *atpB* BI produced a tree (Figure 2.3b) incongruent to that of the 88-taxon *rbcL* BI analyses with the major difference being the placement of cryptogramroids. The cryptogramroids (PP=1.0) were sister to the ceratopteridoids (PP=1.0) as opposed to the rest of the Pteridaceae but this was supported with low posterior probability (PP=0.74). This new clade was sister to the pteridoids (PP=1.0) as previously noted and was somewhat well supported (PP=0.91). The cheilanthoids were sister to the adiantoids. The adiantoids in this analysis was unresolved, with a trichotomy of the vittaroids, *Adiantum* I, and *Adiantum* II. The species *A. tenerum*, which consistently showed affinity for *Adiantum* I in the analyses 88-taxon *rbcL*, now had affinity to *Adiantum* II.

The BI of the 88-taxon *atpB+rbcL* data set recovered yet another different topology which was still incongruent with Schuettpelz et al. (2007). A basal trichotomy comprising the cryptogramroids, the clade containing the ceratopteridoids and pteridoids, and the clade containing the adiantoids and cheilanthoids was recovered in the analysis. The relationship of the ceratopteridoids to the pteridoids (PP=0.52) was not well supported, and neither was a monophyletic *Adiantum* (PP=0.67).

Bayesian Inference of the 220-taxon *rbcL* data set (tree not shown) resolved the five major Pteridaceae clades all with high support (PP=1.0). Their position was in agreement with previous studies and well supported with the exception of the position of the cryptogramroids receiving low support (PP=0.85) as sister to the

Pteridaceae. Also, Bayesian Inference failed to resolve a well supported monophyletic *Adiantum* (PP<0.50) as this analysis recovered a trichotomy comprising *Adiantum* I, *Adiantum* II, and the vittaroids.

Maximum likelihood

Maximum likelihood analyses for both the 88-taxon *rbcL* and the 88-taxon *atpB* data sets produced almost identical topologies to their respective BI analyses. The relationships amongst the major clades in the 88-taxon *rbcL* (Figure 2.3f) analyses were supported with high bootstrap (BS) values, with the exception of the relationship between the vittaroids and *Adiantum* (BS=56%) and also the ceratopteridoids to the pteridoids (BS=54%). Again, the 88-taxon *atpB* (Figure 2.3c) data set showed the cryptogrammooids form a clade with the ceratopteridoids which was sister then to the pteridoids but these relationships were not supported with high bootstrap values (BS=59% and BS=62%, respectively). The cheilanthoids and adiantoids were sister to one another as expected. Maximum likelihood analysis for the 88-taxon *atpB+rbcL* data set (Figure 2.3i) produced a more resolved topology to that of its Bayesian Inference, with the clade containing the ceratopteridoids and pteridoids recovered as sister to the cryptogrammooids but again this was not supported by high bootstrap values (BS <50%).

Within the adiantoids, incongruence was found among the relationships of the genus *Adiantum* and the members of the vittaroids. The 88-taxon *rbcL* and 88-taxon *atpB* analyses resolve *Adiantum* as paraphyletic with vittaroids grouping with *Adiantum* I and not *Adiantum* II. In the 88-taxon *atpB* data set, *A. tenerum* had affinities to the *Adiantum* II as opposed to the *Adiantum* I. A monophyletic *Adiantum* was resolved in the 88-taxon combined gene data set but did not receive high support from bootstrap values (BS<50%).

2.3.3 Phylogenetic relationships of the New Zealand Pteridaceae species

The New Zealand representatives mostly group together within their respective major clades. According to the 220-taxon *rbcL* data set the members of the New Zealand *Cheilanthes* (Figure 2.4a) group with one another and are sister to *C. micropteris*, the type species of *Cheilanthes*, in both the BI and MP analyses. The New Zealand species of the genus *Pellaea* (Figure 2.4a) form a monophyletic group that corresponds to *Pellaea* sect. *Platyloma* (Gastony and Rollo 1995, Kirkpatrick 2007). These species form a clade with some of the members of the genus *Paragymnotopteris* rather than other members of the genus *Pellaea*. However, the New Zealand *Pellaea* and some *Paragymnotopteris* are sister to a clade which contains the type species for *Pellaea*, *P. atropurpurea*. The New Zealand members of the genus *Adiantum* (Figure 2.4b) are paraphyletic with South American *A. raddianum* and *A. cuneatum* being more closely related to the New Zealand representatives rather than to other *Adiantum* species native to South America.

The members of the New Zealand *Pteris* (Figure 2.4c) are polyphyletic with *Pteris tremula* being grouped with the majority of the overseas *Pteris* species (excluding *P. vittata*). *Pteris vittata*, which is thought to have close affinity to the type species of *Pteris*, *P. longifolia* L., is not closely allied to the other sampled members of the genus *Pteris*, suggesting this genus is polyphyletic. It was not surprising, however, that the other native New Zealand representatives of *Pteris* (i.e., *P. macilenta*, *P. saxatilis*, and *P. comans*) are grouped together as they are part of what believed to be a hybrid complex where *P. macilenta* is an allopolyploid of *P. saxatilis* and *P. comans* (Braggins 1975, Brownsey and Smith-Dodsworth 2000). Lastly, the only New Zealand representative of the genus *Anogramma* (Figure 2.4c) was grouped with other members of the subfamily Taenitideae, in which it was grouped with other members of the genus *Anogramma*. It was, however, distinct from the duplicate sample of *A. leptophylla* from Mexico.

Figure 2.4a. The 50% majority rule consensus phylogeny for MP analysis of the 220-taxon *rbcL* data set. The five putative clades are shown but only the relationships within the cheilanths are represented here. Thickened branches indicate well supported bootstrap values (>70%) and good posterior probabilities (>0.95). New Zealand taxa are represented by an * and bold text.

Figure 2.4c. The 50% majority rule consensus phylogeny for MP analysis of the 220-taxon rbcL data set. The five putative clades are shown but only the relationships within the pteridoids and represented here. Thickened branches indicate well supported bootstrap values (>70%) and good posterior probabilities (>0.95). New Zealand taxa are represented by an * and bold text.

2.4 Discussion

2.4.1 Incongruence between single and combined gene phylogenies

The consistent resolution of five major clades within the Pteridaceae in this study was in agreement with the results of Schuettpelz et al. (2007) and Prado et al. (2007). However, there were differences between the topologies of the individual and combined gene analyses. For example, the incongruence that exists among the relationship of the cryptogrammoideae to the Pteridaceae between 88-taxon *rbcL* and 88-taxon *atpB* data sets and also the 88-taxon *atp+rbcL* data set. Although the chloroplast genes, *rbcL* and *atpB*, code for different enzymatic functions, and hence, are subject to different evolutionary constraints, they are independent data sets representing the evolutionary history for that part of the chloroplast genome (Wolf 1997). However, these two genes are expected to reflect the same history as they are both located in the large single-copy region of the plastid genome (Wolf 1997, Savolainen et al. 2000).

The phylogenetic utility of *atpB* has been previously studied among plants (Wolf 1997, Savolainen et al. 2000, Guillon 2007). These studies reported a similar level of incongruence between the single gene analyses of *atpB* and *rbcL*, and they also reported the increased resolution with the combination of *atpB* with other genes. A study on the phylogeny of *Equisetum* compared DNA sequence data from *atpB* with three other chloroplast genes (Guillon 2007). Their results indicated incongruence only in topologies recovered from analyses of the *atpB* sequence data. Comparison of divergence rates and pairwise comparisons of the two genes suggests that *rbcL* has a slightly faster rate of change, suggesting *atpB* could be more reliable at inferring relationships of older divergence than *rbcL* (Wolf, 1997).

These studies also reported that single *rbcL* and combined gene data sets usually provided largely congruent phylogenies as well as increased support when the genes were combined (Wolf 1997, Savolainen et al. 2000). Surprisingly, in the present study there was a lack of resolution and a decrease in support for the placement of cryptogramroids when the two data sets were combined (Figure 2.3g-i). In both studies, Schuettpelz et al. (2007) and Prado et al. (2007), the *rbcL* data alone provides a poorly resolved (MP, Prado) or poorly supported (BI, Schuettpelz and Prado) position of the cryptogramroids in the Pteridaceae phylogeny (Figure 2.1). This was concordant with results from this study. However, signal from the *atpB* sequence data, which favors the affinity of the cryptogramroids to the ceratopteridoids, appeared strong enough to produce an unresolved position for cryptogramroids (BI) or a poorly supported clade containing the cryptogramroids and the pteridoid+ceratopteridoids clade (ML) when the *atpB* and *rbcL* genes were combined.

The multi-gene analyses in Schuettpelz et al. (2007) included another chloroplast gene, *atpA*, and they reported robust support for the relationships between the major Pteridaceae clades. The *atpA* gene has been recently described as a particularly useful marker for detecting phylogenetic signal (Schuettpelz et al. 2006). It appears that the addition of *atpA* sequence data provided even more resolution than just *atpB* and *rbcL* alone or even the combination of the two. It is generally agreed upon that multiple gene analyses aid in providing more resolution to phylogenetic relationships and that single gene phylogenies are that of the gene itself and not necessarily the organismal phylogeny (Wolf 1997, Savolainen et al. 2000, Sàñches-Baracaldo 2004, Smith et al. 2006, Guillon 2007, Schuettpelz et al. 2007, Zhang et al. 2007). The results from this study confirm that it is important to include different chloroplast loci when inferring relationships as each may yield incongruent phylogenies.

2.4.2 Parsimony analyses and Long Branch Attraction

Maximum parsimony analyses produced incongruent results with regard to the relationship of the ceratopteridoids within the Pteridaceae. The 88-taxon *rbcL*, 88-taxon *atpB*, and 88-taxon *atpB+rbcL* data set were not in agreement with phylogenies of previous studies (Prado et al. 2007, Schuettpelz et al. 2007) or with analyses employing different methods in the present study. This difference in topologies seems to be an issue in the methods of analysis and possibly also the taxon set used. The relationship of the ceratopteridoids as sister to the rest of the Pteridaceae as opposed to being sister to the pteridoids was different from the results of Schuettpelz et al. (2007) and Prado et al. (2007). However, incongruent relationships have been previously reported results from parsimony analyses. For example, a study focusing on tree ferns reported similar incongruent topologies when performing maximum parsimony analyses (Korall et al. 2006).

The phenomenon of long-branch attraction (LBA) was the most reasonable explanation for these relationships, as MP analyses are particularly susceptible to LBA (Kennedy et al. 2005). This occurs when taxa within a data set that have particularly long branches are attracted to distant outgroups. Although LBA tends to be a scapegoat for unexplainable topologies in many phylogenetic studies (Anderson and Swofford 2004, Bergsten 2005, Kennedy et al. 2005), there are certain indicators that imply a topology is experiencing LBA effects. It has been suggested that certain data sets under suspicion should be reanalyzed excluding the outgroups (Anderson and Swofford 2004, Bergsten 2005). If the exclusion of the outgroups resolves the correct ingroup topology, then there is a good chance that the data set is experiencing LBA, and furthermore the ingroup-alone topology is more likely to be accurate (Bergsten 2005).

Other indicators that MP analyses are experiencing LBA are simple comparisons of topologies from MP to that of model based analyses such as maximum likelihood and Bayesian Inference (Bergsten 2005). In the present study, comparisons were made of the topologies recovered from MP analyses to the topologies from BI and ML. The comparisons indicate that maximum likelihood and Bayesian Inference, although not strongly supported, are in agreement with one another and topologies recovered from MP were largely incongruent. Given the suspicion of LBA, all three data sets were rerun with the same parsimony parameters but excluding the outgroups. The ceratopteridoids regained their expected affinity to the pteridoid clade. However, the cryptogrammoides tended to have affinity to the pteridoids and/or the ceratopteridoids when outgroups are excluded (unpublished data) even in the 88-taxon *rbcL* data set.

The studies of Schuettpelz et al. (2007) and Prado et al. (2007) resolved the ceratopteridoids as being sister to the pteridoid clade. Schuettpelz et al. (2007) used similar genetic markers for DNA sequence data to that of the present study, yet did not run MP analyses. However, Prado et al. (2007) performed MP analyses and recovered topologies that were in agreement with Schuettpelz et al. (2007) with respect to the position of the ceratopteridoids, but they used different outgroups and different taxa in the data set. To investigate the effects of outgroup taxa on the topology in this study, each data set was rerun using the same parsimony settings but with the current outgroup taxa replaced by the outgroup taxa from Prado et al. (2007). The resulting topology was in agreement with the BI topologies from Schuettpelz et al. (2007). Furthermore, the data set from Prado et al. (2007) was run using the outgroup taxa from this study and the same correct ingroup topology was obtained suggesting that the taxonomic sampling used in Prado et al. (2007) was more conducive to filling in the branches and avoiding effects of LBA. Results from the MP analyses of the expanded *rbcL* data set also suggest that increased taxonomic sampling helped avoid the effects of LBA.

2.4.3 Increased taxonomic sampling

Creating taxonomic classifications that comprise monophyletic groups is regarded by many taxonomists as desirable (Potter and Freudenstein 2005, Stevens 2006). The results from this study indicated that the vittaroids are indeed included as part of the adiantoids. But the relationship between *Adiantum* and the vittaroids in this study remains unclear. The results from the 88-taxon *atpB+rbcL* BI and ML analyses indicate that *Adiantum* was monophyletic though it does not receive good support from either PP or BS values. This is in contrast to the paraphyletic relationship with respect to the vittaroids in the single gene analyses.

Both single gene *rbcL* data from Schuettpelz et al. (2007) and Prado et al. (2007) recovered a paraphyletic relationship for the genus *Adiantum*. In contrast, the multi-gene analyses (*atpB*, *rbcL*, and *atpA*) in Schuettpelz et al. (2007) recovered a monophyletic *Adiantum*, though this was not well supported (Figure 2.1). A latter study by Schuettpelz and Pryer (2007) used the same taxonomic sampling of the adiantoids and the same three chloroplast loci in a larger data set comprising representatives for all leptosporangiate ferns. Their results recovered *Adiantum* as paraphyletic showing the same relationships as those recovered from the single gene *rbcL* analyses in Schuettpelz et al. (2007).

The 220-taxon *rbcL* data set also recovered a monophyletic *Adiantum*. The additional taxonomic sampling included in this data set provided resolution for the monophyly of *Adiantum*, suggesting that resolution and support for relationships can be improved by both increasing sequence data and increasing the number of sequence loci. Although, the 220-taxon *rbcL* data set was a marked improvement on taxonomic sampling, it only sampled 25 of approximately 150 *Adiantum* species and 30 of the predicted 300 species of the vittaroids. As additional species are added to the data set, relationships of the adiantoids should become clearer, as the vast

amount of present incongruence might be a reflection of insufficient sampling. Hence, until sufficient sampling is obtained, taxonomic revisions would be hasty.

2.4.4 Phylogenetic relationships of the New Zealand Pteridaceae

The topologies recovered from this study show that the species of *Adiantum* sampled form two distinct clades. New Zealand representatives of *Adiantum* fall into a clade with the addition of Brazilian *A. raddianum* and *A. cuneatum*. Tyron and Tryon (1982) suggested a classification for the genus *Adiantum* by dividing them into eight groups based loosely on morphological characters (Tryon and Tryon 1982). The New Zealand species were placed into three different groups. *Adiantum aethiopicum*, which also grows in Australia, Norfolk Island, and South Africa, was placed in a group with the type species of *Adiantum*, *A. capillus-veneris*. Also included in this group were *A. raddianum* and *A. cuneatum*. *Adiantum diaphanum*, which also grows in Australia, areas of south-western Asia, and Pacific Islands as far east as Fiji, was placed in a group with *A. pedatum*, which according to the results of the 220-taxon *rbcL* data set, had affinity to *A. capillus-veneris*. The remainder of the New Zealand species, excluding the endemic species *A. viridescens* and *A. cunninghamii*, were placed in a group with *A. pectinatum* (not sampled in this study) which has a distribution predominately in South America. It was clear that this classification based on morphology was not concordant with molecular data, and intense sampling is needed to produce a phylogeny-based classification. However, it is beginning to be apparent that there is support for a possible South West Pacific group within *Adiantum*, and this group appears distinct from that group with affinity to the type, *A. capillus-veneris*.

The New Zealand members of *Pteris* are situated in separate positions within the clade containing the majority of the species in the genus. *Pteris tremula* was grouped with the other *P. tremula* sample and other sampled *Pteris* species, most of which are

from South America. The other three New Zealand species, *P. macilenta*, *P. saxatilis*, and *P. comans* were sister to a clade containing *P. arborea* from South America and *Ochropteris pallens* from Madagascar. The majority of the samples of *Pteris* were placed in a clade separate from *P. vittata*. It has been suggested, though not confirmed by molecular data, that the type specimen for *Pteris* (*P. longifolia* L.) would have affinity to *Pteris vittata* (Tryon and Tryon 1982, Schuettpelz et al. 2007), suggesting polyphyly in the genus. Like the genus *Adiantum*, only a small number of *Pteris* species were sampled for this study. There are approximately 300 species in the genus *Pteris*, and with increased sampling a better understanding of the relationships among the genus would become clearer, particularly amongst the species of *Pteris* that have affinity to *P. vittata*.

The New Zealand *Pellaea* form a monophyletic group within the cheilanthoid clade. This group corresponds to the *Pellaea* section *Platyloma* as described by Tryon and Tryon (1982). Other representatives of the section *Platyloma* include the Australian *P. paradoxa* and *P. nana*. Though not sampled here, they group with the New Zealand representatives (unpublished data Chapter 3). *Pellaea* sect. *Platyloma* is nested within the clade consisting of members of *Pellaea* sect. *Pellaea*, which comprises ferns mostly occurring in South America (Gastony and Rollo 1995, Kirkpatrick 2007). Asian ferns from the genus *Paragymnopteris* had the closest affinity to the New Zealand *Pellaea*. Based on results from Kirkpatrick (2007), Australian *Paraceterach muelleria* is also grouped with members of New Zealand *Pellaea*.

Paraphyletic and polyphyletic relationships are rampant in the cheilanthoid ferns particularly in the genus *Pellaea*, as the representatives of *Pellaea* sampled for this study are highly polyphyletic, being situated in several different positions within the tree. These results support claims that convergent morphologies have complicated generic circumscriptions for these ferns (Tryon and Tryon 1982,

Gastony and Rollo 1995, Kirkpatrick 2007, Zhang et al. 2007). Taxonomic revisions for these ferns have previously been suggested (Kirkpatrick 2007). However, additional sampling will be required in order to accurately represent *Pellaea* ferns and their relationships to the other cheilanthoid ferns on a global level.

The New Zealand species of *Cheilanthes* also occur in Australia and other islands of the South Pacific. Their affinity to the type species for *Cheilanthes* was supported in both the MP and BI of the 220-taxon *rbcL* data set. However, there are approximately 13 other species of *Cheilanthes* that occur in Australia and the South Pacific, none of which were sampled for this study. The inclusion of the South Pacific *Cheilanthes* to this data set would provide a better picture of the relationships of these *Cheilanthes* to the type specimen and hence, other members of the cheilanthoid ferns.

Lastly, the only species of the genus *Anogramma* occurring in New Zealand, *A. leptophylla* (the type species for *Anogramma*), showed affinity for other members in the genus, but it was, however, distinct from the other sample of *A. leptophylla*, from Mexico. The New Zealand sample was more closely related to the African *A. caespitosa* than to the central American *A. guatemalensis* and Mexican *A. leptophylla*. Tryon and Tryon (1982) commented that *A. leptophylla* was the most widely distributed and also the most polymorphic species of the genus (Tryon and Tryon 1982). They also commented that several robust morphological characters of *A. leptophylla* have caused confusion with distinguishing it from *A. guatemalensis* and *A. caespitosa*. The genus was also paraphyletic with members of the genus *Pityrogramma* being nested within the members of *Anogramma*.

2.4.5. Estimated divergence times

The Pteridaceae family is a lineage of polypod ferns within the core leptosporangiate ferns (Pryer et al. 2004). Pryer et al. (2004) inferred the divergence of the core leptosporangiate ferns to have occurred during the Triassic period (~220 mya). The polypod lineage began to diversify in the Jurassic (~183 mya to 160 mya) and contains some of the most species diverse lineages, specifically the Pteridaceae. According to Pryer et al. (2004), this lineage diverged during the late Cretaceous (~76 mya). In addition, Perrie and Brownsey (2007) investigated the divergence times a New Zealand species of each of *Adiantum* and *Pteris* with respect to their closest (available) non-New Zealand relative. Their inferred divergence times were approximately 20 and 30 mya, respectively (Perrie and Brownsey 2007).

Thoughts on the origins for the flora of New Zealand are centered on two main hypotheses. Either the flora in New Zealand has been isolated since New Zealand's separation from Gondwana (~80 mya) or the flora has arrived in New Zealand as a result of long-distance dispersal. The recent divergence of the Pteridaceae ferns supported both by Pryer et al. (2004) and Perrie and Brownsey (2007) imply that they are the result of long-distant dispersal. Although long-distant dispersal has been suggested by several studies (Brownsey 2001, Winkowrth et al. 2002, Perrie et al. 2007, Perrie and Brownsey 2007), the direction of dispersal could not be determined because of the lack of a phylogeny containing large amounts of overseas relatives. The 220-taxon *rbcL* data set shows that the New Zealand Pteridaceae species are nested within the overseas diversity, indicating their respective groups have originated elsewhere and immigrated to New Zealand.

2.4.6. Conclusions

As new taxa from the Pteridaceae family are sequenced from around the world and as new DNA sequence markers are developed, the resolution of the relationships in this family will continue to improve. Large scale analyses, coupled with studies focusing on particular segregates (e.g., *Pellaea*, *Cheilanthes*, *Pteris*, and *Adiantum*) will help to provide a robust phylogeny of the Pteridaceae, not only between the subfamilies, but the relationships among the genera within these subfamilies. The New Zealand Pteridaceae species are representatives from a previously under-sampled part of the world. Their inclusion particularly provided a better picture for the relationships among the genus *Adiantum*, as well as representing the members of *Pellaea* sect. *Platyloma* among the cheilanthoid ferns. The Pteridaceae is a large and diverse family of ferns and quite possibly could continue to grow with regard to size and diversity as new species are continually being discovered (Prado 2006). There are several genera that require taxonomic revision. However it is clear now that until adequate taxonomic sampling is achieved, a revision, based on the currently available data, would be premature.

2.5 References

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Table 2.1. List of taxonomic sampling from the current study, including available voucher information and accession numbers. The New Zealand taxa will have collection numbers until voucher information is available. – indicates where no accession numbers are available.

Species Name	Voucher information	<i>rbcL</i> GenBank Accession	<i>atpB</i> GenBank Accession
New Zealand taxa			
<i>Adiantum aethiopicum</i> L.	LRP3637	--	--
<i>Adiantum capillus-veneris</i> L.	WB10	--	--
<i>Adiantum cunninghamii</i> Hook.	LRP3153	--	--
<i>Adiantum diaphanum</i> Blume	LRP4392	--	--
<i>Adiantum formosum</i> R.Br.	LRP4987	--	--
<i>Adiantum fulvum</i> Raoul	WB02	--	--
<i>Adiantum hispidulum</i> Sw.	LRP4834	--	--
<i>Adiantum pubescens</i> Shukhur	PO20741	--	--
<i>Adiantum raddianum</i> C.Presl	WB09	--	--
<i>Adiantum viridescens</i> Colenso	WB04	--	--
<i>Anogramma leptophyllum</i> (L.) Link	LRP3632	--	--
<i>Cheilanthes distans</i> (R.Br.) Mett.	LRP3646	--	--
<i>Cheilanthes sieberi</i> Kunze	LRP3630	--	--
<i>Pellaea caldirupium</i> Brownsey et Lovis	LRP3884	--	--
<i>Pellaea</i> aff. <i>falcata</i>	LRP3607	--	--
<i>Pellaea falcata</i> (R.Br.) Fee	PO20738	--	--
<i>Pellaea rotundifolia</i> (G.Forst.) Hook.	LRP3972	--	--
<i>Pteris macilenta</i> A.Rich.	LRP4995	--	--
<i>Pteris comans</i> G.Forst.	PO20739	--	--
<i>Pteris tremula</i> R.Br.	LRP3766	--	--
<i>Pteris saxatilis</i> Carse	LRP3662	--	--
Other Pteridaceae taxa sourced from GenBank			
<i>Acrostichum aureum</i> L.	Masuyama et al. (2002)	AB059586	--
<i>Acrostichum danaeifolium</i> Langsd. et Fisch.	Schuettpelz 616 (B)	EF452129	EF452008
<i>Actiniopteris dimorpha</i> Pic. Serm.	Schneider s.n. (GOET)	EF452130	EF452009
<i>Actiniopteris radiata</i> (Sw.) Link	Gastony and Johnson (2001)	AF336100	--
<i>Adiantopsis chlorophylla</i> (Sm.) Fee	Prado and Yano 1047	EF473684	--
<i>Adiantopsis radiata</i> (L.) Fee	Christenhusz 4033 (TUR)	EF452131	EF452010
<i>Adiantum cajennense</i> Willd.	Prado et al. 1201	EF473765	--
<i>Adiantum capillus-veneris</i> L.	Hasebe et al. (1993)	D14880	--
<i>Adiantum capillus-veneris</i> L.	Wolf et al. (2003)	AY178864	AY178864
<i>Adiantum cuneatum</i> Langsd. et Fisch.	Prado and Yano 1078	EF473676	--
<i>Adiantum latifolium</i> Lam.	Prado et al. 1294	EF473677	--
<i>Adiantum malesianum</i> J. Ghatak	Huiet 111 (UC)	EF452132	EF452011
<i>Adiantum obliquum</i> Willd.	Prado et al. 1345	EF473678	--
<i>Adiantum pedatum</i> L.	Hasebe et al. (1994), Huiet 117 (UC)	U05602	EF452012
<i>Adiantum pentadactylon</i> Langsd. et Fisch.	Prado et al. 1085	EF473679	--

Species Name	Voucher information	<i>rbcL</i> GenBank Accession	<i>atpB</i> GenBank Accession
<i>Adiantum peruvianum</i> Klotzsch	Huiet 103 (UC)	EF452133	EF452013
<i>Adiantum raddianum</i> C. Presl	Prado and Yano 1077	EF473680	--
<i>Adiantum raddianum</i> C. Presl	Wolf et al. (1994), Wolf (1997), Wolf 717 (UTC)	U05906	U93840
<i>Adiantum serratodentatum</i> Humb. et Bonpl. ex Willd.	Prado et al. 1545	EF473681	--
<i>Adiantum subcordatum</i> Sw.	Prado and Yano 1075	EF473682	--
<i>Adiantum terminatum</i> Kunze ex Miq.	Prado et al. 1354	EF473683	--
<i>Adiantum tenerum</i> Sw.	Huiet 107 (UC)	EF452134	EF452014
<i>Adiantum tetraphyllum</i> Humb. et Bonpl. ex Willd.	Huiet 105 (UC)	EF452135	EF452015
<i>Adiantum venustum</i> D. Don	Huiet 116 (UC)	EF452136	--
<i>Aleuritopteris albomarginata</i> (C.B. Clarke) Ching	Zhang et al (2005)	AY266411	--
<i>Aleuritopteris argentea</i> (S.G. Gmelin) Fee	Yatskievych 01-23 (MO)	EF452137	EF452016
<i>Aleuritopteris duclouxii</i> (Christ) Ching	Gastony and Rollo (1995)	U27447	--
<i>Aleuritopteris formansana</i> (Hayata) Tagawa	Gangmin Zhang 458	DQ432643	--
<i>Aleuritopteris grisea</i> (Blanf.) Panigrahi	Zhang et al. (2005)	AY299653	--
<i>Aleuritopteris kuhii</i> (Milde) Ching	Zhang et al. (2005)	AY266412	--
<i>Aleuritopteris likiangensis</i> Ching	Gangmin Zhang 278	DQ432645	--
<i>Aleuritopteris niphobola</i> (C. Chr.) Ching	Zhang et al. (2005)	AY266409	--
<i>Aleuritopteris squamosa</i> (Hope et C.H. Wright) Ching	Shiyong Dong 28	DQ432642	--
<i>Aleuritopteris tamburii</i> (Hook) Ching	Gangmin Zhang 434	DQ732644	--
<i>Ananthacorus angustifolius</i> Underw. et Maxon	Crane et al. (1995)	U20932	--
<i>Anetium citrifolium</i> (L.) Splitg.	Crane et al. (1995), Christenhusz 4076 (TUR)	U21284	EF452017
<i>Anogramma caespitosa</i> Pic. Serm.	Nakazato and Gastony (2003)	AY168718	--
<i>Anogramma chaerophylla</i> (Desv.) Link	Nakazato and Gastony (2003)	AY168712	--
<i>Anogramma quatemalensis</i> (Domin) C. Chr.	Nakazato and Gastony (2003)	AY168716	--
<i>Anogramma leptophylla</i> (L.) Link	Nakazato and Gastony (2003)	AY168715	--
<i>Anogramma lorentzii</i> (Hieron.) Diels	Gastony and Johnson (2001)	AF336102	--
<i>Anogramma novogaliciana</i> Mickel	Nakazato and Gastony (2003) Ruanker 1774 (COLO)	AY168714	--
<i>Antrophyum boryanum</i> (Willd.) E.H. Crane	Crane et al. (1995)	U20930	--
<i>Antrophyum ensiforme</i> Hook.	Crane et al. (1995)	U20931	--
<i>Antrophyum latifolium</i> Blume	Ranker 1774 (COLO)	EF452138	EF452018
<i>Antrophyum plantagineum</i> (Cav.) Kaulf.	Crane et al. (1995)	U21285	--
<i>Antrophyum reticulatum</i> (G. Forst.) Kaulf.	Hasebe et al. (1994)	U05604	--
<i>Argyrochosma fendleri</i> (Kunze) Windham	Gastony and Rollo (1995)	U27727	--

Species Name	Voucher information	<i>rbcL</i> GenBank Accession	<i>atpB</i> GenBank Accession
<i>Argyrochosma limitanea</i> (Maxon) Windham	Schuettpelz 472 (DUKE)	EF452139	EF452019
<i>Asplenium unilaterale</i> Lam.	Ranker 2072 (COLO)	EF452140	EF452020
<i>Astrolepis sinuate</i> (Lag. ex Sw.) D.M. Benham et Windham	Schueettpelz 310 (DUKE)	EF452141	EF452021
<i>Blechnum occidentale</i> L.	Wolf et al. (1994), Wolf (1997), Wolf 289 (UTC)	U05910	U93838
<i>Bommeria ehrenbergiana</i> (Kotzsch) Underw.	Gastony and Rollo (1995)	U19497	--
<i>Bommeria elegans</i> (Davenp.) Ranker & Haufler	Gastony & Rollo (1995)	U27729	--
<i>Bommeria hispida</i> (Mett. ex Kuhn) Underw.	Schuettpelz 467 (DUKE)	EF452142	EF452022
<i>Ceratopteris pteridoides</i> (Hook.) Hieron.	Masuyama et al. (2002)	AB059584	--
<i>Ceratopteris richardii</i> Brongn.	Masysyama et al. (2002), Pryer et al. (2004), Killip 44595 (GH)	AB059585	AY612691
<i>Ceratopteris thalictroides</i> (L.) Brongn.	Masuyama et al. (2002)	AB059573	--
<i>Cheilanthes alabamensis</i> (Buckley) Kunze	Schuettpelz 319 (DUKE)	EF452143	EF452023
<i>Cheilanthes allosuroides</i> Mett.	Gastony and Rollo (1995)	U27239	--
<i>Cheilanthes aurea</i> Baker	Gastony and Rollo (1995)	U28786	--
<i>Cheilanthes bonariensis</i> (Willd.) Proctor	Gastony and Rollo (1995)	U19499	--
<i>Cheilanthes californica</i> (Hook.) Mett.	Gastony and Johnson (2001)	AF336101	--
<i>Cheilanthes chinensis</i> (Baker) Domin	Gangmin Zhang 227	DQ432651	--
<i>Cheilanthes eatonii</i> Baker	Schuettpelz 323 (DUKE)	EF452144	EF452024
<i>Cheilanthes flexuosa</i> Kunze	Forza and Mello-Silva 1503	EF473686	--
<i>Cheilanthes goyazensis</i> (Taub.) Domin	Prado and B.-Blubaugh 1403	EF473687	--
<i>Cheilanthes horridula</i> Maxon	Gastony and Rollo (1995)	U27448	--
<i>Cheilanthes insignis</i> Ching	Zhang et al. (2005)	AY166413	--
<i>Cheilanthes intramarginalis</i> (Kaulf. ex Link) Trevis.	Gastony and Rollo (1995)	U27449	--
<i>Cheilanthes lanosa</i> (Michx.) D.C.Eaton	Gastony and Rollo (1995)	U27205	--
<i>Cheilanthes micropteris</i> Sw.	Deginani 1363 (MO)	EF452145	--
<i>Cheilanthes nitidula</i> Hook.	Schneider s.n. (GOET)	EF452146	EF452025
<i>Cheilanthes viridis</i> Sw.	Janssen 2701 (P)	EF452147	EF452026
<i>Cheiloplecton rigidum</i> (Sw.) Fee	Gastony and Rollo (1995)	U29133	--
<i>Cheilosoria chusana</i> (Hook.) Ching et K.H. Shing	Gangmin Zhang 641	DQ432650	--
<i>Cheilosoria hancockii</i> (Baker) Ching et K.H. Shing	Gangmin Zhang 282	DQ432649	--
<i>Cheilosoria patula</i> (Baker) P.S.Wang	Gangmin Zhang 228	DQ432663	--
<i>Coniogramme fraxinea</i> (D.Don) Fee ex Diels	Korall et al. (2006), Pryer et al. (2004), Korall et al. (2006)	AM177359	AY612693
<i>Coniogramme japonica</i> (Thunb.) Diels	Hasebe et al. (1994)	U05611	--
<i>Cosentinia vellea</i> (Aiton) Tod.	Nakazato and Gastony (2003)	AY168720	--

Species Name	Voucher information	<i>rbcL</i> GenBank Accession	<i>atpB</i> GenBank Accession
<i>Cryptogramma brunoniana</i> Wall. ex Hook. et Grev.	Zhang et al. (2005)	AY266407	--
<i>Cryptogramma stelleri</i> Prantl	Gangmin Zhang 306	DQ432660	--
<i>Cryptogramma crispa</i> (L.) R.Br. ex Hook.	Christenhusz 3871 (TUR)	EF452148	EF452027
<i>Cystopteris reevesiana</i> Lellinger	Schuettelz 419 (DUKE)	EF452149	EF452028
<i>Davallia solida</i> (G.Forst.) Sw.	Tsutsumi and Kato (2005), Schuettelz 300 (DUKE)	AB212712	EF452029
<i>Dennstaedtia punctilobula</i> (Michx.) T.Moore	Wolf et al. (1994), Wolf, 1997, Paris s.n. (UTC)	U05918	U93836
<i>Didymochlaena truncatula</i> (Sw.) J.Sm.	Smith and Cranfill (2002), Schuettelz 267 (DUKE)	AF425105	EF452030
<i>Doryopteris collina</i> (Raddi) J.Sm.	Prado and B.-Blubaugh 1402	EF472688	--
<i>Doryopteris concolor</i> (Langsd. et Fisch.) Kuhn	Zhang et al. (2005)	AY266414	--
<i>Doryopteris decora</i> Brack.	Gastony and Rollo (1995)	U27446	--
<i>Doryopteris lomariacea</i> Klotzsch	Prado and Yano 1045	EF473689	--
<i>Doryopteris ludens</i> (Wall. ex. Hook.) J.Sm.	Schneider s.n. (GOET)	EF452150	EF452031
<i>Doryopteris nobilis</i> (T.Moore) C.Chr.	Prado et al. 1119	EF473690	--
<i>Doryopteris ornithopus</i> (Hook. et Baker) J.Sm.	Prado and B.-Blubaugh 1399	EF473691	--
<i>Doryopteris paradoxa</i> (Fee) Chirst.	Prado et al. 1131	EF473692	--
<i>Doryopteris pedata</i> (L.) Fee	Gastony and Rollo (1995)	U27206	--
<i>Doryopteris pentagona</i> Pic. Serm.	Prado et al. 1100	EF473693	--
<i>Doryopteris rediviva</i> Fee	Prado et al. 1107	EF473694	--
<i>Doryopteris sagittifolia</i> (Raddi) J.Sm.	Schuettelz 562 (GOET)	EF452151	EF452032
<i>Dryopteris aemula</i> (Aiton) Kuntze	Geiger and Ranker (2005), Christenhusz 3866 (TUR)	AY268881	EF452033
<i>Eriosorus cheilanthoides</i> (Sw.) A.F.Tyron	Moran 7579 (NY)	EF452152	EF452034
<i>Eriosorus flexuosus</i> (Humb. et Bonpl.) Copel.	Nakazato and Gastony (2003)	AY168709	--
<i>Eriosorus myriophyllus</i> (Sw.) Copel.	Prado and Yano 1033	EF473710	--
<i>Haplopteris anguste-elongata</i> (Hayata) E.H.Crane	Crane et al. (1995)	U21291	--
<i>Haplopteris elongata</i> (Sw.) E.H.Crane	Hueit 122 (UC)	EF452153	EF452035
<i>Haplopteris ensiformis</i> (Sw.) E.H.Crane	Crane et al. (1995)	U21290	--
<i>Haplopteris flexuosa</i> (Fee) E.H.Crane	Hasebe et al. (1994)	U05656	--
<i>Haplopteris zosterifolia</i> (Willd.) E.H.Crane	Crane et al. (1995)	U21296	--
<i>Hecistopteris pumila</i> (Spreng.) J.Sm.	3976 (TUR)	U21286	EF452036
<i>Hemionitis levyi</i> E.Fourn.	Gastony and Rollo (1995)	U27725	--
<i>Hemionitis palmata</i> L.	Ranker and Geiger (unpublished), Schuettelz 297 (DUKE)	AY357708	EF452037
<i>Hemionitis rufa</i> (L.) Sw.	Ranker and Geiger (unpublished)	AY357707	--
<i>Hemionitis tomentosa</i> (Lam.) Raddi	Ranker and Geiger (unpublished)	AY357709	--

Species Name	Voucher information	<i>rbcL</i> GenBank Accession	<i>atpB</i> GenBank Accession
<i>Jamesonia blepharum</i> A.F.Tryon	Schuettzel 269 (DUKE)	EF452154	--
<i>Jamesonia canescens</i> Kunze	Nakazato and Gastony (2003)	AY168710	--
<i>Jamesonia osteniana</i> (Dutra) Gastony	Nakazato and Gastony (2003)	AY168711	--
<i>Jamesonia verticalis</i> Kunze	Moran 7593 (NY)	EF452155	EF452038
<i>Leptolepidium dalhousiae</i> (Hook.) K.H.Shing & S.K.Wu	Gangmin Zhang 328	DQ432646	--
<i>Llavea cordifolia</i> Lag.	Gastony and Rollo (1995), Schuettzel 377 (DUKE)	U27726	EF452039
<i>Microlepia platyphylla</i> (D.Don) J.Sm.	Wolf (1995), Wolf (1997), Wolf 596 (UTC)	U18642	U93832
<i>Monachosorum henryi</i> Christ	Wold et al. (1994), Pryer et al. (2004), Korall et al. (2006)	U05932	AY612706
<i>Monogramma</i> sp. (<i>dareicarpa</i>)	Ranker 1778 (COLO)	EF452156	--
<i>Monogramma graminea</i> (Poir.) Schkuhr	Janssen 2692 (P)	EF452157	EF4522040
<i>Nephrolepis cordifolia</i> (L.) C.Presl	Wolf et al. (1994), Wolf 309 (UTC)	U05933	EF452041
<i>Neurocallis praestantissima</i> Bory ex Fee	Christenhusz 3997 (TUR)	EF452158	EF452042
<i>Notholaena aschenborniana</i> Klotzsch	Schuettzel 476 (DUKE)	EF452159	EF452043
<i>Notholaena delicatula</i> Maxon et Weath.	Gastony and Rollo (1995)	U19500	--
<i>Notholaena fendleri</i> Kunze	Gastony and Rollo (1995)	U27727	--
<i>Notholaena rosei</i> Maxon	Gastony and Rollo (1995)	U27728	--
<i>Notholaena sulphurea</i> (Cav.) J.Sm.	Gastony and Rollo (1995)	U28254	--
<i>Ochropteris pallens</i> (Sw.) J.Sm.	Janssen 2677 (P)	EF452160	EF452044
<i>Onychium contiguum</i> Wall. ex C.Hope	Zhang et al. (2005)	AY266416	--
<i>Onychium japonicum</i> (Thunb.) Kunze	Hasebe et al. (1994), Schneider s.n. (GOET)	U05641	EF452045
<i>Onychium lucidum</i> (D.Don) Spreng.	Gastony and Johnson (2001)	AF360359	--
<i>Onychium plumosum</i> Ching	Zhang et al. (2005)	AY266408	--
<i>Onychium tenuifrons</i> Ching	Zhang et al. (2005)	AY266415	--
<i>Paragymnopteris bipinnata</i> (H.Christ) K.H. Shing	Gangmin Zhang 292	DQ432254	--
<i>Paragymnopteris dalavayi</i> (Baker) K.H.Shing	Gangmin Zhang 268	DQ432654	--
<i>Paragymnopteris marantae</i> (L.) K.H.Shing	Yatskievych 02-35 (KUN)	EF452161	EF452046
<i>Paragymnopteris sargentii</i> (H.Christ) K.H. Shing	Gangmin Zhang 341	DQ432653	--
<i>Paragymnopteris vestita</i> (Wall. ex Presl) K.H. Shing	Gangmin Zhang 640	DQ432652	--
<i>Parahemionitis arifolia</i> (Burm.f.) Panigrahi	Ranker and Geiger (unpublished)	AY357706	--
<i>Pellaea andromedifolia</i> (Kaulf.) Fee	Gastony and Rollo (1995)	U19501	--
<i>Pellaea atropurpurea</i> (L.) Link	Schuettzel 312 (DUKE)	EF452162	--
<i>Pellaea boivinii</i> Hook.	Gastony and Rollo (1995)	U29132	--

Species Name	Voucher information	<i>rbcL</i> GenBank Accession	<i>atpB</i> GenBank Accession
<i>Pellaea cordifolia</i> (Sesse et Moc.) A.R.Sm.	Gastony and Rollo (1995)	U28253	--
<i>Pellaea cymbiformis</i> J. Prado	Prado and B.-Blubaugh 1404	EF473697	--
<i>Pellaea gleichenioides</i> (Hook.) Christ	Prado and B.-Blubaugh 1398	EF473698	--
<i>Pellaea intermedia</i> Mett. ex Kuhn	Schuettelpelz 481 (DUKE)	EF452163	EF452047
<i>Pellaea pauperula</i> (H.Christ) Hall et Lellinger	Gangmin Zhang 260	DQ432640	--
<i>Pellaea pinnata</i> (Kaulf.) Prantl	Prado and B.-Blubaugh 1407	EF473699	--
<i>Pellaea pringlei</i> Davenp.	Gastony and Rollo (1995)	U28787	--
<i>Pellaea riedilii</i> Baker	Forza and Mello-Silva 1515	EF473703	--
<i>Pellaea rotundifolia</i> (G.Forst.) Hook.	Gastony and Rollo (1995)	U28788	--
<i>Pellaea trichophylla</i> (Baker) Ching	Gangmin Zhang 266	DQ432639	--
<i>Pellaea truncata</i> Goodd.	Schuettelpelz 430 (DUKE)	EF452164	EF452048
<i>Pentagramma triangularis</i> (Kaulf.) Yatsk., Windham et E.Wollenw.	Schuettelpelz 445 (DUKE)	EF452165	EF452049
<i>Pityrogramma austroamericana</i> Domin	Schuettelpelz 301 (DUKE)	EF452166	EF452050
<i>Pityrogramma calomelanos</i> (L.) Link	Gastony and Johnson (2001)	AF336103	--
<i>Pityrogramma jamesonii</i> (Baker) Domin	Moran 7592 (NY)	EF452167	--
<i>Pityrogramma trifoliata</i> (L.) R.M.Tryon	Gastony and Johnson (2001)	AF336104	--
<i>Platyzoma microphyllum</i> R.Br.	Nakazato and Gastony (2003), Kato 303 (TI)	AY168721	EF452051
<i>Polytaenium cajanense</i> (Desv.) Benedict	Crane et al. (1995), Schuettelpelz 211 (DUKE)	U20934	EF452052
<i>Polytaenium lanceolatum</i> (L.) Benedict	Crane et al. (1995)	U21287	--
<i>Polytaenium lineatum</i> (Sw.) J.Sm.	Crane et al. (1995)	U20935	--
<i>Pteridium esculentum</i> (G.Forst.) Nakai	Wolf et al. (1994), Wolf (1997), Smith s.n. (UC)	U05940	U93834
<i>Pteris arborea</i> L.	Christenhusz 4050 (TUR)	EF452168	EF452053
<i>Pteris argyraea</i> T.Moore	Schuettelpelz 542 (GOET)	EF42169	EF452054
<i>Pteris brasiliensis</i> Raddi	Prado et al. 1086	EF473702	--
<i>Pteris cretica</i> L.	Schuettelpelz 597 (DUKE)	EF452170	EF452055
<i>Pteris decurrens</i> C.Presl.	Prado and Yano 1082	EF473703	--
<i>Pteris deflexa</i> Link	Prado et al. 1089	EF473704	--
<i>Pteris denticulata</i> Sw.	Prado et al. 1084	EF473705	--
<i>Pteris fauriei</i> Hieron.	Hasebe et al. (1994)	U05647	--
<i>Pteris lechleri</i> Mett.	Prado et al. 1093	EF473706	--
<i>Pteris leptophylla</i> Sw.	Boldrin et al. 160	EF473707	--
<i>Pteris multifida</i> Poir.	Schuettelpelz 591 (GOET)	EF452171	EF452056
<i>Pteris propinqua</i> J.Agardh	Schuettelpelz 268 (GOET)	EF452172	EF452057
<i>Pteris quadriaurita</i> Retz.	Schuettelpelz 546 (GOET)	EF452173	EF452058
<i>Pteris splendens</i> Kaulf.	Prado 1131a.	EF473708	--
<i>Pteris tremula</i> R.Br.	Schuettelpelz 620 (B)	EF452174	EF452059

Species Name	Voucher information	<i>rbcL</i> GenBank Accession	<i>atpB</i> GenBank Accession
<i>Pteris vittata</i> L.	Wolf et al. (1994), Schuettzel 249 (DUKE)	U05941	EF452060
<i>Pterozonium brevifrons</i> (A.C.Sm.) Lellinger	Schuettzel 285 (DUKE)	EF452175	EF452061
<i>Radiovittaria gardneriana</i> (Fee) E.H.Crane	Crane et al. (1995), Schuettzel 249 (DUKE)	U21294	EF452062
<i>Radiovittaria minima</i> (Baker) E.H.Crane	Crane et al. (1995)	U21288	--
<i>Radiovittaria remota</i> (Fee) E.H.Crane	Crane et al. (1995)	U21289	--
<i>Radiovittaria stipitata</i> (Kunze) E.H.Crane	Crane et al. (1995)	U21293	--
<i>Rheopteris cheesmaniae</i> Alston	Croft 1749 (A, K)	EF452176	EF452063
<i>Sinopteris albofusca</i> (Baker) Ching	Gastony and Rollo (1995)	U19498	--
<i>Sinopteris grevilleoides</i> (H.Christ) C. Chr. et Ching	Gangmin Zhang 639	DQ432648	--
<i>Taenitis blechnoides</i> (Willd.) Sw.	Hasebe et al. (1994)	U05654	--
<i>Thelypteris palustris</i> Schott	Wolf et al. (1994), Pryer et al. (2004), Wolf 284 (UTC)	U05947	AY612713
<i>Trachypteris pinnata</i> (Hook.f.) C.Chr.	Gastony and Rollo (1995)	U27450	--
<i>Vittaria appalachiana</i> Farrar et Mickel	Crane (1997)	U88961	--
<i>Vittaria dimorpha</i> Mull.	Crane et al. (1995)	U21292	--
<i>Vittaria graminifolia</i> Kaulf.	Crane et al. (1995), Schuettzel 227 (DUKE)	U21295	--
<i>Vittaria isoetifolia</i> Bory	Crane et al. (1995)	U20936	--
<i>Vittaria lineata</i> (L.) Sm.	Crane et al. (1995)	U20937	--

Chapter 3

Morphological intermediates cause taxonomic confusion in New Zealand *Pellaea*

3.1 Introduction

The genus *Pellaea*, as defined by Tryon and Tryon (1982), comprises approximately 40 species. It is centered mainly in the United States, Mexico and South America, but it also extends through India and into the Pacific including Australia and New Zealand. Tryon and Tryon (1982) divided the genus into four sections: *Pellaea* (20 species in America and one in Africa), *Ormopteris* (five to seven species of South America, mostly in Brazil), *Holcochlaena* (about 10 species from Africa and three extending to India and Sri Lanka), and *Platyloma* (three species distributed in India, Sri Lanka, Tasmania, Australia, New Zealand, and New Caledonia. *Pellaea* sect. *Platyloma* has recently been expanded to include the new species *P. calidirupium* (Brownsey and Lovis 1990) and *P. nana* (Bostock et al. 1998). The

phylogenetic position of *Pellaea* sect. *Platyloma* is nested within *Pellaea* sect. *Pellaea*, with an affinity to the Australian *Paraceterach muelleri* and Asian species of the genus *Paragymnopteris*, according to Kirkpatrick (2007).

The New Zealand representatives of *Pellaea* sect. *Platyloma* are *Pellaea rotundifolia* (Forst.) Hook., *P. falcata* (R. Br.) Fee and *P. calidirupium* Brownsey et Lovis, as recorded in Brownsey and Smith-Dodsworth (2000). *Pellaea rotundifolia* is endemic to New Zealand and is quite commonly found throughout the North and South Islands. The distribution of *P. calidirupium* is predominately on the South Island although it has also been found in areas around Wellington and Auckland on the North Island (Brownsey and Lovis 1990). The rarer *P. falcata* is thought to grow on off shore islands (e.g., Kermadec Islands, Three Kings Island) and coastal patches on the mainland north of Auckland, according to Brownsey and Smith-Dodsworth (2000). However, its distribution throughout New Zealand is uncertain (for distribution maps see Appendix A).

Both *Pellaea calidirupium* and *P. falcata* are found growing outside of New Zealand. In Australia, these species are mostly restricted to the east coast in Queensland, New South Wales and Victoria, and Tasmania. *Pellaea falcata* is also found in New Caledonia, Lord Howe Island and the Kermadec Islands; on the latter it is the only *Pellaea* species present (Sykes et al. 2000). The other members of section *Platyloma* growing outside of New Zealand include *P. nana*, and *P. paradoxa*. Both of these occur in Australia and the latter is also found on Lord Howe Island (Bostock et al. 1998). Interestingly, the species shared between New Zealand and Australia, New Zealand plants are tetraploid whereas Australian plants are diploid (Kokubugata et al. 2006).

The genus *Pellaea* is characterized as terrestrial or rupestral ferns with scaly creeping rhizomes. Their fronds are pinnate, scaly and the veins are free. The sori are continuous along pinnae margins though often absent at apices. Brownsey and Smith-Dodsworth (2000) also note that this genus is distinct in New Zealand with pinnate fronds with unprotected sori on pinnae margins and that the three species are not clearly defined and require further investigation.

The three species in New Zealand are most often differentiated from one another by three main morphological characteristics: extent of marginal sori, nature of rachis scales, and pinnule length and shape. *Pellaea rotundifolia* typically has sori absent from the pinnae apices, spreading scales on the rachis, and round to oblong shaped pinnae, 8 to 20mm in length. *Pellaea falcata* also has sori absent from the pinnae apices, spreading scales, but mostly oblong shaped pinnae 15 to 40mm in length. Plants of *P. calidirupium* differ in having sori continuous around their margins and appressed scales, with round to oblong pinnae 8 to 40mm in length (Brownsey and Smith-Dodsworth 2000).

Although *P. calidirupium* is usually easily distinguished by its appressed rachis scales, it is often difficult to distinguish *P. rotundifolia* from *P. falcata* particularly because of plants with intermediate morphologies. In New Zealand intermediate plants, which will hereafter be referred to as *P. aff. falcata*, have caused taxonomic confusion and led to the suspicion of *P. falcata* growing beyond its distribution as recognized in Brownsey and Smith-Dodsworth (2000). These *P. aff. falcata* plants more often identified as *P. falcata* than *P. rotundifolia* on herbarium specimens, have been reported from as far south as Geraldine on the South Island according to herbarium records held in CHR (e.g., CHR 510910; herbarium abbreviations follow Holmgren et

al. 1990). Morphologically, they have pinnae ranging from 15 to 40mm and their pinnules are mostly oblong rather than round. It is unknown whether these *P. aff. falcata* plants are more closely related to *P. falcata*, *P. rotundifolia*, or whether they are a new *Pellaea* species.

DNA sequence data from non-coding *trnL-trnF* locus (*trnL* intron, *trnL* 3'-exon, and *trnL-trnF* intergenic spacer), *rps4* gene, and the *rps4-trnS* intergenic spacer (IGS) were obtained and analysed to determine the relationships between the New Zealand *Pellaea* species. A specific goal was to determine the relationships of *P. aff. falcata* to the other New Zealand *Pellaea* species. The *trnL-trnF* locus evolves quickly and is particularly useful for investigating interspecific variation (Perrie and Brownsey 2005b). The *rps4* and the *rps4-trnS* IGS are another widely used gene and spacer for phylogenetic studies (Sánchez-Baracaldo 2004, Kirkpatrick 2007). Both of these regions had DNA sequence data available on GenBank for many species, for the basis of comparison and the use as outgroups. This coupled with their phylogenetic properties make them ideal markers for studying the New Zealand *Pellaea*.

Using morphology to classify cheilanthoid ferns has been misleading as shown by recent molecular based studies of these ferns (Gastony and Rollo 1995, Kirkpatrick 2007, Zhang et al. 2007). The cause of this morphological homoplasy has been attributed to convergent adaptation to xeric habitats (Copeland 1947, Tyrone et al. 1990, Gastony and Rollo 1995). Though the members making up the New Zealand *Pellaea* are few, this explanation appears opposite to what is observed in New Zealand, where plants with a wide range of pinnule morphologies are found growing in the same population.

Because clear species boundaries are lacking for the majority of the members of *Pellaea* sect. *Platyloma*, accurate identification of these species can be difficult. It has been suggested that problems identifying *Pellaea* members extends beyond New Zealand and it is a trans-Tasman problem with the genus (Peter de Lange, personal communication). Furthermore, it is clear that in New Zealand traditional taxonomic characters are inadequate for consistently discerning *P. aff. falcata* from either *P. rotundifolia* or *P. falcata*. Finding a character for consistent species identification in New Zealand and between the other members of *Pellaea* sect. *Platyloma* is desirable, as utilizing molecular data to positively confirm identifications is not always feasible.

The aim of this study is to determine the relationships of the New Zealand *Pellaea* species through the use of DNA sequence data. The following questions were addressed: 1) What are the relationships of the morphologically intermediate *P. aff. falcata* plants? 2) How does morphological variation correspond to the variation found with the molecular data? 3) What are the relationships of the New Zealand members to the overseas representative of *Pellaea* sect. *Platyloma*?

3.2 Materials & Methods

3.2.1 Sample collection

This study examines 35 exemplars from the genus *Pellaea* in New Zealand. Twenty samples of *Pellaea rotundifolia* and five samples of *P. calidirupium* were collected from several wild populations from both the North and South Islands (Figure 3.1). Samples of *P. rotundifolia* were collected to encompass the diversity found in pinnule morphology. Five of these samples had a

Figure 3.1. Distribution map of *Pellaea* samples collected for this study. Colors represent the haplotype: red = 1, blue = 2, yellow = 3, and green = 4. The shapes correspond to pinnule morphology: circle = *P. rotundifolia*, square = *P. aff. falcata*, triangle = *P. calidrupium*, and oval = *P. falcata*. Samples beginning with P are in the WELT herbarium. WB, LRP, and EK are collection numbers for Whitney Bouma, Leon Perrie, and Ewen Cameron respectively.

Table 3.1. List of taxa collected for this study. Collection numbers, morphology, and haplotype are included as it corresponds to Figure 3.1. Collection number prefixes represent the collector's initials: WB- Whitney Bouma, LRP- Leon Perrie, and EK – Ewen Cameron, respectively.

Collection No.	Species	haplotype	Collection Location
WB14	<i>Pellaea</i> aff. <i>falcata</i>	2	Kitchener Park, Fielding
WB51	<i>Pellaea</i> aff. <i>falcata</i>	1	Waiuku Forest
WB52	<i>Pellaea</i> aff. <i>falcata</i>	1	Waiuku Forest
LRP3607	<i>Pellaea</i> aff. <i>falcata</i>	1	Wairarapa
WB06	<i>Pellaea</i> aff. <i>falcata</i>	1	Trentham Park, Upper Hutt, Barton's Bush
WB08	<i>Pellaea</i> aff. <i>falcata</i>	1	Otari Wilton Bush, Karori Wellington
WB12	<i>Pellaea</i> aff. <i>falcata</i>	1	Talbot Forest Scenic Reserve, Geraldine
WB31	<i>Pellaea calidirupium</i>	3	Mt. Wellington, Auckland
WB32	<i>Pellaea calidirupium</i>	3	Mt. Wellington, Auckland
LRP3884	<i>Pellaea calidirupium</i>	3	Port Hills, Christchurch
LRP4623	<i>Pellaea calidirupium</i>	3	Alexandra
EK14556	<i>Pellaea calidirupium</i>	3	Mt. Wellington, Auckland
P020736	<i>Pellaea falcata</i>	4	Kermadec Islands
P020738	<i>Pellaea falcata</i>	4	Kermadec Islands
WB55	<i>Pellaea falcata</i>	1	Tasmania
WB01	<i>Pellaea rotundifolia</i>	1	Wellington
WB11	<i>Pellaea rotundifolia</i>	2	Talbot Forest Scenic Reserve, Geraldine
WB13	<i>Pellaea rotundifolia</i>	2	Kitchener Park, Fielding
WB16	<i>Pellaea rotundifolia</i>	1	Waitomo Walkway, Waitomo
WB23	<i>Pellaea rotundifolia</i>	1	Pukemokemoke, Hamilton
WB30	<i>Pellaea rotundifolia</i>	1	Mt. Wellington, Auckland
WB33	<i>Pellaea rotundifolia</i>	1	Mt. Wellington, Auckland
WB34	<i>Pellaea rotundifolia</i>	1	Mt. Wellington, Auckland
WB35	<i>Pellaea rotundifolia</i>	1	Mt. Wellington, Auckland
WB50	<i>Pellaea rotundifolia</i>	1	Kawiti Caves
WB53	<i>Pellaea rotundifolia</i>	1	Waiuku Forest
WB54	<i>Pellaea rotundifolia</i>	1	Waiuku Forest
LRP4119	<i>Pellaea rotundifolia</i>	1	Rotoroa
LRP3972	<i>Pellaea rotundifolia</i>	1	Kelceys Bush, Hunters
LRP4064	<i>Pellaea rotundifolia</i>	2	Livingstone
LRP3895	<i>Pellaea rotundifolia</i>	1	Banks Peninsula, Herbert
LRP3677	<i>Pellaea rotundifolia</i>	1	Whangaroa
LRP4326	<i>Pellaea rotundifolia</i>	1	Ohakune
LRP4993	<i>Pellaea rotundifolia</i>	1	Eastern Wairarapa, Pongaroa
EK14557	<i>Pellaea rotundifolia</i>	1	Mt. Wellington, Auckland

pinnule length longer than that typical for *P. rotundifolia*, while two other samples were within the range of typical *P. rotundifolia* but had strongly oblong pinnules. These seven samples were treated here as *P. aff. falcata*. Two samples of the rarer *P. falcata* were obtained from the Kermadec Islands, as collection efforts found no populations growing on mainland New

Zealand. Positive identifications were made using Brownsey and Smith-Dodsworth (2000). Dried silica gel tissue of *P. falcata* from Tasmania was obtained for comparison with *P. falcata* from New Zealand.

3.2.2 Morphology

Morphological measurements were taken from all *Pellaea* samples collected for this study and an additional 53 *Pellaea* samples held in WELT Herbarium. The three longest pinnules, typically towards the middle of the lamina, were measured. Measurements consisted of pinnule length and pinnule width. Measurements were averaged and plotted to determine the extent to which separate taxonomic groups differed with respect to pinnule measurements.

Figure 3.2. Fronds typical of *P. falcata*, *P. rotundifolia*, and *P. calidrupium*, respectively. silhouettes are by Patrick Brownsey as shown in Brownsey and Smith-Dodsworth (2000).

Figure 3.3. Frond silhouettes representing the variation in frond length and shape of those plant recognized as *Pellaea* aff. *falcata*.

Measurements were also taken of spore size from New Zealand and Australian *Pellaea* specimens for a preliminary comparison of spore size and ploidy level. The spores from two *P. rotundifolia* samples with typical *rotundifolia*-like pinnule morphology and one with *P. aff. falcata* morphology were measured. In addition, spores from two New Zealand and one Australian *P. calidirupium*, and one New Zealand and two Australian *P. falcata* were measured. The length and width of the exine of ten spores from each sample were measured under oil immersion in 50:50 glycerol: water (magnification x1000).

3.2.3 DNA isolation/PCR/sequencing

Total DNA was extracted from silica dried tissue, or fresh when available, according to a modified CTAB protocol (Doyle & Doyle, 1990).

The *trnL-trnF* locus was amplified with the primers, Fern1 (5'-GGCAGCCCCCARATTCAGGGRAACC-3') and F (5'-ATTGAACTGGTGACACGAG-3') in 20 µl total volume PCR mixtures

(Taberlet et al. 1991, Trewick et al. 2002). PCR mixtures consisted of the following reagents: 1M betaine, 10X NH₄ reaction buffer (BioLine), 1.5mM MgCl (BioLine), 250μM dNTPs (BioLine), 10pmols of each forward and reverse primer, 1U of Taq DNA Polymerase (BioLine), and 50ng of DNA template. Thermocycler conditions consisted of an initial denaturing of 95°C for 2 minutes, 34 cycles of 95°C for 1 minute, 56°C for 1 minute, 72°C for 1 minute, and a final 72°C extension for 5 minutes.

The *rps4* gene and *rps4-trnS* IGS were amplified using the primers T1, (5'-ATGTCMCGTTAYCGAGGRCCTCGT-3'), and F1, (5'-TACCGAGGGTTCTGAATC-3'), in 20 μl total volume PCR mixtures (Nadot et al. 1994, Souza-Chies et al. 1997). PCR mixtures consisted of the following reagents: 1M betaine, 10X NH₄ reaction buffer (BioLine), 2.0mM MgCl (BioLine), 250μM dNTPs (BioLine), 10pmols of each forward and reverse primer, 1U of Taq DNA Polymerase (BioLine) taq, and 50ng of DNA template. PCR thermocycler conditions followed the protocol from Small et al. (2005), consisting of 30 cycles of 95°C for 1 minute, 50°C for 1 minute followed by a slow ramp (1°C/8 s) to 65°C, and finally 65°C for 4 minutes (Small et al. 2005).

PCR products were visualized with agarose gel electrophoresis. Successfully amplified PCR products were purified with 0.2U of SAP (shrimp alkaline phosphatase, USB Corp., Cleveland, USA) and 1U EXO (exonuclease I, USB Corp., Cleveland, USA) to remove any residual reagents. Thermocycler conditions consisted of 37°C for 30 minutes and 80°C for 15 minutes. Cleaned products were sequenced at the Allan Wilson Center Genome Service with an ABI3730 Genetic Analyzer (Allan Wilson Centre Genome Service, Palmerston North, New Zealand). Newly obtained DNA sequences for the New Zealand *Pellaea* and the Tasmanian *P. falcata* will be submitted to GenBank (species

and collection information are found in Table 3.1; newly generated DNA sequence data is in the CD appendix).

3.2.4 DNA sequence alignments

DNA sequence data was organized into four data sets for phylogenetic analyses. The chloroplast *trnL-trnF* locus was amplified and sequenced for each sample collected. The *trnL-trnF* locus data set contained the 35 New Zealand *Pellaea* exemplars aligned with *P. falcata* from Tasmania. The most closely related cheilanthoid ferns to the New Zealand *Pellaea*, *Paragymnopteris bipinnata* and *Paragymnopteris vestita*, were chosen as outgroups, selected from topologies presented in Kirkpatrick (2007). These outgroups had both the *trnL-trnF* locus as well as the *rps4* gene and *rps4* IGS DNA sequences available for this study.

A second data set was created from the *trnL-trnF* IGS for which sequences of Australian *P. falcata* and *P. nana*, referred to as *P. falcata* var. *nana* in Kirkpatrick (2007), were available on GenBank. This alignment contained a representative for each of the four different haplotypes present in New Zealand *Pellaea*, combined with the Australian *P. falcata* and *P. nana*. This data set had a total of six ingroup sequences and the two *Paragymnopteris* outgroup sequences.

A third data set was constructed consisting of the DNA sequence data from the *rps4* and *rps4-trnS* IGS. This data set included the same taxa used in the *trnL-trnF* IGS data set. This dataset again, had a total of six ingroup sequences and the two *Paragymnopteris* outgroup sequences. A partition homogeneity test using PAUP* version 4.b10 (Swofford 2002) was conducted to test for conflict between the three loci. A concatenated data set containing

the *trnL-trnF* IGS, *rps4* gene, and *rps4-trnS* IGS sequence data was also constructed including the same six taxa and two *Paragymnopteris* outgroups.

Newly generated DNA sequences for both *trnL-trnF* locus and *rps4* gene and *rps4-trnS* IGS were edited by eye using BioEdit version 5.0.9 (Hall 1999). Edited alignments were constructed using Clustal X version 1.83 (Thompson et al. 1997). These alignments were imported into MEGA version 4.0 (Tamura et al. 2007) and combined with imported sequences from GenBank. Summary statistics on alignments were calculated using MEGA v 4.0 (Table 3.2).

3.2.5 Phylogenetic analysis

PAUP* version 4.b10 was used to implement maximum parsimony analyses on each of the four data sets. The relationships among the *Pellaea trnL-trnF* locus, the *rps4* gene, and *rps4-trnS* IGS sequence data were inferred using an exhaustive search under a parsimony criterion. Gaps in the alignment were treated as missing data. For each of the four DNA sequence data sets, networks were constructed using the program TCS with statistical parsimony (Clement et al. 2000) and Splitstree 4.b4 with neighbor-net (Huson 1998); http://wwwab.informatik.unituebingen.de/software/jsplits/welcome_en.html. Settings in TCS consisted of treating gaps as missing.

3.3 Results

3.3.1 Morphology

Examples of the pinnule morphology of *Pellaea* in New Zealand are represented in Figure 3.2 and 3.3. Of the 82 *Pellaea* specimens measured,

results showed that there is an overlap with respect to the pinnule morphology of the different taxa (Figure 3.4). Exemplars labeled *P. falcata* in the WELT herbarium along with *P. falcata* specimens from the Kermadec Islands had average pinnule length measurements ranging from 14mm to 39mm. WELT herbarium records that were unidentified were considered exemplars of *P. aff. falcata*. These, along with *P. aff. falcata* collected for this study, had average pinnule measurement of 14mm to 34mm. Specimens of *P. rotundifolia* had a range of 7mm to 23mm.

Figure 3.4. Graph representing pinnule measurements of length and width for New Zealand *Pellaea*. The red and green bars on the left represent the currently recognized range for pinnule length of *P. falcata* and *P. rotundifolia*.

The spore measurement of New Zealand and Australian *Pellaea* specimens showed that New Zealand spores were on average larger than Australian spores though some overlap exists when New Zealand *P. rotundifolia* is included. Figure 3.5 shows that although the spores of the New Zealand *P. falcata* sample were larger, there was just as much difference between the average measurements of the two Australian samples (length/width = 33.9 μm /30.2 μm , 38.5 μm /34.9 μm) than between that of the spores of the New Zealand sample (43.6 μm /40.9 μm). The measurements of spores from *P.*

calidirupium consisted of only one Australian sample; however, its average size of spores (31.2µm/30.0 µm) was considerably smaller than the two New Zealand samples measured (46.5µm/42.3µm, 39.3µm/36.5µm).

Figure 3.5. Graph of average spore length and width measurements for New Zealand *Pellaea* and their Australian counterparts.

3.3.2 Alignments

Summary statistics (Table 3.2) for the *trnL-trnF* locus produced an alignment length of 892 base pairs. The ingroup taxa exhibited nine variable sites of which all were also parsimony informative. The *trnL-trnF* locus sequence data (tree not shown) showed one base pair transversion among all the representatives of *P. rotundifolia* signifying haplotype variation among this species (Figure 3.9). The *P. falcata* sample obtained from Tasmania was identical to the most common of the haplotypes of New Zealand *P. rotundifolia*. However, the samples of *P. falcata* from the Kermadec Island differed at six base pair positions to the rest of the alignment. The samples of *P. calidirupium* differed at four positions, sharing two derived characters with *P. falcata* from the Kermadec Islands. The morphologically intermediate

samples labeled *P. aff. falcata* had identical haplotypes to those found in *P. rotundifolia*.

Table 3.2. Summary statistics for the four data sets analyzed in this study by maximum parsimony analysis.

Data set	Alignment Length (bp)	Variable sites	Parsimony Informative sites	No. of MP trees	MP Tree length
<i>trnL-trnF</i> locus	892	9	9	NA	NA
<i>trnL-trnF</i> IGS	350	9	2	5	43
<i>rps4+rps4</i> IGS	989	13	5	2	98
<i>trnL-trnF+rps4</i>	1339	22	7	10	142

The *trnL-trnF* IGS data set alignment was 350 base pairs in length. The ingroup taxa had nine variable sites with two of them being parsimony informative. The network (Figure 3.6) produced similar relationships compared to the *trnL-trnF* locus despite the addition of Australian *P. falcata* and *P. nana*. However, conflict was detected in the alignment as Australian *P. falcata* shared one derived character, G at position 9, with the New Zealand *P. calidurupium*.

Figure 3.6. Neighbournet network representing the *trnL-trnF* IGS sequence data. Each substitution is labeled in its respective node. Note the conflict between Australian *P. falcata* and New Zealand *P. calidurupium*.

The *rps4* gene and *rps4-trnS* IGS alignment was 989 base pairs in length. The ingroup taxa contained 13 variable sites and five parsimony informative sites. Figure 3.7 represents the network obtained from TCS. It shows a base pair substitution distinguished haplotypes among the exemplars of *P. rotundifolia*. The relationships differed from the *trnL-trnF* IGS in that *P. falcata* from Australia has affinity to *P. calidurupium* from New Zealand as opposed to Australian *P. nana*.

Figure 3.7. Neighbournet network representing the *rps4* and *rps4-trnS* IGS sequence data. Each substitution is labeled in its respective node.

The results of the partition homogeneity test ($p < 0.36$) allowed for a concatenated data set. The alignment was 1339 base pairs in length and the ingroup taxa contained 22 variable sites and seven parsimony informative sites. Figure 3.8 represents the TCS network for the concatenated data set. The relationships are congruent with that of the *rps4* gene and *rps4-trnS* IGS

Figure 3.8. Neighbournet network representing the concatenated sequence data. Each substitution is labeled in its respective node.

network. Again, *P. falcata* from Australia has affinity to *P. calidurupium* from New Zealand as opposed to *P. nana* from Australia.

3.3.3 Phylogenetic analyses

Exhaustive parsimony searches on the *trnL-trnF* IGS data set produced five trees of 43 steps in length. The *rps4* gene and *rps4-trnS* IGS data set recovered two trees 98 steps in length. The concatenated data set recovered ten trees, 142 steps in length. Similar relationships among the *Pellaea* haplotypes were inferred from maximum parsimony, statistical parsimony, and neighbor-net

with each of the data sets. The relationships inferred from the different data sets were mostly in agreement. Differences were observed between the *rps4* gene and *rps4-trnS* IGS to that of the *trnL-trnF* IGS where it was unclear if Australian *P. falcata* were more closely related to Australian *P. nana* or to New Zealand's *P. calidirupium*. However, these relationships were further resolved in the analyses of the concatenated data set, where Australian *P. falcata* was more closely related to New Zealand's *P. calidirupium* than to Australian *P. nana* (Figure 3.9).

Figure 3.9. One of the two most parsimonious trees from maximum parsimony analyses of the concatenated data set. Bootstrap values are shown above the branches.

3.4 Discussion

3.4.1 Morphological vs. molecular data

Plants of *Pellaea rotundifolia* and *P. falcata* exhibit a wide range of pinnule morphologies throughout New Zealand, where plants intermediate in morphology between *P. rotundifolia* and *P. falcata* occur with plants more typical of *P. rotundifolia*. Where this occurs, suspicion is raised that these intermediate plants constitute different species. The results from pinnule measurements confirm that specimens identified according to Brownsey and Smith-Dodsworth (2000) cannot be separated by pinnule length. However, chloroplast DNA sequence data of the *trnL-trnF* locus and *rps4* gene and *rps4-trnS* IGS suggest that these morphologically intermediate plants are molecularly identical to *P. rotundifolia* and distinct by 10 base pairs from *P. falcata* from the Kermadec Islands.

It is not uncommon for morphological characters to disagree with molecular data as has been the case in studies on cheilanthoid ferns (Gastony and Rollo 1995, Zhang et al. 2007) and also other New Zealand ferns. Studies of New Zealand *Asplenium hookerianum* also displayed pinnule morphology not concordant with variation in the *trnL-trnF* locus. This study found nine distinct haplotypes among the sampled specimens of *Asplenium hookerianum*, but no clear pattern grouping narrow or broad-pinnuled individuals with any of the haplotypes. Hence, recognizing two distinct species, one with narrow pinnules and the other with broad, was not justified (Perrie and Brownsey 2005a). The potential for morphological differences in pinnule length corresponding to the two haplotypes found among the plants of *P. rotundifolia* was investigated, but again, there was no pattern between the two characters. It is clear that pinnule morphology is insufficient for discriminating *P. falcata*

and *P. rotundifolia*. The average pinnule length measurement of *P. falcata* from the Kermadec Islands (23mm) is shorter than *P. falcata* from Tasmania (35mm) and many of the *P. aff. falcata* specimens (33mm) that were molecularly determined to be more closely related or identical to *P. rotundifolia*.

Further investigations into the haplotype variation present in *Asplenium hookerianum* found a total of 25 haplotypes throughout New Zealand. Those haplotypes corresponded to specific geographical regions (Shepherd et al. 2007). Although the sample set for this study includes *P. rotundifolia* samples from throughout the country, more thorough sampling is required to assess whether additional haplotypes exist among this species and whether the haplotypes are linked to geographic regions. Rather, it seems according to the results of the current study, *P. rotundifolia* is a polymorphic species with a diverse range of pinnule morphologies that do not necessarily signify separate evolutionary lineages.

An issue with the polymorphic *P. rotundifolia* arises when plants are misidentified as *P. falcata*. The New Zealand Department of Conservation currently has labeled *P. falcata* as chronically threatened and subject to gradual decline (de Lange et al. 2004). For species conservation it is important to find a reliable way to determine identities for these plants and to ensure that appropriate action is being taken for the correct species. The collection efforts for this study found no populations of *P. falcata* on the mainland. Therefore, the confirmation that the *P. aff. falcata* plants were more closely related or possibly identical to *P. rotundifolia* raises concern that *P. falcata* might be rarer than previously believed.

Results from *trnL-trnF* locus and *rps4* gene and *rps4-trnS* IGS sequence data revealed that *Pellaea falcata* from Tasmania is molecularly identical to *P.*

rotundifolia compounds the problem of circumscribing *P. falcata*. It also suggests that problems identifying *P. falcata* plants extend beyond New Zealand to other regions of the South Pacific where *P. falcata* is known to grow. *Pellaea rotundifolia* is believed to be endemic to New Zealand. The specimen from Tasmania with identical chloroplast sequences to *P. rotundifolia* suggests the latter may actually extend outside New Zealand. Whether or not this has been a recent introduction or a case of misidentifying *P. rotundifolia* in Tasmania, due to the variability of its pinnule morphology, remains unknown. Unfortunately, the extent of this issue could not be resolved in the current study as the problem has only become apparent. Clearly though, this section of *Pellaea* warrants further investigations.

3.4.2. *Pellaea falcata* and *Pellaea* sect. *Platyloma*

The networks from the *trnL-trnF* IGS data set recovered conflict in the data set over whether Australian *P. falcata* was more closely related to *P. calidrupium* or to Australian *P. nana*. However, maximum parsimony analyses and networks of the *rps4* gene and *rps4-trnS* IGS and the concatenated data sets resolved the relationship supporting Australian *P. falcata* as more closely related to the New Zealand *P. calidrupium* than to *P. nana* from Australia. Furthermore, exhaustive parsimony searches support that *P. falcata* from the Kermadec Islands is more closely related to *P. calidrupium* from New Zealand than to *P. falcata* from Australia.

It might be expected that *P. falcata* from Australia would be more closely related to its New Zealand counterpart. However, *P. falcata* from the Kermadec islands differs from Australian *P. falcata* by nine base pairs, four of which uniquely characterize *P. falcata* from the Kermadec Islands. This suggests it may be a distinct species, although it remains unknown if it is

restricted to the Kermadec Islands or if it occurs in other areas of the South Pacific. Extensive collecting and sequencing will be necessary to determine these relationships, but nonetheless these results compound the problem of circumscribing *P. falcata*.

Also investigated in this study were the relationships between the other members of *Pellaea* sect. *Platyloma*. Australian *P. nana* was previously considered a variety of *P. falcata* (Bostock et al. 1998). These results showed that *P. nana* is quite distinct from Australian *P. falcata* and confirmed that should it be treated as a distinct species. Indeed, relationships inferred from the concatenated data sets show that *P. nana* is actually more closely related to New Zealand *P. rotundifolia* than to the other members of *Pellaea*.

Not included in this study were Australian samples of *P. calidirupium* and *P. paradoxa*. It is uncertain whether the Australian *P. calidirupium* would group with New Zealand *P. calidirupium* according to DNA sequence data. It has been noted that *P. calidirupium* lacks continual sori around pinnule margins and that morphologically it appears intermediate between *P. paradoxa* and *P. falcata* (Bostock et al. 1998). *Pellaea paradoxa* would be expected to group with representatives of *P. calidirupium* according to morphological descriptions. However, it has become quite clear that morphology is often not a reliable means of inferring relationships between the members of *Pellaea* sect. *Platyloma*.

3.4.3. Future directions

It had been mentioned before that the distinction between the members of the New Zealand *Pellaea* are not clearly defined and are in need of redefinition (Brownsey and Smith-Dodsworth 2000). With only three known species

present in New Zealand, this task was deemed straightforward. With the results of DNA sequence data combined with preliminary morphological analyses, it is now clear that the problem is far greater than anticipated. Furthermore, the problem of recovering clear evolutionary lines between the members of *Pellaea* no doubt extends beyond those in New Zealand to include the entire *Pellaea* section *Platyloma* of the South Pacific. Several issues should be addressed in future studies of New Zealand *Pellaea* and also *Pellaea* sect. *Platyloma* in general.

The species *Pellaea falcata* appears to be ill-defined and its distribution is unclear not only in New Zealand but in the South Pacific. Additional sampling is required in areas that report having *P. falcata* to determine if they are in fact allied to *P. falcata* (the type locality is Sydney, Australia), "*P. falcata*" from the Kermadec Islands, *P. rotundifolia* (including morphologically intermediate *P. aff. falcata* plants), *P. nana*, or some additional taxon.

It should be determined if other molecular markers (chloroplast and additional genomes, e.g., mtDNA or rDNA) are in agreement with the *trnL-trnF* locus and *rps4* gene and *rps4-trnS* IGS sequence data, in suggesting that *P. aff. falcata* plants with longer pinnules are the same species as *P. rotundifolia*. An intron of the nuclear LEAFY locus was sequenced to determine if data from the nuclear genome inferred the same relationships as the chloroplast genome. Unfortunately, the LEAFY intron offered no insights to the relationships as the sequences generated showed an immense amount of heterozygosity among the exemplars of New Zealand *P. rotundifolia* (unpublished data). The extent of the distribution of *P. rotundifolia* in the Pacific region (i.e. its endemism) should also be determined with sampling in locations where *P. falcata* grows or is said to grow, i.e., Tasmania, Australia, Lord Howe Island, New Caledonia, Kermadec Islands.

To accurately infer the relationships among the members of *Pellaea* sect. *Platyloma*, Australian samples of *P. calidirupium* and *P. paradoxa* should be incorporated into further phylogenetic analyses. In addition to molecular analyses, morphological investigations should be conducted to determine if a viable character exist to aid in identifying members of *Pellaea* sect. *Platyloma*. In the current study, preliminary spore measurements were made to determine if they would be a helpful character in discerning the diploid Australian species of *Pellaea* from the tetraploid New Zealand species. The measurement averages suggest that New Zealand spores are larger than Australian spores, which is consistent with previous studies reporting that spore size corresponds to ploidy level where larger spores are indicative of a higher ploidy level (Barrington et al. 1986, Huang et al. 2006). Future investigations of spore size will require a larger sample set to determine if this difference is statistically significant and is thus an appropriate character that will help discern the relationships amongst the Australasian *Pellaea*.

In conclusion, preliminary results from morphological and chloroplast DNA sequence data suggest that the morphologically intermediate *Pellaea* aff. *falcata* is molecularly identical or very closely related to *P. rotundifolia*. *P. aff. falcata* appears to have a distribution that extends outside of New Zealand, questioning the endemic status of *P. rotundifolia*. *Pellaea falcata*, as a species, is ill-defined, both morphologically and with respect to its distribution, and is in need of extensive sampling and taxonomic revisions. The plants labeled *P. falcata* from the Kermadec Islands appear to be a distinct species of *Pellaea*, but again, further sampling in other areas of the South Pacific are required to confirm this. Taxonomic and phylogenetic issues with the cheilanthoid ferns apparently are not just an issue at generic levels, and clearly extend to groups comprising only a handful of species. Establishing sound species-level

classifications is a necessary step for resolving the higher-level taxonomy of contentious fern groups such as *Pellaea*.

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Chapter 4

A molecular phylogeny for New Zealand's *Adiantum* ferns from analyses of chloroplast DNA sequences and another look at the *Adiantum hispidulum* Swartz complex.

4.1 Introduction

The genus *Adiantum* comprises over 200 species within the Pteridaceae family. It has a global distribution except in areas of extreme cold and dry climates (Tryon and Tryon 1982). This genus is characterized by tufted or creeping scaly rhizomes. The fronds are pinnate to 4-pinnate, also being glabrous or hairy. The sori are borne abaxially on strongly reflexed, usually kidney-

shaped marginal lobes, called false indusia (Brownsey and Smith-Dodsworth 2000).

Adiantum has been recognized as an old and isolated genus with its evolutionary ties to the Pteridaceae based on soral characters and chromosome number (Tryon and Tryon 1982). Although this genus is one of the better understood within the family Pteridaceae, no comprehensive infra-generic classification has been generated for *Adiantum*. Previous studies on the genus have been focused on specific segregates (Parris and Croxall 1974, Parris 1980, Paris and Windham 1988, Large and Braggins 1993, Korpelainen et al. 2005). The last effort to classify *Adiantum* on a global scale was by Tryon and Tryon in 1982 where the genus was split into eight groups on the basis of morphological characters. The authors admitted that although the groups in their classification brought together related species, some species were doubtfully placed and the relationships of the groups to one another were uncertain (Tryon and Tryon 1982).

In New Zealand, seven native *Adiantum* species are recognized: *A. aethiopicum* L., *A. diaphanum* Blume, *A. formosum* R.Br., *A. hispidulum* Sw., *A. cunninghamii* Hook., *A. fulvum* Raoul, and *A. viridescens* Colenso. The latter three are endemic to New Zealand (Brownsey and Smith-Dodsworth 2000). Previously only *A. cunninghamii* and *A. fulvum* were recognized as endemic in New Zealand, but the discovery of an *Adiantum* intermediate between *A. cunninghamii* and *A. fulvum*, with respect to indumentum, was morphologically determined by Parris (1974) to be a distinct species, *A. viridescens*. The endemic status of *A. cunninghamii* was questioned because of a morphologically similar fern growing in Australia. Recently, the Australian counterpart was included under the name *A. silvaticum* (Bostock et al. 1998).

However, it has been noted that it is strikingly similar to New Zealand's *A. viridescens* (Parris and Croxall 1974).

Most of the native species are distributed on both the North and South Islands. However, some species are rarer and are restricted to the North Island. *Adiantum formosum* is very common in Australia, but is the rarest of the New Zealand *Adiantum*, surviving only around the Manawatu Gorge. It has been recorded from Northland, but no longer occurs there (Brownsey and Smith-Dodsworth 2000). Two other *Adiantum* species, *A. capillus-veneris* (type species for *Adiantum*) and *A. raddianum*, have become naturalized in New Zealand and although they are included in this study, the focus is directed towards the native species.

Also restricted to the North Island in New Zealand is *Adiantum hispidulum*. This fern is found growing in coastal areas and is one of the few ferns to grow well in open sunny conditions (Brownsey and Smith-Dodsworth 2000). Outside of New Zealand, this fern grows in Africa, India, south-east Asia, Australia, and various Pacific Islands (Bostock et al. 1998). *Adiantum hispidulum* has been previously termed a polymorphic species with two morphs growing in New Zealand as part of the *Adiantum hispidulum* complex, consisting of *Adiantum hispidulum sensu stricto* and *A. pubescens* Schkuhr (Parris 1980, Large and Braggins 1993). These two taxa were considered distinct by the degree of indumentum and also frond formation.

A study by Parris in 1980 analyzed the two taxa morphologically. Based on her findings on differences in hair type and length and frond formation, she suggested recognizing two distinct species: *A. hispidulum* having short, stout, rigid hairs and fronds that were either 2-3 pinnate or pedate; and *A. pubescens* having longer, thin, sub-rigid hairs and fronds that were always pedate.

Parris (1980) also stated that recognizing only a single polymorphic species would be a step backwards.

Large and Braggins (1993) carried out a much more extensive morphological analysis of *Adiantum hispidulum* and *A. pubescens*. Of all the morphological characters considered in the study, the only consistent character distinguishing the two ferns was hair length and type (Large and Braggins 1993). Another character, frond shape, had been previously utilized for separating the two taxa by Parris (1980). However, *A. pubescens* and *A. hispidulum* were found to be present in either pinnate or pedate form. This study characterized the two taxa by the following criteria: *Adiantum hispidulum* had short stiff hairs 63-815µm in length, with pigmented enlarged basal cells, and most often pinnate frond formation; whereas, *A. pubescens* had long lax hairs, 251-1003µm in length, with narrow basal cells, and most often pedate frond formation.

Large and Braggins (1993) concluded that there was a distinction between the two taxa in New Zealand. However, because it was based on only one character and there was a lack of information based on foreign material, *A. pubescens* should be considered a variety of *A. hispidulum*; *A. hispidulum* var. *pubescens* (Schukuhr) Large et Braggins. Yet, since the publication of both of these studies, neither *A. pubescens* or *A. hispidulum* var. *pubescens* have been widely adopted in New Zealand and still only *A. hispidulum* is recognized (Brownsey and Smith-Dodsworth 2000).

With the advent of molecular techniques it is possible to further investigate classifications previously based on morphological characters. The chloroplast *trnL-trnF* locus (*trnL* intron, *trnL* 3'exon, and *trnL-trnF* intergenic spacer), *rps4* gene and *rps4-trnS* intergenic spacer (IGS), *rbcl* gene, and *atpB* gene were

selected for investigations of the relationships among the members of the New Zealand *Adiantum*. For a detailed investigation of the *A. hispidulum* Swartz complex, the *trnL-trnF* locus was selected based on its phylogenetic properties. This region evolves relatively quickly and accumulates base pair substitutions and indels, making it an ideal locus for detecting variation at the interspecific and intraspecific taxonomic levels.

The aim of this study was to determine the relationships of the New Zealand *Adiantum* using DNA sequence data. The following questions were addressed: 1) What are the relationships between the New Zealand members of the genus *Adiantum*? 2) Does DNA sequence data show genetic variation between the representatives of the *Adiantum hispidulum* complex? 3) Does hair length and type correspond to molecular variation amongst the members of the *A. hispidulum* complex?

4.2 Materials and Methods

4.2.1 Data collection

This study examines 28 exemplars from the genus *Adiantum* as it is represented in New Zealand. Duplicate samples of each species were collected from several wild populations from both the North and South Islands (Figure 4.1) to confirm positive identification of the species. A total of 11 exemplars of the *A. hispidulum* complex were collected for the investigation of morphological and genetic variation. Positive identifications were made using Brownsey and Smith-Dodsworth (2000).

Figure 4.1. Distribution of the *Adiantum* species collected for this study. Not shown on this map is sample PO20141, *A. hipidulum* (var. *pubescens*), which was collected from the Kermadec Islands which can be found in Figure 2.2 in this study. WB and LRP prefixes refer to collection numbers for Whitney Bouma and Leon Perrie, respectively.

4.2.2 Morphology

Using the morphological descriptions from Large and Braggins (1993), regarding length and rigidity of hairs between *A. hispidulum* and *A. (hispidulum var.) pubescens*, all *A. hispidulum* samples collected for this study as well as the *A. hispidulum* specimens held in the WELT Herbarium were visually examined and categorized as having *hispidulum*-like or *pubescens*-like hairs.

4.2.3 DNA isolation/PCR/sequencing

Total DNA was extracted from silica gel dried or fresh when available, tissue, according to a modified CTAB protocol (Doyle & Doyle, 1990).

The *trnL-trnF* locus was amplified with the Fern 1 (5'-GGCAGCCCCCARATTCAGGGAACC-3') and F (5'-ATTTGAACTGGTGACACGAG-3') primers in 20µl total volume reactions (Taberlet et al. 1991, Trewick et al. 2002). PCR mixtures consisted of the following reagents: 1M betaine, 10X NH₄ reaction buffer (160mM (NH₄)₂SO₄, 670mM Tris-HCl (pH8.8 at 25°C), 0.1% Tween-20, BioLine), 1.5mM MgCl₂ (BioLine), 250µM dNTPs (BioLine), 10pmols of each forward and reverse primer, 1U of Taq DNA Polymerase (BioLine), and approximately 50ng of DNA template. Thermocycler conditions consisted of an initial denaturing of 95°C for 2 minutes, 34°C cycles of 95°C for 1 minute, 56°C for 1 minute, 72°C for 1 minute, and a final 72°C for 5 minutes.

The *rps4* gene and *rps4-trnS* intergenic spacer were amplified using the following primers: T1 (5'-ATGTCMCGTTAYCGAGGRCCTCGT-3') and F1 (5'-TACCGAGGGTTCTGAATC-3') primers, in 20µl total volume mixtures (Taberlet et al. 1991). PCR mixtures consisted of the following reagents: 1M

betaine, 10X NH₄ reaction buffer (BioLine), 2.0mM MgCl₂ (BioLine), 250μM dNTPs (BioLine), 10pmols of each forward and reverse primer, 1U of Taq DNA Polymerase (BioLine), and approximately 50ng of DNA template. PCR thermocycler conditions were selected according to Small et al, (2005) of 30 cycles of 95°C for 1 minute, 50°C for 1 minute followed by a slow ramp (1°C/8 s) to 65°C, and finally 65°C for 4 minutes.

Primers 1F (5'-ATGTCACCACAAACGGAGACTAAAGC-3') and 1361R (5'-TCAGGACTCCACTTACTAGCTTCACG-3') were used for amplifying the *rbcL* chloroplast gene (Nadot et al. 1994, Souza-Chies et al. 1997). PCR amplifications, in a total volume of 20 μl, were made with the following reagents: 1M betaine, 10X NH₄ reaction buffer (BioLine), 1.5mM MgCl₂ (BioLine), 250μM dNTPs (BioLine), 10pmols of each forward and reverse primer, 1U of Taq DNA Polymerase (BioLine), and approximately 50ng of DNA template. Thermocycler conditions consisted of an initial denaturing of 95°C for 2 minutes, 34 cycles of 95°C for 1 minute, 50°C for 1 minute, 72°C for 1 minute, and a final 72°C for 5 minutes. The *atpB* gene was amplified using the primers 672F (5'-TTGATACGGGAGCYCCTCTWAGTGT-3') and 384R (5'-GAATCCCAAACCTATTCGATTAGG-3') PCR amplification mix and thermocycler settings were the same as for *rbcL* (Schuettpelz and Pryer 2007).

PCR products were visualized by agarose gel electrophoresis. Successfully amplified PCR products were purified with 0.2 U of SAP (shrimp alkaline phosphatase, SAP, USB Corp., Cleveland, USA) and 1 U EXO (exonuclease I, Exo I, USB Corp., Cleveland, USA) to remove any residual reagents. Thermocycler conditions consisted of 37°C for 30 minutes followed by inactivation of enzymes at 80°C for 15 minutes. Cleaned products were sequenced at the Allan Wilson Centre Genome Service with an ABI3730 Genetic Analyzers (Allan Wilson Centre Genome Service, Palmerston North,

New Zealand). Newly obtained DNA sequences for the New Zealand *Adiantum* will be submitted to GenBank (species collection information is available in Table 4.1 and DNA sequence detail are in the CD appendix).

Table 4.1. List of *Adiantum* taxa collected for this study including collection location. Duplicate samples were collected for each of the native species for trnL-trnF locus DNA sequencing. The native *Adiantum* indicated with an * have *trnL-trnF* locus, *rps4* and *rps4-trnS*IGS, *atpB*, and *rbcL* DNA sequence data available in the CD appendix. Collections beginning with P are held in the WELT herbarium. WB and LRP prefixes are collection numbers for Whitney Bouma and Leon Perrie respectively.

Collection No.	Species	Collection Location
LRP3637*	<i>Adiantum aethiopicum</i>	Pahaoa Valley, Wairarapa
LRP2813	<i>Adiantum aethiopicum</i>	Logues Bush, Wellsford
WB10	<i>Adiantum capillus-veneris</i>	Hataitai, Wellington
LRP4815*	<i>Adiantum cunninghamii</i>	Opuia, Bay of Islands
LRP3153	<i>Adiantum cunninghamii</i>	Golden Bay, North-West Nelson
LRP4989	<i>Adiantum diaphanum</i>	Woodville
LRP4392*	<i>Adiantum diaphanum</i>	Whanarua Bay, Northland
LRP4766	<i>Adiantum formosum</i>	Manawatu Gorge
LRP4987*	<i>Adiantum formosum</i>	Woodville, Wairarapa
LRP3669	<i>Adiantum fulvum</i>	Whangaroa
WB02*	<i>Adiantum fulvum</i>	Plimmerton, Wellington
WB24	<i>Adiantum hispidulum</i>	Cathedral Cove, Coromandel
WB25	<i>Adiantum hispidulum</i>	Cathedral Cove, Coromandel
WB45	<i>Adiantum hispidulum</i>	Whale Bay, Northland
WB48	<i>Adiantum hispidulum</i>	Whangaroa, Northland
WB49	<i>Adiantum hispidulum</i>	Kawakawa, Northland
LRP4393	<i>Adiantum hispidulum</i>	Te Kaha, East Cape
LRP4834	<i>Adiantum hispidulum sens. lat.</i>	Opuia, Bay of Islands
LRP4994*	<i>Adiantum hispidulum sens. lat.</i>	Aorangi Range, Wairarapa
P020741	<i>Adiantum hispidulum sens. lat.</i>	Kermadec Islands

LRP5195	<i>Adiantum hispidulum sens. lat.</i>	Hawai, Bay of Plenty
LRP5192	<i>Adiantum hispidulum sens. lat.</i>	Waiwera, Auckland
LRP4796	<i>Adiantum raddianum</i>	Pahao Gorge
WB09	<i>Adiantum raddianum</i>	Hataitai, Wellington
LRP4552	<i>Adiantum viridescens</i>	Eltham, Wanganui
WB04	<i>Adiantum viridescens</i>	Plimmerton, Wellington

4.2.4 Alignments

The sequence data for each of the four data sets were edited by eye using BioEdit version 5.0.9 (Hall 1999) and aligned in ClustalX version 1.83 (Thompson et al. 1997). For the non-coding regions in the *trnL-trnF* locus and *rps4-trnS* IGS, manual adjustments to the alignment were made to minimize insertions and deletions. Regions that could not be unambiguously aligned were excluded, and indels were treated as missing data. A partition homogeneity test was run on PAUP* version 4.b10 (Swofford 2002) to test for conflict between the four data sets. The alignments were then imported into MEGA version 4.0 (Tamura et al. 2007) and combined to form a concatenated data set. *Adiantum capillus-veneris* was originally selected as the outgroup. However, broader sampling of the Pteridaceae (Chapter 2) indicated *A. formosum* was the most distantly related of the native New Zealand *Adiantum* and therefore, it was chosen as the outgroup instead. The final alignment contained eight taxa, representing the species of *Adiantum* native in New Zealand and also the adventive *A. raddianum*.

For the exemplars of the *Adiantum hispidulum* complex, sequences from the *trnL-trnF* locus were combined in a data set with sequence data from *Adiantum raddianum*, which was selected as an outgroup again based on results from the analysis above. These sequences were again edited by eye using BioEdit version 5.0.9 and aligned using ClustalX version 1.83. The

alignments were imported into MEGA version 4.0 and manual adjustments were made to minimize insertions and deletions.

4.2.5 *Phylogenetic analysis*

Phylogenetic analyses under the maximum parsimony and maximum likelihood criteria were conducted using PAUP version 4.0b10 (Swofford 2002). Exhaustive parsimony searches were conducted on each of the individual data sets (*trnL-trnF* locus, *rps4* gene and *rps4-trnS* IGS, *rbcL*, and *atpB*) to identify any incongruence between them. Heuristic searches under parsimony criteria were performed with 100 random addition sequence replicates and TBR branch swapping. Bootstrap values were assessed using 200 pseudoreplicates, each with 100 random sequence addition replicates.

Maximum likelihood analyses were implemented using the appropriate model of evolution as assessed by ModelTest version 3.6 (Posada and Buckley 2004) according to the Akaike Information criteria. Maximum likelihood settings ran for 100 replicates of random sequence addition. Bootstrapping was again performed to assess the confidence with settings consisting of 100 pseudoreplicates, one random sequence addition, and TBR branch swapping.

MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001) was used to carry out Bayesian analyses (nst=6, rates=invgamma, and default priors) on all four of the data sets in this study. Analyses were run for 5,000,000 generations, with sampling from every 1000th generation, and the initial 50% discarded as burn-in. Standard deviations of split frequencies were checked at 2,500,000 generations to ensure a value of <0.01 (suggesting a convergence to stationary distribution had been achieved).

Splitstrees 4.b4 (Huson 1998); http://wwwab.informatik.unituebingen.de/software/jsplits/welcome_en.html, was used to construct a neighbor-net network in order to visualize the haplotype variation in the *A. hispidulum* Swartz complex.

4.3 Results

4.3.1 Morphology

Three distinct hair types were found amongst the samples of *Adiantum hispidulum*. The most common hair type was referred to as *hispidulum*-like or type 1, with sparse, short stiff hairs and having pigmented, enlarged basal cells. Another hair type is what would be considered *pubescens*-like, or type 2, with dense long lax hairs and narrow basal cells. The third hair type, type 3, was more like *A. hispidulum*, but with the hairs being more abundant, much shorter and more like a spike (Figure 4.3).

4.3.2 Alignment

Alignment statistics for the individual data sets can be found in Table 4.2. The results of the partition homogeneity test ($p=0.52$) allowed a concatenated data set. The concatenated alignment was 4013 base pairs in length. The alignment including the outgroup contained 543 variable sites, 158 of them being parsimony informative. Seven distinct haplotypes were recovered in the alignment corresponding to each of the species included in the alignment with the exception of the species *A. viridescens* and *A. fulvum*, which were identical at all sequenced loci.

Type 1 hairs

Type 2 hairs

Type 3 hairs

Figure 4.3. Photographs of the three hair types found on the abaxial side of pinnules among the *Adiantum hispidulum* complex. Each pair of photographs, magnification 70X, is of two different specimens exhibiting the respective hair type.

Table 4.2. Summary statistics for the four data sets analyzed in this study by maximum parsimony analysis.

Data set	Alignment Length (bp)	Variable sites	Parsimony Informative sites	No. of MP trees	MP Tree length
<i>trnL-trnF</i> locus	916	162	35	1	77
<i>rbcL</i>	1143	88	23	23	101
<i>rps4</i> gene & <i>rps4-trnS</i> IGS	843	207	85	1	280
<i>atpB</i>	1111	71	15	1	180
concatenated	4013	528	158	2	642

MP- maximum parsimony

The *trnL-trnF* locus data set of representatives of the *A. hispidulum* complex had an alignment length of 922 basepairs. The ingroup taxa, excluding *A. raddianum*, had four variable sites of which all were parsimony informative at positions 230, 534, 650, and 693. These defined three distinct haplotypes within *A. hispidulum*.

4.3.3 Phylogenetic analysis

The parsimony exhaustive searches of each of the individual data sets (*trnL-trnF* locus, *rps4* gene and *rps4-trnS* IGS, *rbcL*, and *atpB*) recovered trees that were congruent with one another, other than for the relationships of *A. hispidulum* and *A. raddianum* to the rest of the New Zealand *Adiantum* (trees not shown). Each of the individual data sets recovered a single tree with the exception of *rbcL* which recovered 23 trees.

The topologies recovered from the analyses of the concatenated data set were congruent across the different tree-building methods used, and were also congruent with topologies recovered from the individual analyses. Figure 4.4 represents one of the two most parsimonious trees recovered from the maximum parsimony analysis each with a length of 642 steps. Because the

Figure 4.4. One of two most maximum parsimony trees for the concatenated data set. Bootstrap values, posterior probabilities, and maximum likelihood bootstrap values are above each branch, respectively.

topologies between the three methods were congruent, the trees recovered from the maximum likelihood and Bayesian analysis are not shown. The relationships in Figure 4.4 show that *A. viridescens* and *A. fulvum*, which are molecularly identical, are sister to *A. cunninghamii*. These are in turn sister to

A. diaphanum, and *A. aethiopicum* is sister to this clade. The position of each of these species was supported with high bootstrap values (BS>95%) and posterior probabilities of 1.0. The phylogenetic placement of *A. raddianum* and *A. hispidulum* was sister to the clade containing the other *Adiantum* species in each of the tree-building analyses. However, the relationship and grouping of *A. raddianum* with *A. hispidulum* was never well supported by either bootstrap values or posterior probabilities.

The neighbornet network for the representatives of the *A. hispidulum* complex showed no conflict and recovered the three haplotypes (Figure 4.5).

Figure 4.5. Neighbornet network representing the three hair types and the haplotype variation among the representatives of the *Adiantum hispidulum* complex. The base pair substitutions are labeled above the corresponding node.

4.4 Discussion

4.4.1 Relationships of New Zealand *Adiantum*

The relationships among the New Zealand *Adiantum*, from the results of phylogenetic analyses, show that most of the members of this genus in New Zealand are distantly related to one another with a large amount of genetic variation present between the different species. Comparing morphological characters, selected from the species key in Brownsey and Smith-Dodsworth (2000), to the phylogenetic relationships, it appears that homoplasy has occurred with respect to these characters among the species of New Zealand

Adiantum. Morphologically, *A. cunninghamii*, *A. fulvum*, *A. diaphanum*, *A. hispidulum*, and *A. viridescens* are grouped together on the basis of having oblong lamina which is attached to the stalk at one corner (Brownsey and Smith-Dodsworth 2000), where as *Adiantum aethiopicum* and *A. raddianum* both have centrally attached stalks, but are distantly related to one another according to chloroplast sequence data suggesting convergent or parallel evolution of the central attachment of the lamina to the stalk. A large scale morphological investigation on character homoplasy in grammitid ferns suggests that some the most commonly used morphological characters used for taxonomic recognition are those which exhibit high levels of homoplasy (Ranker et al. 2004).

The grouping of *Adiantum* proposed by Tryon and Tryon (1982) placed both *Adiantum raddianum* and *A. aethiopicum* in the *A. capillus-veneris* group (Tryon and Tryon 1982). However, according to the topology recovered from a large scale investigation of Pteridaceae (Chapter 2) based on *rbcL* DNA sequence data, *A. raddianum* and *A. aethiopicum* are quite distantly related to *A. capillus-veneris*. In addition, the results from this study using several chloroplast loci, confirm this relationship as *A. aethiopicum* is in a clade with *A. cunninghamii*, *A. diaphanum*, *A. fulvum*, and *A. viridescens* as opposed to being placed with *A. raddianum*. *Adiantum raddianum* is native to South America yet has affinity to New Zealand species of *Adiantum*. Whether or not *A. raddianum* originated in New Zealand or in South America is unknown. Additional taxonomic sampling will be necessary to determine if additional species will have affinity to *A. raddianum* or the New Zealand *Adiantum*.

Adiantum cunninghamii, *A. fulvum*, and *A. viridescens* all have glabrous reflexed indusia, in addition to an oblong lamina attached to a stalk at one corner, where as, *A. hispidulum* has hairy reflexed indusia, while still having an

oblong lamina attaching to the stalk at one corner (Brownsey and Smith-Dodsworth 2000). *Adiantum hispidulum* is consistently recovered as having affinity to *A. raddianum* though there is a lack of morphological characters to group them together. According to the topology based on *rbcL* sequence data (Chapter 2), *A. hispidulum* is recovered as having affinity to the *A. cunninghamii*, *A. fulvum*, and *A. viridescens*, although not supported with bootstrap values or posterior probabilities. The topologies recovered from this study confirm the affinity of *A. hispidulum* to the New Zealand *Adiantum*, yet morphological and molecular data fail to establish its exact relationship to the other New Zealand *Adiantum* species.

Most interesting is *Adiantum formosum* which had the most different genetic distance compared to the other native New Zealand taxa and its distribution only extends to Australia. The study investigating the Pteridaceae with *rbcL* DNA sequences (Chapter 2) included 24 representatives of the genus *Adiantum* mostly from the South Pacific and South America. The relationship of *A. formosum* to the other *Adiantum* species was sister to the remainder of the South-West Pacific *Adiantum* with high support from both bootstrap values and posterior probabilities. According to the groups suggested by Tryon and Tryon (1982), *A. formosum* is placed in a group with *A. fulvum* and *A. hispidulum*, and would mostly likely be expanded to include *A. viridescens* and *A. cunninghamii*. Based on morphological characters, *A. formosum* shares several characters with the species mentioned above, including oblong lamina attached to the stalk at one corner and glabrous indusial. In addition, this grouping is consistent with chromosome numbers reporting *A. fulvum*, *A. cunninghamii*, and *A. fulvum* having $n=58$ (Dawson et al. 2000). Tryon and Tryon (1982) also mention other *Adiantum* species that would be included in this group mostly from South America. Unfortunately DNA sequence data from these species was not available for the former analyses to determine if

they would have affinities to *A. formosum*. Until further taxonomic sampling can be incorporated, the origins and relationships of *A. formosum* to other *Adiantum* species in the South-West Pacific remain unclear.

4.4.2 *Adiantum hispidulum* complex

The recognition in the *Adiantum hispidulum* complex of either a distinct species *A. pubescens* Schkuhr or a variety *A. hispidulum* var. *pubescens* (Schkuhr) Large et Braggins, has not been addressed since the publications of Parris (1980) and Large and Braggins (1993). Based on the morphological observations of the 64 *A. hispidulum* specimens collected around New Zealand and off shore islands, it is clear that three distinguishable hair types are present. Large and Braggins (1993) determined that differences in hair type were not a result of environmental factors but influenced by genetic factors. The *trnL-trnF* DNA sequence data shows that these three hair types are consistent with haplotype variation at the *trnL-trnF* locus, with the exception of one specimen, suggesting that there are three separate taxa within the *A. hispidulum* complex as opposed to two. Two of the three distinct groups correspond to previous descriptions of *A. hispidulum*. Plants with type 1 hairs fit with the descriptions of *A. hispidulum* (Parris 1980, Large and Braggins 1993). Plants with type 2 hairs conform to those described as *A. pubescens* or *A. hispidulum* var. *pubescens* (Parris 1980, Large and Braggins 1993). The plants with type three hairs have no mention in the known literature of *A. hispidulum*. Parris (1980) describes several other hair types found amongst overseas representatives of *A. hispidulum*, but none match the description of hairs found on these plants. Furthermore, Large and Braggins (1993) discuss the variability of hair length amongst plants of *A. hispidulum*. Yet, they do not describe hairs that would be descriptive of type 3 hairs.

The sample set obtained for *A. hispidulum* was small but still an almost perfect correspondence between hair type and haplotype was recovered. The plant that did not fit this pattern was an anomaly already as this plant was collected a great distance beyond the normal distribution of *A. hispidulum* in New Zealand (Aorangi Range, Wairarapa). Also, when it was found its appearance was unlike that of which would be seen with typical *A. hispidulum* plants (see appendix C). Although it cannot be ignored that this sample does not fit the pattern, further investigations of this disjunct population would be worthwhile.

The collection locations of these non-*hispidulum*-like specimens, i.e. type 2 and type 3 hairs, showed no obvious geographical pattern (Figure 4.2). This supports the finding of Large and Braggins (1993) that *A. hispidulum* and *A. (hispidulum* var.) *pubescens* are not geographically or ecologically separated. However, the distribution of *pubescens*-like plants and the plants with the third hair type in New Zealand are unclear and more thorough sampling is required.

Adiantum hispidulum also occurs in Australia where four distinct varieties are recognized (Bostock et al. 1998). The criteria for distinguishing the Australian varieties is contingent among more than just indumentum characters though two varieties seem to loosely conform to descriptions of New Zealand *A. hispidulum* (Australian *A. hispidulum* var. *minus*) and *A. pubescens* (Australian *A. hispidulum* var. *hypoglaucum*). Bostock et al. (1998) describe *A. hispidulum* var. *hypoglaucum* as having silky, pubescent, pale crowded lax hairs. *Adiantum hispidulum* var. *minus* was described as having pubescent pale hairs that were stiffly erect. The other two Australian varieties (*A. hispidulum* var. *whitei* and *A. hispidulum* var. *hispidulum*) are both described as being sparsely pubescent. Based on the vague descriptions of the latter two, it is not possible

Figure 4.2. The distribution of the collection details for all specimens of the *Adiantum hispidulum* complex from the WELT herbarium. Each color represents the hair type observed: blue- type 1, red-type 2, and yellow- type 3. Note specimen LRP4994 which occurs beyond the range typical of that of *A. hispidulum*.

to determine if they would conform to type 1 or type 3 hairs. No known molecular or even morphological investigations have been done with respect to the Australian representatives of *A. hispidulum* to determine their relationships to one another and also their relationships to those occurring in New Zealand.

The main problem with classifying these taxa is determining what criteria are required for recognizing a new species. Large and Braggins (1993) argue that differences in hairs, coupled with frond formation, which is quite variable anyway, is not enough to denote separate species. However, the addition of molecular data in support of three distinct haplotypes, corresponding to the three distinct hair types, provides a second character in support of distinct taxa. Similar problems were encountered in a study on *Asplenium nidus*, where morphological characters were insufficient for species recognition between sympatric species. However, evidence from *rbcL* DNA sequence data and crossing experiments supported that three distinct, reproductively isolated species did exist though they were indistinguishable by morphological characters (Yatabe et al. 2001). Although it is unknown if the *Adiantum hispidulum* taxa are commonly found growing in sympatry through out New Zealand, crossing experiments could give insight as to whether these taxa are reproductively isolated and could constitute distinct species.

The genetic variation present in the *trnL-trnF* locus was small; one base pair substitution distinguished those plants with type 2 hairs from type 1 hairs (the most common haplotype), and three base pair substitutions for those with type 3 hairs from type 1 hairs. However, when compared to the genetic variation found between the species *A. cunninghamii*, *A. viridescens*, and *A. fulvum*, *A. cunninghamii* is distinct by the same amount of base pair

substitutions as is found between the haplotypes of *A. hispidulum*. The most prominent distinguishing morphological character for *A. cunninghamii* is a glabrous stipe and rachis, as compared to the hairy stipe and rachis of *A. viridescens* and *A. fulvum*. Although other characters have been recommended for distinguishing between the two species (e.g., lamina color and pinnule shape) these are often variable with respect to individual plants (Parris and Croxall 1974).

In addition, *A. fulvum* and *A. viridescens* are considered distinct species on the basis of one morphological characteristic, the presence of hair on the abaxial pinnae, and are molecularly identical according to multiple chloroplast loci. If these two species continue to be considered distinct species it might be consistent to consider recognizing two if not three distinct species among the *A. hispidulum* taxa corresponding to hair type and their respective haplotype. Nonetheless, the genetic variation coupled with differences in hair type present among the representatives of *A. hispidulum* warrants reinvestigation of these taxa and their status as either varieties or distinct species. Also, some level of consistency will need to be developed with the treatment of hairs with respect to the recognition of taxa within this genus in New Zealand. It would benefit future studies of these taxa if investigation were expanded beyond New Zealand and into other areas of the South West Pacific where the *A. hispidulum* complex is known to grow.

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Chapter 5

5.1 General Discussion

5.1.1 New Zealand Pteridaceae

The investigation into the phylogenetic relationships of the New Zealand Pteridaceae to their overseas relatives produced topologies suggesting that New Zealand Pteridaceae species were generally part of predominately South West Pacific groups within their respective genera. For example, results from the 220-taxon *rbcL* data set showed that the New Zealand species from the genera *Adiantum*, *Cheilanthes*, and *Pellaea* were generally more closely related to one another than to overseas relatives. The exception to this pattern was *Pteris tremula*, which was more closely related to overseas species than to the other New Zealand *Pteris*. The New Zealand members of the genera *Pellaea* and *Cheilanthes* formed monophyletic groups nested within other members of the cheilanthoid ferns (Chapter 2). The New Zealand *Pellaea* was sister to members of *Paragymnopteris* as opposed to other members of the genus

Pellaea. The New Zealand *Cheilanthes* did have affinity to other members of the genus *Cheilanthes*; however, this genus as a whole was highly polyphyletic. These results were not surprising because the genera comprising the cheilanthoid ferns are notorious for their confusing taxonomy, and are consistently resolved as paraphyletic and polyphyletic (Gastony and Rollo 1995, Kirkpatrick 2007, Zhang et al. 2007). Previous molecular studies of *Pellaea* and *Cheilanthes*, along with the results from Chapter 2, confirm that these genera are polyphyletic (Kirkpatrick 2007, Zhang et al. 2007). However, *Pellaea* sect. *Platyloma*, defined by Tryon and Tryon (1982), grouped together the members of the Australasian *Pellaea* in a monophyletic group indicating a possible new genus if taxonomic revisions are imposed. A revision of this nature has been suggested by Kirkpatrick (2007) where the species within *Pellaea* sect. *Platyloma* would constitute the new genus, *Platyloma*.

The New Zealand *Cheilanthes* were also more closely related to one another than to other non-New Zealand members of *Cheilanthes*. They formed a clade with the type species for *Cheilanthes*, *C. micropteris*. Although these two representatives of New Zealand *Cheilanthes* do group together, there are 13 other species of *Cheilanthes* that occur in the Australasian region (Bostock et al. 1998). These additional species would need to be incorporated into phylogenetic analyses to accurately determine their affinity to either the New Zealand *Cheilanthes* or to the overseas relatives, and hence, whether they support a South West Pacific group.

The New Zealand *Adiantum* species formed a group with the addition of the South American *Adiantum raddianum* and *A. cuneatum*. The affinity of *A. raddianum* and *A. cuneatum* to the New Zealand *Adiantum* as opposed to other South American *Adiantum* was surprising. Based on morphological characteristics, *A. raddianum* has traditionally been grouped with both *A.*

aethiopicum and *A. capillus-veneris* (Tryon and Tryon 1982). However, results from Chapter 2 and Chapter 4 recover that *A. raddianum* is distantly related to *A. capillus-veneris*, and although situated in the same clade as *A. aethiopicum*, has closest affinity to *A. hispidulum*. With the present data, it is unclear if *A. raddianum* has origins in South America and has immigrated to New Zealand or if it originated in New Zealand and dispersed to South America. As more of the 150 species of *Adiantum* are sampled, the relationships between the constituents of the two main clades will become more obvious.

The members of the New Zealand *Pteris* were polyphyletic, with *P. tremula* found to be more closely related to *Pteris* species from South America than to the other New Zealand *P. comans*, *P. saxatilis*, and *P. macilenta*. *Pteris tremula* is found throughout the South Pacific (Bostock et al. 1998). The majority of the *Pteris* species found in New Zealand and Australia have a vast distribution through the tropics. Only one endemic species occurs in Australia and two endemic species in New Zealand (Bostock et al. 1998, Brownsey and Smith-Dodsworth 2000). The endemic species in New Zealand are believed to be part of an allopolyploid complex, where endemic *P. macilenta* is a hybrid between Pacific *P. comans* and endemic *P. saxatilis*. Furthermore, the relationships between *Pteris* on a global level were unresolved in the *rbcL* MP analysis presented in Chapter 2 causing uncertainty among the relationships at the species level, as well as, the relationships of the New Zealand *Pteris* to its overseas members.

5.1.2 *Pellaea*

The morphologically-intermediate *Pellaea* aff. *falcata* plants were recovered as being more closely related to *P. rotundifolia* than to *P. falcata*, suggesting that *P. rotundifolia* may be a polymorphic species throughout New Zealand

(Chapter 3). Also, DNA sequence data recovered a sample of *P. falcata* collected from Tasmania to be molecularly identical to *P. rotundifolia*, raising concern that the *P. aff. falcata* plants are occurring outside of New Zealand. The biggest implication of this discovery is the difficulty in determining what is *P. falcata* in New Zealand and also other areas in the South Pacific.

The genetically different *Pellaea falcata* from the Kermadec Islands may be a new distinct species. However, until a more extensive sampling of these "*P. falcata*" plants is done, it will remain unclear whether this *Pellaea* species only occurs on the Kermadec Islands or whether it also exists on the mainland of New Zealand or other South Pacific Islands. Based on the finding of this thesis research, it is apparent that the problems with identifying *Pellaea* species in New Zealand is far greater than previously thought and also that this is an issue that extends beyond New Zealand to encompass Australia, and other South Pacific Islands.

5.1.3 *Adiantum*

The polymorphic *Adiantum hispidulum* is in need of reinvestigation based on the amount of haplotype variation detected amongst the 11 samples sequenced for this study. The three haplotypes corresponded to three distinct hair types that were visually distinguishable; these were: type 1- sparse, short stiff hairs, type 2- dense long lax hairs, and type 3- abundant, shorter than *A. hispidulum*, spike-like hairs. More extensive sampling of these plants will be necessary to not only to confirm the pattern found between haplotypes and hair type but also to better determine the distributions of these types throughout New Zealand. Based on previous species treatments within *Adiantum* in New Zealand, particularly *A. fulvum* and *A. viridescens* (Parris and Croxall 1974), single morphological characters were deemed appropriate

to denote separate species. Given the morphological differences found among hairs coupled with haplotype variation, the plants recognized in the *A. hispidulum* complex warrant further investigation or at most a confirmation of their status as either varieties or separate species.

5.1.4 Taxonomic sampling

A main problem encountered with many phylogenetic investigations is obtaining an adequate amount of taxonomic sampling to represent a realistic amount of biodiversity. Many studies have emphasized the importance of the taxonomic sample set, where even the addition or deletion of a single taxon can change the inferred phylogenetic relationships (Poe 2003, Soltis et al. 2004, Martin et al. 2005). With many of the findings reported in this study, it has become clear that increased taxonomic sampling would improve both the clarity and the confidence of the results. The results from Chapter 2 confirm previous classifications of the Pteridaceae with respect to recovering five major monophyletic clades (i.e., cryptogrammoids, ceratopteridoids, cheilanthoids, pteridoids, and the adiantoids). But incongruent topologies recovered between the chloroplast genes *atpB* and *rbcL* offered little insight into confident relationships at the generic level. The Pteridaceae is a very large and very diverse fern family that has a vast world wide distribution, which only compounds obtaining a sample set that is adequate for inferring robust relationships.

For example, previous studies reported recovering the genus *Adiantum* as paraphyletic with respect to the Vittarioid ferns (Prado et al. 2007, Schuettpelz and Pryer 2007, Schuettpelz et al. 2007). As reported by the results recovered from the 220-taxon *rbcL* data set, with the addition of the New Zealand species of *Adiantum*, the genus appears to be monophyletic. Nonetheless, the

vittaroids have affinity to *Adiantum* and should be included in the Pteridaceae family, but whether they are derived from within *Adiantum*, hence making this genus paraphyletic, is unclear. Also, the problems with the species of *Pellaea* in the South Pacific will begin to be clarified with increased sampling. Geuten et al. (2007) stress that rather than focusing on increasing the number of characters, more emphasis should be placed on choosing the appropriate taxa set to maximize the improvement and accuracy of inferred evolutionary relationships.

5.1.5 New Zealand biodiversity

The Pteridaceae in New Zealand is among the largest fern families in New Zealand (Breitwieser et al. in press). Accurately determining the species diversity within the family, as well as the relationships between these species is an important step towards cataloguing fern biodiversity in New Zealand. The advancement of molecular technologies has provided the tools to detect variation among species that lack distinguishable morphological characteristics used to traditionally classify fern species. Specifically, several morphologically-intermediate *Pellaea* plants have led to the mis-identification of *P. falcata* populations throughout New Zealand. It appears from both the collection efforts and the results from this study that *P. falcata* may be more threatened than previously thought. Having molecular markers available to accurately distinguish these plants will aid efforts of conservationists as they help to identify any possible remaining populations left in New Zealand. Furthermore, molecular haplotype variation within the samples of *A. hispidulum* could lead to additional species diversity within *Adiantum*.

Gaining an understanding of the relationships of the New Zealand ferns to their overseas relatives, will help shed light on the origins of the flora in New

Zealand. Inference from previous studies (Pryer et al. 2004, Perrie and Brownsey 2007) suggested that the members of the Pteridaceae in New Zealand are of recent origins, and are most likely a result of long distance dispersal. The relationships shown in the topologies recovered from the 220-taxon *rbcL* data set suggest that the direction of dispersal for the species of Pteridaceae is immigration into New Zealand. The results from this study will aid in further investigations of both the biogeographic history of Pteridaceae ferns in New Zealand, as well as, accurately describing species within the genera *Pellaea* and *Adiantum*.

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Appendix A – Distribution maps of New Zealand Pteridaceae

Map data based on collection information from the WELT, CHR, and AK herbaria and includes cultivated specimens. Note that voucher information of *Pellaea falcata* was mapped as originally determined.

Figure A1. *Adiantum cunninghamii*

Figure A2. *Adiantum aethiopicum*

Figure A3. *Adiantum raddianum*

Figure A4. *Adiantum diaphanum*

Figure A6. *Adiantum formosum*

Figure A7. *Adiantum fulvum*

Figure A8. *Adiantum hispidulum*

Figure A9. *Adiantum viridescens*

Figure A10. *Annogramma leptophylla*

Figure A11. *Cheilanthes distans*

Figure A12. *Cheilanthes siberi*

Figure A13. *Pellaea calidurupium*

Figure A14. *Pellaea falcata*

Figure A15. *Pellaea rotundifolia*

Figure A16. *Pteris comans*

Figure A17. *Pteris macilenta*

Figure A18. *Pteris saxatilis*

Appendix B – Transition vs. transversion graphs for *rbcL* and *atpB* data sets

Figure B1. Graph of transition/transversion vs. sequence divergence for the 88- taxon *atpB* data set.

Figure B2. Graph of transition/transversion vs. sequence divergence for the 88- taxon *rbcL* data set.

Appendix C – Specimen LRP4994: *Adiantum hispidulum*
(var. *pubescens*)

Figure C1. Herbarium specimen from WELT herbarium of the *Adiantum hispidulum* sample, LRP4994.

CD Appendix – Chloroplast sequence data and Nexus files