Declaration

I can confirm that is my own work and the use of all material from other sources has been properly and fully acknowledged.

Mazhani Binti Muhammad

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Abstract

Ethnobotanical knowledge of plants' medicinal use could make a contribution to bioprospecting by identifying plants to target for drug discovery. In recent years, methods to investigate the medicinal uses of flowering plants using a phylogenetic framework have been developed. Drugs derived from higher plants are prevalent, and ferns are relatively neglected. Thus, this thesis investigates the evolutionary patterns amongst fern species that are used medicinally using phylogenetic tools at a range of taxonomic and spatial scales, from global to regional scales, for the first time.

Dense sampling at species levels may be critical for comparative studies, thus an updated fern megaphylogeny focusing on four gene regions, *rbcL, rps4, atpA* and *atpB* was reconstructed. This large-scale phylogeny comprises more than 3500 fern species in 273 genera and 47 families, covering over a quarter of extant global fern species. To evaluate the medicinal importance of ferns, a database based on a comprehensive review of records published in books, journals or in online sources including databases was assembled. The use database comprised 3220 use-reports for 442 species, and showed that approximately 5% of total estimated extant fern species have a documented therapeutic use, but only 189 species have become the focus of screening concerning their bioactivity properties.

Using a comprehensive phylogenetic tree and medicinal data from the database, species used in traditional medicine were shown to be significantly dispersed across the fern phylogeny, contrary to previous findings in many similar studies of flowering plants. Whatever the hierarchical level or spatial scale investigated, the results consistently found evidence of phylogenetic overdispersion of medicinal use, suggesting more exploration of medicinal uses in a phylogenetic context is required before the generality of this approach is globally accepted. Finally, safety and toxicity of ferns that are used as medicine and food were also explored, since fern consumption can pose a serious health risk. The distributions of toxic compounds and arsenic hyperaccumulation in ferns were investigated using taxonomic and phylogenetic approaches in order to access the risk to consumers. Overall, assessment of toxicity in ferns is still limited and awareness needs to be raised about the health risks associated with the use of ferns.

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Chapter 1. General Introduction

1.1 General introduction

1.1.1 What are medicinal plants?

Medicinal plants are defined as plants that contain substances or properties which can be used for therapeutic purposes. They are generally considered to play a beneficial role in health care (Srivastava et al., 1996; Odugbemi, 2008). Whether the use of medicinal plants in traditional systems are efficacious or not is controversial (Calixto, 2000; Saslis-Lagoudakis et al., 2012). The continued use of plants in traditional cultures maybe because of their perceived magical or symbolic importance. There maybe no scientific proof of efficacy, but beliefs are passed verbally from one generation to the next generation by elders or shamans (Chin, 2005; Van Wyk & Wink, 2004).

It is estimated that more than 50,000 species of Angiosperms have been used for medicinal purposes (Schippmann et al., 2002) in the form of crude drugs, as tinctures, powders, teas, poultices or other herbal formulations (Balunas & Kinghorn, 2005; Lewis & Elvin-Lewis, 1995). The majority may have been used in the treatment and prevention of disease for thousands of years (Samuelson, 1999) and many have been documented in local folk pharmacopeias or in ancient literature (Ebbell, 1937; Unschuld, 1986; Saito, 2000). According to the World Health Organization (WHO), many developing countries still rely on traditional medicinal plants for their healthcare needs (Gurib-Fakim, 2006; Hamann, 1991; Van Wyk & Wink, 2004). The importance of medicinal plants has gained considerable attention amongst the scientific community and led to growth of ethnomedicinal and ethnobotanical research in many countries since documentation of medicinal plants is not only crucial for conservation but also assists in the search for new drugs (Cox & Balick, 1994; Balick & Cox, 1996; Heinrich, 2000; Fabricant & Farnsworth, 2001; Sheng-Ji, 2001; Heinrich & Bremner, 2006; Gu et al., 2014). The value of medicinal plants to human livelihoods is significant since collecting and trading medicinal plants provides an additional source of income for local communities (Hamilton, 2004).

The Doctrine of Signatures may seem to be an irrational concept found in many of the healing cultures around the world. The concept is based on the appearance of plants providing signs or clues to their medicinal properties. For example, bile and jaundice are believed to be treated by yellow flowering plants, and the human shape of particular roots is associated with the female form and fertility. It has been suggested that the placebo effect may apply to plants perceived in this way when those species with characteristics resembling the disease treated maybe seen to be more effective by patients (Bennett, 2007). However, sometimes, plants identified through Doctrine of Signature are proven efficacious. For example, *Chelidonium majus* has yellow flowers and a yellow alkaloid containing latex that has successfully been used to treat jaundice (Gurib-Fakim, 2006; Van Wyk & Wink, 2004).

1.1.2 Medicinal plants and the pharmaceutical industry

In a survey published in 2001, Fabricant and Farnsworth described 122 compounds from only 94 species of plants that are used globally as drugs (Fabricant & Farnsworth, 2001). It seems that plants are important sources of leads for the pharmaceutical industry since plants are able to synthesize an enormous variety of potentially medicinal molecules (Ahmad et al., 2006). For example, the discovery of taxol in the bark of trees of genus *Taxus* provided a new chemotherapy drug to treat ovarian and breast cancer (Cragg, 1998; Goodman & Walsh, 2001). Other drugs derived from medicinal plants include campthothecins from *Camptotheca acuminata* used for cancer treatment (Sriram et al., 2005), etopside from *Podophyllum peltatum* used to treat variety of tumors (Van Maanen et al., 1988) and tiotropium, a derivative of atropine from *Atropa belladonna* used to treat chronic obstructive pulmonary disease (Barnes et al., 1995; Balunas & Kinghorn, 2005). With more than 400,000 species of land plant (Govaerts, 2001), there may be an abundance of potential drug leads remaining to be discovered.

Ethnobotanical data could make a tremendous contribution to bioprospecting by identifying plants for drug discovery. Fabricant and Farnsworth (2001) showed of the 122 drug compounds from plants and demonstrated that 80% have identical or related ethnomedicinal uses. However, ethnobotanical bioprospecting has been criticized because some traditionally used medicinal plants may not be efficacious, resulting in disappointing outcomes (Firn, 2003; Saslis-Lagoudakis et al., 2012). In addition to this, the ethnomedicinal information collection process from primary sources requires extended periods of ethnobotanists' time, thus increasing the financial cost (Cox & Balick, 1994). Furthermore, difficulties in agreeing prior informed consent and negotiating benefit sharing for traditional knowledge acts to limit international research (Ningthoujam et al., 2012). Nevertheless, surveying and understanding how medicinal plants are selected for traditional use, and predicting which plants might yield important new compounds is important.

1.1.3 Medicinal plants and public health

The popularity of traditional medicine globally is associated with ease of access, ‘low cost’ and the assumption of safety. This assumption is based on the perception that plants are ‘natural’, but there is evidence that many plants contain carcinogenic and toxic compounds. These toxins have proven to be associated with numerous health problems. For instance, pyrrrolizidine alkaloids (PAs) were the amongst the first compounds demonstrated to have carcinogenic activities (Bull, 1955; Mattocks, 1968; Hirono, 1987; Chan et al., 2003; Chen et al., 2010) and they are present in various plants, some of which are used as food and herbal remedies (Hirono, 1987). Since then, many carcinogenic principles were isolated from plants such as Ptaquiloside (PTA) that has been found in a variety of ferns and which can cause cancer in farm animals (Saito et al., 1989; Saito et al., 1990), and aristolochic acid in species of *Aristolochia* that is associated with a high incidence of upper urinary tract cancer (Michl et al., 2013). Reliance on medicinal plants in developing countries raises concerns about exposure to toxins since many traditional herbal medicines known to contain toxic compounds are still used (Roeder & Wiedenfeld, 2013) but cases of negative side effects from consuming these plants as herbal medicines are rarely reported in the literature. Apart from naturally-occurring carcinogenic chemical compounds present in some medicinal plants, contamination by heavy metals such as cadmium, chromium, lead and arsenic should also be a major concern. For example, several medicinal plants in Brazil (Caldas & Machado, 2004) and some herbal dietary supplements in Mexico (García-Rico et al., 2007) have been shown to contain dangerous amounts of heavy metals resulting in serious health problems.

In addition to the health risks associated with traditional medicinal plant use if the medicinal species are toxic, adverse effects of herbal products may also result from other factors. From the cultivation of medicinal plants to the final herbal products, factors such as adulteration, substitution, contamination, misidentification, lack of standardization or incorrect preparation may influence their quality and safety (Woo, 2000; Zhang et al., 2012). For example, seven cases of toxicity were identified in infants following the home administration of star anise tea, attributable to the adulteration of Chinese star anise (*Illicium verum*) with toxic Japanese star anise (*Illicium anisatum*) (Ize-Ludlow et al., 2004). With the great growth and worldwide interest in using complementary and alternative medicine (CAM) involving herbal medicines (Barnes et al., 2004; Hyodo et al., 2005; Hornerber et al., 2011), authorities have been charged with ensuring the safety of imported plant medicines through newly enacted legislation. In the United Kingdom, for instance, the Medicines and Healthcare Products Regulatory Agency (MHRA) is responsible for herbal medicine safety, while in other countries, such as Malaysia and Singapore, the regulation of herbal medicines is controlled by their Ministries of Health (Woo, 2000; Jayaraj, 2010). Nevertheless, risks associated with herbal medicines still occur if individual consumers who have bought products from overseas for their own use are unaware of ‘hidden’ ingredients which may cause serious toxic effects (Miller, 1998; Coghlan et al., 2012). Therefore, greater control over the safety of herbal medicines could be achieved not only through a global improvement in regulatory processes, but also through greater awareness among herbalists and the public.

1.1.4 Ferns as medicinal plants

It has been reported that different fern species have been used for the health benefits they provide and to treat various ailments throughout the world since antiquity (Winter & Amoroso, 2003; Van Wyk & Wink, 2004; Chin, 2005; Srivastava, 2007; Mannan et al., 2008). These uses have either been transmitted orally, from generation to generation, or recorded in pharmacopoeia (Chin, 2005; Van Wyk & Wink, 2004). Despite literature describing medicinal use of ferns for a variety of diseases, interesting biological activities of few ferns are known. Antioxidant activity has been reported in various ferns such as *Adiantum capillus-veneris* (Kumar, 2009), *Blechnum orientale, Stenoclaena palustris* and *Dicranopteris linearis* (Lai et al., 2010; Lai & Lim, 2011; Chai et al., 2012) while antimicrobial activity has been reported in *Osmunda regalis* (Thomas, 2011), *Athyrium filix-femina* and *Pleopeltis macrocarpa* (Kumarpal, 2013). Some of these biological activities support the traditional use of fern species.

1.2 New methods for investigating medicinal plants

This study uses a phylogenetic framework to interrogate published use data, newly compiled here, and explore the evolutionary patterns amongst fern species that are used medicinally and the distribution of known toxins in ferns. The use of phylogeny alongside existing use data is emerging as a new method in medicinal plant research (Saslis-Lagoudakis et al., 2015).

1.2.1 Applications of phylogenetic methods

Phylogenetic predictivity has been a principle used to justify phylogenetic research, for example in conservation biology (Vane-Wright et al., 1991; Faith, 1992) as well as the possibility of using phylogenies to guide bioprospecting (Smith & Wheeler, 2006). The application of phylogeny to understanding medicinal plants has included studies of the distribution of phytochemicals (Wink, 2003; Wink & Mohamed, 2003; Muellner et al., 2005) and studies of the ethnobotanical use in a phylogentic context (Saslis-Lagoudakis et al., 2011; Saslis-Lagoudakis et al., 2012; Yessoufou et al., 2015). Whilst phylogenetic methods are emerging as a way of understanding how organisms have and could be used, phylogeny has hardly been used to predict health risks of exposure to plants or animals. The investigation of the phylogeny of the diatom genus *Pseudo-nitzschia* is one exception (Trainer et al., 2012).

Combined phylogenetic and phytochemical studies have revealed that there is strong phylogenetic signal in the distribution of chemical constituents in plants (Wink, 2003; Rønsted et al., 2008; Rønsted et al., 2012), which can be applied in finding new sources of natural products. Nevertheless, chemical data for the majority of species are unavailable and can be costly to generate. Recent studies also have shown that species with traditional medicinal use are not randomly scattered within plant lineages. These studies have shown that phylogenetic tools can reveal whether plant lineages are independently selected, and whether related plants find a similar use (Lukhoba et al., 2006; Forest et al., 2007; Saslis-Lagoudakis et al., 2011; Saslis-Lagoudakis et al., 2012; Yessoufou et al., 2015; Ernst et al., 2016). These findings were interpreted to indicate that closely related plant species share the same medicinal properties, implying phylogenies combined with traditional knowledge could be useful for bioprospecting.

1.2.2 Meta-analysis: compilation and interrogation of published use data

As ethnobotanical data are scattered in various publications in different languages with limited accessibility and application, initiatives are needed to collect all the data into a centralized database for easy reference (Gaikwad et al., 2011). Nowadays, with the increasingly rapid development of information technology (IT), computerized databases have become an indispensable tool for managing, compiling, disseminating and analyzing large amounts of data in diverse arenas.

Several of the published studies using phylogeny have depended on the compilation and use of published data. For example, studies using data sets compiled from literature have tested whether there are global patterns of plant use between distantly related peoples (Saslis-Lagoudaskis et al, 2012), and determined the relative importance of different drivers of plant selection at national levels (de la Torre et al., 2012; de Madeiros et al. 2013; Saslis-Lagoudaskis et al, 2014). Indeed, there has been a call for meta-analysis in ethnobotany, as it is increasingly recognized that there may be patterns in use, which are informative of the processes acting on cultures and plants (Albuquerque & de Medeiros, 2012; de Medeiros et al, 2013).

1.3 A summary of aims and an overview of the structure of this thesis

This thesis describes the finding of three years of study of medicinal ferns. The relative lack of interest in medicinal ferns, compared to flowering plants, means that a synthesis of the relatively dispersed literature describing medicinal fern use can provide new insights into the ways in which ferns have been selected and used in traditional medicine, and the implications to public health of their use. A new fern megaphylogeny and databases have been reconstructed to explore the patterns of fern species that are used medicinally throughout the globe and at the same time predict the risk related with medicinal species.

Previous studies have greatly improved our understanding of fern evolution and provided a backbone for modern classification. Phylogeny reconstructed using molecular, morphological or combined molecular and morphological data have revealed many major branches of the fern tree of life and in this light the classification of ferns has been revised and updated (Smith et al., 2006a; Christenhusz et al., 2011). The most comprehensive fern phylogeny to date represents over one-fifth of extant global fern diversity, so understanding of the relationships between ferns is fairly good (Lehtonen, 2011). However, many species are unplaced and these could include economically important species. In addition, there are still some controversies in the exact position of several families and some genera are still poorly defined thus further studies for certain families must be undertaken. One of the factors contributing to the lack of clarity regarding fern relationships is probably a lack of data. Therefore a more comprehensive approach with a larger dataset and more taxa could contribute to more robust phylogeny and provide a pivotal platform to for future hypothesis-driven ecological, biogeographical and other related studies of ferns. As publically available sequence data are accumulating from ongoing phylogenetic studies, a global analysis using a denser sampling than a previous fern megaphylogeny (Lehtonen, 2011) was performed (Chapter 2). This ‘supermatrix’ analysis not only permits exploration of large-scale evolutionary patterns in ferns, but also permitted further evaluation of their utilities (Chapter 4 and Chapter 5).

Existing data on medicinal use of fern species was compiled into a single source of comprehensive literature in one database (Chapter 3). The application of this database is to facilitate the storage of data for fern species throughout the globe, access to, and use any relevant data for further analyses (Chapter 4 and Chapter 5). A review of the pharmacological of medicinally-used ferns was also made (Chapter 3), to point out the gap between modern pharmacological knowledge and traditional uses of ferns.

The phylogenetic relatedness of ferns that are used medicinally were explored for the first time in Chapter 4. This investigation focussed on exploring patterns at different a range of spatial scales, from global to continental and regional/national scales by using methods from phylogenetic community ecology.

It is desirable to recognize which fern species that are used medicinally have been shown to contain toxic properties. Finally, Chapter 5 highlights specifically the presence of PTA and arsenic hyperaccumulation in ferns that are used for human consumption as a food or medicine. Many of these species have close relatives that may also be toxic. By using data on medicinal use of ferns from Chapter 3 and the reconstructed fern phylogeny from Chapter 2, Chapter 5 also investigates the phylogenetic distribution of toxic plant as this might suggest which groups are likely to have the same properties and should be focused on further investigations.

To summarize, this thesis comprises four main chapters that include data analysis:

- Chapter 2 presents a phylogeny based on a supermatrix approach focusing on four gene regions; *rbcL, rps4, atpA,* and *atpB*, mostly based on published sequences available in Genbank;
- Chapter 3 consists of the compilation of a database to describe the traditional use of medicinal ferns and what is known about their pharmacology;
- Chapter 4 focuses on an investigation of large-scale phylogenetic patterns amongst fern species that are used medicinally;
- Chapter 5 reviews the toxicity and carcinogenicity of ferns, focussing on PTA and arsenic hyperaccumulation by fern species, especially on species that are used as traditional medicine or eaten as vegetables. The chapter assesses the distribution of known toxins, taking a taxonomic and phylogenetic approach to predicting risks associated with species that have not been screened for toxins.

Chapter 2. A comprehensively sampled fern tree of life

2.1 Introduction

Ferns (monilophytes sensu (Pryer et al., 2001)), also known as pteridophytes, comprise approximately 9,000 to 12,000 species (Smith et al., 2006; Lehtonen, 2011; Christenhusz & Chase, 2014) and are sister to the seed plants (Pryer et al., 2001). In a recent classification, ca. 280 genera in 45 families were recognized (Christenhusz et al., 2011). Since then, two new families have been created; Hemidictyaceae (Christenhusz & Schneider, 2011) and Arthropteridaceae (Liu et al., 2013) and changing generic limits informed by phylogenetic studies have resulted in an increase in the number of genera to 287 genera (Appendix 2.1).

Higher-level classification of ferns and their allies was unstable until the first molecular phylogenies were published in the mid-1990s (Wolf et al., 1994; Gastony & Rollo, 1995; Hasebe et al., 1995; Pryer et al., 1995; Wolf, 1995). Since then, numerous phylogenetic studies based on DNA sequences or combined molecular and morphological data have either densely sampled genera or families (e.g. Hennequin et al., 2003 (*Hymenophyllum*); Rouhan et al., 2004 (*Elaphoglossum*); Hauk et al., 2003 (Ophioglossaceae); Korall et al., 2007 (Cyatheaceae)) or sought a robust backbone for the fern phylogeny by sampling exemplar species of higher taxa (Pryer et al., 2001; Pryer et al., 2004; Qiu et al., 2006; Schuettpelz et al., 2006; Qiu et al., 2007; Schneider et al., 2009; Rai & Graham, 2010). The latter have consistently shown ferns comprise five major extant lineages, horsetails, psilotophytes (whisk ferns), ophioglossoid ferns, marattioid ferns and leptosprangiate ferns, contradicting traditional classifications which placed horsetails and whisk ferns together with Lycopodiales and Selaginellales, the "fern allies" (Bidin, 1986; Parris & Latiff, 1997). As a result, the focus of phylogenetic enquiry and phylogenetically informed classifications has become the monophyletic 'monilophyte' or 'fern' clade (Christenhusz et al., 2011; Lehtonen, 2011; Christenhusz & Chase, 2014). Ongoing phylogenetic studies at family/genus level, or testing the backbone fern phylogeny by sampling exemplar species or higher taxa continues to generate new sequence data. Nevertheless, relationships between several major clades still remain unresolved and polyphyletic and paraphyletic genera are common in many lineages, so that generic delimitation within the important families still needs further investigation.

Whist backbone and clade-specific phylogenies can be the basis of revised classifications, a need for large, densely sampled phylogenies to address broad evolutionary questions is increasingly recognized (Smith et al., 2009; Legume Phylogeny Working Group, 2013; Särkinen et al., 2013; Antonelli et al., 2014; Qian & Zhang, 2014; Qian & Jin, 2015). New analytical approaches have been developed for reconstruction of extremely large phylogenetic trees using publically available molecular data; the so-called 'supermatrix' or 'mega-phylogeny' analyses (Driskell et al., 2004; de Queiroz & Gatesy, 2007; Smith et al., 2009; Lehtonen, 2011; Lewis et al., 2013; Qian & Zhang, 2014; Qian & Jin, 2015). Massive molecular data sets can be retrieved from databases such as Genbank, combined into a single, large and comprehensive matrix, and used to infer phylogenies to address large-scale evolutionary questions. A mega-phylogeny for ferns exists. Lehtonen (2011) generated a phylogeny representing more than one-fifth of extant global fern diversity representing 2656 taxa based on four plastid genes (*rbcL, rps4, atpA*, and *atpB*) using parsimony and maximum likelihood analyses. Nevertheless, several phylogenetic relationships still could not be completely resolved and many comparative studies often require more species than are available in previous phylogenies (Pearse & Purvis, 2013). For instance, for community ecology, some species in which ecologist are interested is not available in such trees, making difficult to test any hypotheses in macroevolutionary studies (Pearse & Purvis, 2013). Furthermore, many species names in previous ferns megaphylogeny also were not currently accepted names, since the names applied to terminal taxa were those used on genbank.

2.1.1 Objectives

Lehtonen’s (2011) study is the benchmark for fern megaphylogeny. The primary objective of this study is to update Lehtonen’s megaphylogeny by adding all the available data for the same regions; *rbcL, rps4, atpA,* and *atpB*. Chloroplast regions were selected in this study since they have been widely used in the majority of fern phylogenetic studies. Furthermore, these genes are variable enough to provide good resolution across the taxonomic diversity of fern taxa (Lehtonen, 2011; Prado et al., 2007). In the five years since his work, many additional sequences have been generated, but to date, there has been no update published. The objective of this study is to generate a more densely sampled tree which can be used for further analysis in Chapter 4 and Chapter 5. In order to generate a phylogeny fit for purpose, it is also necessary to sequence de novo some medicinal ferns not represented in Genbank.

Ultimately, the tree would be made available so it could be used in other evolutionary research on ferns, such as in ecological and community phylogenetic and biogeographical studies. To maximise the value of the phylogeny, nomenclatural changes are made. Standardization of species nomenclature is essential when the study involves sequence data deposited over a period of time when many nomenclature changes have taken place. Genbank names are not currently accepted names, and this limits the utility of any phylogeny with names following Genbank. Hence, a second objective is that the nomenclature will be updated, rather than using names as available on Genbank, to produce a more user-friendly phylogeny.

The topology of the phylogeny will be checked by comparison to existing phylogenetic hypotheses. This ensures the phylogeny is fit for future use, and does not include any anomalous relationships. This study also aims to investigate whether there are taxonomic or geographic biases in the availability of sequence data for the ferns, and to suggest a sampling strategy to address these biases.

2.2 Materials and Methods

2.2.1 Data assembly and taxonomic sampling

Four chloroplast protein-coding genes*, rbcL, rps4, atpA,* and *atpB,* were selected for this study. These are genes which have been used extensively to infer fern phylogeny in recent years (Pryer et al., 2001; Pryer et al., 2004; Schneider et al., 2004; Schuettpelz et al., 2006; Schuettpelz & Pryer, 2007; Lehtonen, 2011). All sequence data available by November 2014 were downloaded for the root node “Moniliformopses’ from Genbank using Geneious Basic (http://www.geneious.com/download). These data were then grouped into folders by gene region.

For each gene region, hybrid taxa and unidentified species (species identified to genus level only e.g., *Adiantopsis* sp. MLP-2011-1) were eliminated. If multiple or duplicate sequences were available for the same taxon, only one sequence, the longest most complete one was retained. In total, 3713 fern sequences including ten generated de novo for this study (Section 2.2.2) were accepted and six outgroups, one lycophyte and five seed plants, were included in the analysis (Appendix 2.2).

A list of accepted names of ferns in the Genbank (NCBI) list was updated since many species names did not follow the latest fern taxonomy and nomenclature. At family and genus levels, the NCBI list was restructured based on Christenhusz et al. (2011) (Appendix 2.1), with some minor changes in family and generic classification to reflect more recent studies (Appendix 2.3). At species level, names in the NCBI list were checked and matched with the currently accepted name based on the three international authority plant databases; Tropicos (2016), Catalogue of Life (2016) and IPNI (2005) in order to apply revised synonymy and to standardize spelling of plant names. If the taxon name appeared two or more times with different authors in Tropicos (2016) or Catalogue of Life (2016), the taxon name used was the one in the publication linked to Genbank's sequence record.

2.2.2 DNA isolation and amplification

New sequences were generated to make the phylogeny fit for further analysis in Chapter 4. The species sequenced are detailed in Table 2.1. Genomic DNA was extracted from 0.3 to 0.5 g of leaf tissue from herbarium or silica-gel dried material using a modification of the CTAB procedure (Doyle & Dickson, 1987) (Appendix 2.4).

Table 2.1 List of generated sequences included in the phylogenetic analysis.

Taxon	Collection site/Location	Voucher specimen number
Alsophila latebrosa	Taman Paku pakis, UKM, Malaysia	MM001 (UKMB)
Alsophila contaminans	Taman Paku pakis, UKM, Malaysia	MM003 (UKMB)
Cheilanthes tenuifolia	Taman Paku pakis, UKM, Malaysia	MM004 (UKMB)
Dicranopteris curranii	New Guinea	MK13594 (UC)
Diplazium cordifolium	Taman Paku pakis, UKM, Malaysia	MM005 (UKMB)
Gleichenia vulcanica	New Guinea	ML2530 (UC)
Hypolepis repens	Las Casas de la Selva, Puerto Rico	Every24 (RNG)
Lygodium flexuosum	Taman Paku pakis, UKM, Malaysia	MM006 (UKMB)
Lygodium longifolium	Taman Paku pakis, UKM, Malaysia	MM007 (UKMB)
Sphaeropteris moluccana	Taman Paku pakis, UKM, Malaysia	MM002 (UKMB)

The *rbcL* gene region was amplified using the primers shown in Table 2.2. The PCR amplification was conducted in 20 µl reactions containing 19 µl of PCR Mastermix (0.75 µL of each primer at 10 µM, 0.5 µL of 2.5 µg/µL BSA solution, 0.2 µL 5% DMSO, 6.8 µL purified water and 10 µL Biomix Red) and 1 µl of template DNA (~ 10-200 ng). The PCR protocol included 5 min initial denaturation (94° C), and followed by 33 cycles of 45 sec denaturation (94°C), 30 sec annealing (45°C), and 2 min elongation (72°C) with a final elongation step of 5 min (72°C). PCR products were cleaned with the QIAquick PCR Purification Kit (Qiagen) following the manufacturer's protocol with a final elution of 30 µL EB buffer. All purified PCR products were sent to Source Bioscience (Oxford, United Kingdom) to be sequenced. Contiguous sequences were assembled using the SeqMan program (DNASTAR, Lasergene). All sequences were blasted in Genbank in order to confirm their identity.

Table 2.2 All the primers used in this study are from Schuettpelz and Pryer (2007).

Region	Name	Sequence 5' - 3'
rbcL	ESRBCL1F^{AS}	ATGTCACCACAAACGGAGACTAAAGC
	ESRBCL1361R^{AS}	TCAGGACTCCACTTACTAGCTTCACG
	ESRBCL628F^{S}	CCATTYATGCGTTGGAGAGATCG
	ESRBCL654R^{S}	GAARCGATCTCTCCAACGCAT

2.2.3 Sequence alignment and phylogenetic analysis

Sequences for each dataset were aligned in MUSCLE (Edgar, 2004) under default settings. The alignments then were checked and edited manually using BioEdit 7.25 (Hall, 1999). The 5′ and 3′ ends containing high proportions of missing data were trimmed using TrimAI (Capella-Gutiérrez et al., 2009).

The four alignments were concatenated to form a multigene matrix using Mesquite 2.74 (Maddison & Maddison, 2001). Two methods, Maximum Likelihood (ML) and Bayesian Inference (BI) are currently the most widely used methods to infer large phylogenetic relationships. These methods introduce an evolutionary-model based on statistical methods which have the potential to search and evaluate different possible trees. However, running BI over a thousand species can take a very long time compared with ML using shortcuts, as recent improvements of the RaxML algorithm provide reasonable time for large dataset to be analysed (Roquet et al., 2012). Therefore, phylogenetic analysis for the combined four gene dataset in this study was conducted under the Maximum Likelihood (ML) criterion and the GTRGAMMA Model in RaxMLv7.2.8 (Stamatakis et al., 2008) implemented in the CIPRES web-portal. Branch support was assessed using 100 replicates of non-parametric bootstrap analysis.

2.2.4 Assessment of taxonomic bias in data availability

To explore taxonomic sampling biases, the number of species in the dataset and the estimated diversity for each order and family were recorded. In most cases, estimates of species numbers for each order and family followed Smith et al. (2006), except where family limits had changed since this treatment. Estimated diversity for these families was recalculated based on more recent publications. General regression for all analyses were carried out in Microsoft Excel 2007 using log-transformed values for total number of taxa represented in the phylogeny as the dependent (y) variable, and total estimated diversity as the independent (x) variable.

An audit of fern nucleotide sequence data against the current taxonomy held in the dataset was also conducted to identify genera for which no nucleotide sequence data from any genomic regions were available in Genbank. These genera were compiled based on the list of accepted names of fern genera (Appendix 2.1). The geographical range information derived from published papers was also added to the list of missing genera.

2.3 Results and Discussion

2.3.1 Availability of sequence data and nomenclatural updates

Five coding regions, *rbcL, atpA, atpB*, *rps4* and *matK*, were most often used for phylogeny reconstruction. The most frequently sequenced gene region was *rbcL* with 7627 sequences available, followed by *rps4* (3480), *atpB* (2311) and *atpA* (1016) (November, 2015). The *matK* region, with 922 sequences, was the least represented. The avaibility of *rbcL* in Genbank might be because of its universality and easy of amplification and alignment (Kress et al., 2005). Furthermore, *rbcL* has been shown to be useful in addressing phylogenetic relationships at a variety of taxonomic levels for ferns (Gastony & Rollo, 1995; Hasebe et al., 1995; Dubuisson, 1997; Lu et al., 2007; Prado et al., 2007). *rbcL* was one of the two regions selected as core barcoding regions (CBOL Plant Working Group, 2009). In contrast, *matK,* the other region selected as core barcode, is much less frequently sequenced. *matK* is embedded within a *trnK* intron and can be amplified using primers targeting the flanking *trnK* exons. Difficulties in amplication due to absence of the flanking *trnK* exons in 90% of fern species (Duffy et al., 2009; Li et al., 2011) is probably one of the reasons why *matK* is less available in Genbank compared to other regions. The implications of different gene sequence availability across land plants are felt in the representation of ferns in land plant megaphylogenies. For example, Zanne et al. (2014) sampled seven regions (18S *rDNA*, 26S *rDNA*, *ITS*, *matK*, *rbcL*, *atpB*, and *trnL-F*) but included only 518 fern species far fewer than the 3713 included.

This number, 3719 including outgroup taxa, remained after removing the multilple accessions of the same taxon and unidentified taxa. The numbers of characters and taxa for each alignment and for the final matrix are shown in Table 2.3. The matrix of all taxa by four genes had 7875 missing gene sequence entries (53% missing data) and nearly half of the sampled taxa were represented by single gene (45%). Only 11% represented by all four genes. Most taxa were represented by the *rbcL* gene (94%) which has been shown to resolve some difficult problems in the phylogeny of green plants, even without other data (Chase et al., 1993; Smith et al., 2009). Though half of the genes sequences are missing from this supermatrix, an entire tree can be reconstructed robustly in such cases, if enough characters are included in the analysis (Wiens, 2003; Philippe et al., 2004; Wiens, 2006).

Table 2.3 Alignment details in this study.

Dataset	Characters (bp)	Taxa
rbcL	1426	3477
rps4	427	1570
atpB	1278	1065
atpA	1505	889
Combined	4636	3719

The phylogeny in this study updates Lehtonen's (2011) megaphylogeny by incorporating 1057 more species from publically available databases. Hence, the taxonomic sampling employed here was broader than the previous best-sampled fern phylogenetic analysis and can be used to test the monophyly of currently accepted genera and families. It also makes 60 nomenclatural updates at generic level (Appendix 2.3) and 480 updates from Genbank names following recent publications, so that the tree presented uses currently accepted names of species. There were 26 unresolved names applied to accessions in Genbank. For the purposes of interpretation, the sequences were considered to represent genera according to the most recent treatment. For example, the unresolved name *Parathelypteris beddomei* is considered here to represent a species of *Amauropelta* based on Almeida et al., (2016) (Appendix 2.2.). Furthermore, our finding also found that *Parathelypteris beddomei* is nested within in *Amauropelta* clade.

Changes in taxonomic names for ferns result from major changes in fern classification in recent studies. As large-scale name changes are still ongoing, especially in several controversial genera, large databases such as Genbank are prone to accumulate incorrect, ambiguous or outdated taxon names. Since the supermatrix method depends on analysis of a massive amount of sequence data that are currently publically available, it is vulnerable to the outdated name or synonomy issue. We considered it important to update the taxon names based on clearly cited publications. Genera or other higher taxa may appear non-monophyletic in the supermatrix tree, especially to non-taxonomists, when species are not updated with the current accepted name. For instance, in the previous supermatrix study, *Hymenophyllum exiguum* (Hymenophyllaceae) was not updated with the current accepted name even though there was already a taxonomic revision of this family (Ebihara et al., 2006). This species was embedded in *Didymoglossum* apparently rendering the genus paraphyletic. By using the accepted names in this study, it will make the investigation of the monophyly of currently accepted genera and families much easier. As an alternative to the approach taken here, a single source of accepted names could be used, for instance, the Taxonomic Name Resolution Service or TNRS, an application for correcting and standardizing taxonomic names. Using a single source of accepted names and software of this kind may facilitate the further interpretation and use of the phylogeny since the software can also be used to interrogate other databases. On the other hand, the single sources of taxonomic data that can be exploited using software such as TRNS often reflect the outdated taxonomic opinion.

2.3.2 Phylogenetic analyses and tree topology

The concatenated dataset for 3719 terminals included 4636 characters, of which 3496 were variable and 2981 were parsimony informative (Dataset F1). The resulting phylogeny included 47 families and 273 genera of ferns, and is presented here as a phylogram (Figure 2.1), with bootstrap values and named monophyletic families collapsed (Figure 2.2) and as a tree file (Dataset F2). At the outset, it was not expected that the phylogeny would have a more resolved or better supported backbone than the several phylogenies using exemplars and many more regions to reconstruct higher level relationships. However, it is desirable that our topology is congruent with previous studies.

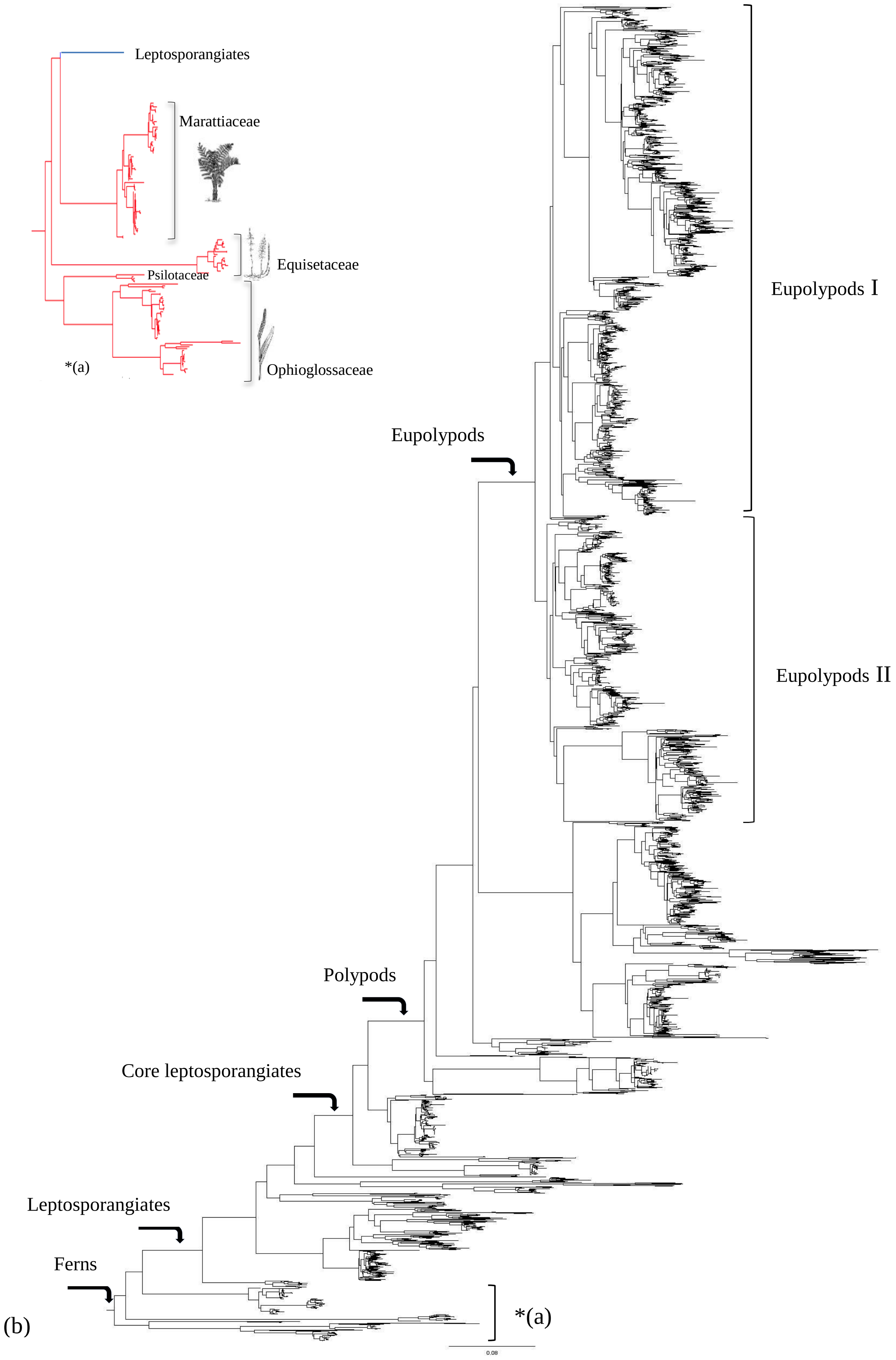


Figure 2.1 Phylogram of ferns based on maximum likelihood analysis. The early divergences are expanded in the inset (a) and all fern lineages indicated in (b). The full tree with taxon names is available as a Nexus file, Dataset F2, in the electronic Appendices.

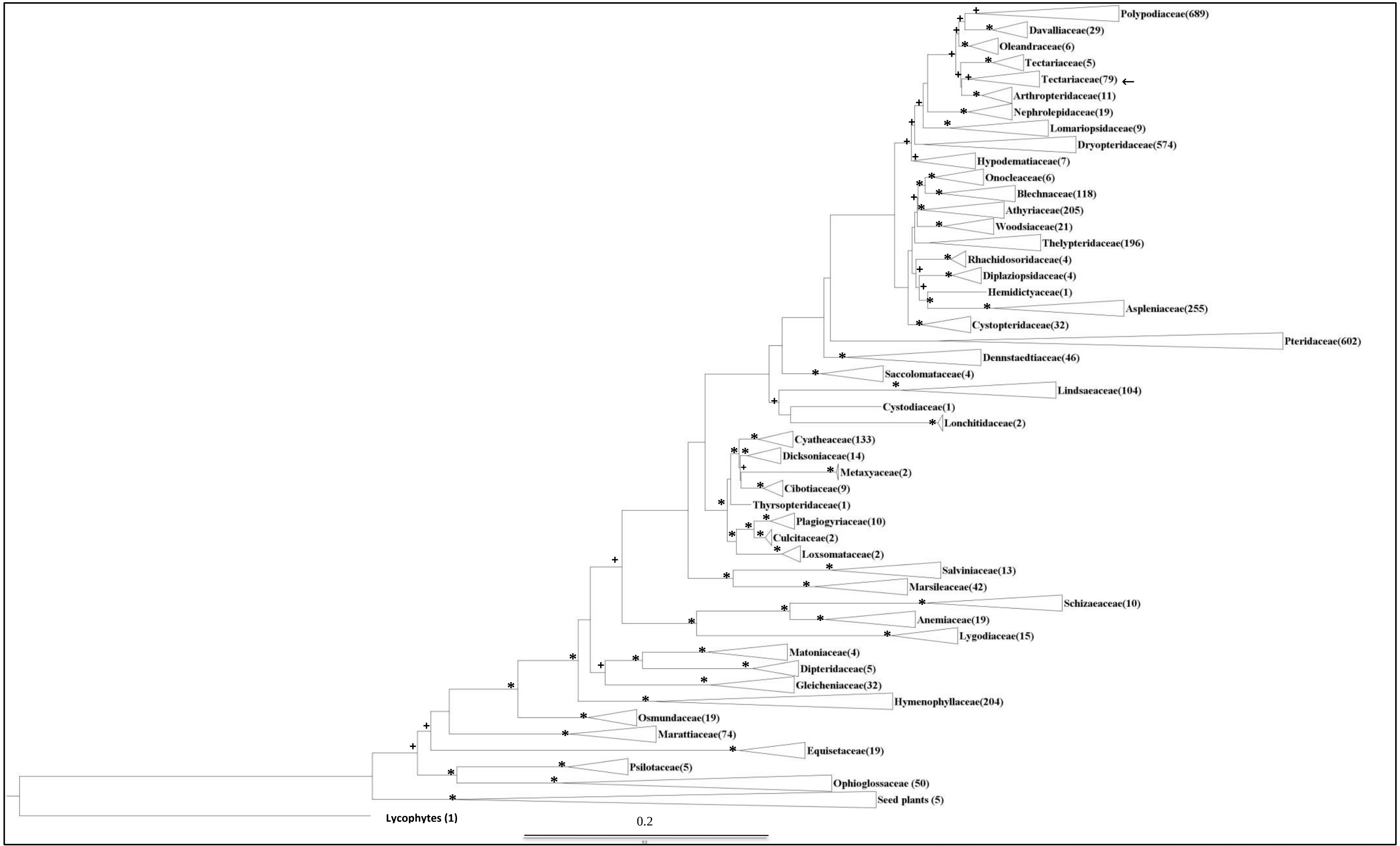


Figure 2.2 Maximum likelihood phylogeny.Maximum likelihood bootstrap values are indicated next to nodes, and nodes are collapsed to show families. Bootstrap values ≥90 are abbreviated (*) and bootstrap values ≥ 50 ≤90 are abbreviated (+). The family which was not monophyletic in this study are highlighted with ←. The numbers of species represented by each collapsed node are given after the family name.

Overall, the phylogeny is consistent with the prevailing view of fern higher level relationships (Hasebe et al., 1995; Pryer et al., 2001; Pryer et al., 2004; Schneider et al., 2004; Schuettpelz et al., 2006; Schuettpelz & Pryer, 2007; Lehtonen, 2011). In the early divergences within ferns, the controversial family Equisetaceae is found resolved as sister to Marattiaceae (BS=62) and the osmundaceous ferns are well supported as sister to the rest (BS=99) consistent with previous fern phylogenetic studies (Pryer et al., 2001; Pryer et al., 2004; Schneider et al., 2004; Wikström & Pryer, 2005; Schuettpelz et al., 2006; Schuettpelz & Pryer, 2007). However, this finding contradicts some recent studies based on nuclear and mitochondarial loci, that discovered strong support for Equisetales as sister to the rest of ferns (Knie et al., 2015; Rothfels et al., 2015). These patterns suggest that multigene datasets incorporating nuclear and mitochondrial sequence could improve support, and resolve uncertainties in monilophyte phylogeny. Therefore, it is critical to test plastid phylogeny with nuclear and mitochondrial genes on further fern phylogeny studies toward a more complete understanding of ferns relationships. Our analysis identified the schizaeiod ferns (Lygodiaceae, Anemiaceae and Schizaeaceae) as a well-supported (BS=81) sister to a large clade, the so-called ‘core leptosporangiates’ that consist of heterosporous, tree and polypod ferns. Within polypods, most of the clades are well supported, but several relationships among them are poorly supported (BS<50). The majority of the families in the polypods are separated into two large eupolypod clades; ‘eupolypods I’ and ‘eupolypods II’ corresponding with previous studies (Schneider et al., 2004; Lehtonen, 2011). Pteridaceae is resolved as sister to the whole of the eupolypod clade, congruent with the previous published study of Schuettpelz and Pryer (2007), however this relationship is poorly supported (BS=26). Most short backbone internodes, particularly in eupolypods II, find poor support since they introduce challenging situations for phylogenetic inference. The ‘ancient rapid radiations’, long branches that are intercalated with short internal nodes, are also a prominent feature in previous fern phylogenetic studies (Schuettpelz & Pryer, 2007; Lehtonen, 2011). That multigene studies provide improved support at deeper nodes is exemplified by the study by Rai and Graham (2010) using a large set of plastid genes (17 genes) but relatively few exemplar taxa to represent fern lineages. Increasing the number of gene regions in this phylogeny could improve the poor resolution or low support in the deeper nodes. However, data mining in Genbank for more regions for large-scale fern phylogeny reconstruction will generate matrices with a high proportion of missing data. In the context of supermatrix analyses, these missing data will result in a decline in support values (Sanderson & Wojciechowski, 2000). In addition, some analytical shortcuts in large datasets with high proportions of missing data present serious computational challenges that may compromise some approaches and results (Lehtonen, 2011). In any case, for many applications of phylogeny, phylogenetic uncertainty in the backbone has relatively little impact on analysis outcomes, at least compared to uncertain placement of missing species (Rangel et al., 2015).

Family-level relationships and family delimitations in this study are also largely consistent with the recently published linear fern classification based on phylogeny (Christenhusz & Schneider, 2011; Christenhusz et al., 2011). All families are monophyletic in our study, except Tectariaceae. The position of Arthropteridaceae nested within Tectariaceae does not correspond to the currently accepted family sensu Liu et al. (2013). The sampling of Arthropteridaceae and Tectariaceae in previous studies has not revealed this relationship. Liu’s study included eight species of *Tectaria* compared to ours which includes 68, suggesting conflict with Liu et al.’s (2013) classification is attributed to denser sampling not problems with our phylogenetic reconstruction. Overall, 41 out of 47 families have high bootstrap support (BS=>95).

Most of the clades recovered here are consistent with currently accepted generic limits and exceptions have been previously reported. In Ophioglossaceae, *Ophioglossum lusitanum* is grouped together with *Helmintostachys zeylanica* as in Lehtonen (2011) while in Gleicheniaceae, *Gleichenia* is not resolved as monophyletic although other genera in this family are resolved as monophyletic. *Dennstaedtia* is found to be strongly paraphyletic (BS=100), due to the inclusion of the monophyletic *Microlepia* as in previous studies (Schuettpelz & Pryer, 2007; Lehtonen, 2011).

In Pteridaceae, numerous of the pteridoid genera are monophyletic including *Adiantum* which was not well resolved in previous studies (Prado et al., 2007; Schuettpelz & Pryer, 2007; Schuettpelz et al., 2007; Bouma et al., 2010). However, generic limits need reconsideration where polyphyly seems to be rampant in several of the largest genera within this family. Eight genera, *Anogramma*, *Monogramma*, *Doryopteris*, *Cheilanthes, Notholaena, Pellaea, Paragymnopteris* and *Aleuritopteris,* are found not monophyletic; all these genera have already been highlighted as problematic in previous studies (Prado et al., 2007; Schuettpelz & Pryer, 2007; Schuettpelz et al., 2007; Rothfels et al., 2008; Bouma et al., 2010; Lehtonen, 2011).

Increased sampling with updated nomenclature in this study supports recent generic reclassifications. The placement of *Pleocnemia* in Dryopteridaceae is consistent with the current study by Liu et al. (2014). *Dracoglossum*, previously placed in Dryopteridaceae (Christenhusz, 2007), is found sister to *Lomariopsis*. This is congruent with the study by Christenhusz et al. (2013) which suggested that *Dracoglossum* should be placed in Lomariopsidaceae. Several genera in this study such as *Amauropelta* (including some *Parathelypteris* species), *Steiropteris, Coryphopteris* (including some *Parathelypteris* species), *Oreopteris, Metahelypteris, Cyclogramma, Macrothelypteris, Phegopteris, Pseudophegopteris* and *Stegnogramma* are shown to be monophyletic, these results also support current generic limits in Thelypteridaceae proposed by Almeida et al. (2016), contradicting Lehtonen's megaphylogeny (Lehtonen, 2011) which showed a polyphyletic *Thelypteris*. However, the *Cyclosorus* clade is still polyphyletic and requires further attention.

Within Polypodiaceae, most genera are resolved as monopyletic, including *Zygophlebia*, contradicting previous studies which found *Zygophlebia* nested within *Enterosora*, or sister to it (Sundue et al., 2010; Sundue et al., 2014). Delimitation of *Lepisorus* changed when *Paragramma* was placed in synonymy with *Lepisorus* (Hennipman et al., 1990). In this study, *Paragramma longifolia (Lepisorus longifolius)* is resolved as the sister to all *Lepisorus*, congruent with the previous phylogeny sampling fewer lepisoroids (Kreier et al., 2008; Wang et al., 2010a; Wang et al., 2010b) thus supporting the acceptance of *Paragramma* as a genus. There are still many genera found to be polyphyletic, such as *Grammitis, Oreogrammitis* and *Radiogrammitis*. These genera were also polyphyletic in a recent less inclusive study (Sundue et al., 2014).

The majority (99%) of sequence data in this study was retrieved from Genbank, and it is well known that some erroneous identifications are present in Genbank. Several species are found placed in clades phylogenetically distant from their congeners. For instance, three notable misplaced samples are *Vandenboschia speciosa* found sister to the core leptosporangiates and *Taenitis pinnata* and *Thelypteris augescens* are found embedded in *Pteris* clade. When blasted in NCBI, none of these problematic sequences find sequence similarity with their congeners, which suggest a wrong identification or laboratory error for these sequences. Furthermore, poor sequence alignment also may contribute to incorrect placement of taxa. The detailed analysis of alignment conditions was not further explored here because of the complexity of assessing length variation in many thousands of sequences. For future work, these problematic species should be pruned from the tree. Duplicate or more sequences for sequence entries should also be included, rather just one sequence per taxon, to increase the possibility of detecting any erroneous identification or laboratory error and to highlight putatively non-monophyletic species.

2.3.3 Assessment of taxonomic bias in missing data

This study includes 3713 out of the 9490 fern species recognised by Smith et al. (2006) and in more recent publications, representing 39% of the total diversity. This is a major increase in the number of species sampled in comparison with the previous fern megaphylogeny of Lehtonen (2011). This improvement will facilitate a variety of further analysis at the species level. Overall, the taxonomic coverage of individual orders and families varied greatly (Table 2.4 and Table 2.5). At ordinal level, Osmundales, Equisetales, Ophioglossales and Salviniales were best sampled with more than 60% of taxa sampled. Three orders, Gleicheniales, Schiziaeales and Cyatheales had less than 30% sampling (Table 2.4).

Table 2.4 Taxonomic coverage and residual analysis at order-level. Estimated diversity per order were recorded following recent publications in family level (Christenhusz et al., 2011). Residual values are –ve for orders sampled less than ferns overall; a positive value indicates orders sampled more.

Order	**Taxa included in this present study**	**Estimated diversity**	**% taxa in tree**	**Residuals**
Equisetales	19	23	83	0.2
Ophioglossales	50	80	63	0.1
Psilotales	5	12	42	-0.2
Marattiales	74	150	49	0.0
Osmundales	19	20	95	0.2
Hymenophyllales	204	600	34	-0.1
Gleicheniales	41	140	29	-0.2
Schizaeales	44	155	28	-0.2
Salviniales	55	91	60	0.1
Cyatheales	173	663	26	-0.2
Polypodiales	3029	7556	40	0.2
Total	3713	9490	39	

Table 2.5 Taxonomic coverage and residual analysis at family-level. Taxonomy follows Christenhusz et al., (2011) with updates following Christenhusz & Schneider, (2011), Rothfels et al., (2012) and Liu et al., (2013). Estimated diversity for each family were recorded based on several publications; [1]Smith et al., (2006), [2]Des Maraies et al., (2003), [3]Lehtonen et al. (2012), [4] Korall et al. (2006), [5]Rothfels et al. (2013),[6]Rothfels et al., (2012),[7]Nooteboom (2012),[8]Christenhusz et al. (2013),[9]Hovenkamp and Miyamoto (2005), [10]Moran et al. (2014), [11]Hovenkamp and Ho (2012). Residual values are –ve for families sampled less than ferns overall; a positive value indicates families sampled more.

Families	**Taxa included in this present study**	**Estimated diversity**	**% taxa in tree**	**Residuals**
Equisetaceae [2]	19	23	83	0.2
Ophioglossaceae[1]	50	80	63	0.1
Psilotaceae[1]	5	12	42	-0.2
Marattiaceae[1]	74	150	49	0.1
Osmundaceae[1]	19	20	95	0.2
Hymenophyllaceae[1]	204	600	34	0.0
Gleicheniaceae[1]	32	125	26	-0.2
Dipteridaceae[1]	5	11	45	-0.1
Matoniaceae[1]	4	4	100	0.1
Lygodiaceae[1]	15	25	60	0.0
Schizaeaceae[1]	10	30	33	-0.2
Anemiaceae [1]	19	100	19	-0.4
Marsileaceae[1]	42	75	56	0.1
Salviniaceae[1]	13	16	81	0.1
Thyrsopteridaceae[1]	1	1	100	0.0
Loxsomataceae[1]	2	2	100	0.1
Culcitaceae[1]	2	2	100	0.1
Plagiogyriaceae[1]	10	15	67	0.0
Cibotiaceae[1]	9	11	82	0.1
Cyatheaceae[1]	133	600	22	-0.2
Dicksoniaceae[1]	14	30	47	-0.1
Metaxyaceae[1]	2	2	100	0.1
Lonchitidaceae [3]	2	2	100	0.1
Saccolomataceae[1]	4	12	33	-0.3
Cystodiaceae[4]	1	1	100	0.0
Lindsaeaceae[1]	104	200	52	0.1
Dennstaedtiaceae[1]	46	170	27	-0.2
Pteridaceae[1]	602	950	63	0.3
Cystopteridaceae[5]	32	38	84	0.2
Aspleniaceae[1]	255	700	36	0.1
Diplaziopsidaceae[6]	4	6	67	0.0
Thelypteridaceae[1]	196	950	21	-0.2
Woodsiaceae[6]	21	35	60	0.1
Rhachidosoridaceae[6]	4	7	57	-0.1
Onocleaceae[6]	6	10	60	0.0
Blechnaceae[1]	118	200	59	0.2
Athyriaceae[6]	205	600	34	0.0
Hypodematiaceae[7]	7	15	47	-0.1
Dryopteridaceae[1]	574	1700	34	0.1
Lomariopsidaceae[8]	9	40	23	-0.4
Nephrolepidaceae[9]	19	29	66	0.1
Tectariaceae[10]	84	290	29	-0.1
Oleandraceae[11]	6	20	30	-0.3
Davalliaceae[1]	29	65	45	0.0
Polypodiaceae[1]	689	1500	46	0.2
Hemidictyaceae[6]	1	1	100	0.0
Arthropteridaceae[10]	11	15	73	0.1
Total	3713	9490	39	

Of the 47 families presented here, five small families and three monotypic families were fully sampled while the largest families such as Dryopteridaceae and Polypodiaceae stand out with more than 30% of the known diversity sequenced respectively (Table 2.5). Despite the density of sampling in this study, several larger families remained poorly sampled. For instance, Thelypteridaceae is a widespread family with ca. 950 species worldwide (Smith et al., 2006). Several relationships within this family, particularly in the christelloid clade (e.g. *Christella* and *Sphaerostephanos*) are still not fully resolved as in previous studies (Almeida et al., 2016) though only 21% of these species are represented (Table 2.5). Therefore, improved understanding of the genera within this family as well as other problematic genera is still needed, by adding more additional taxa in sampling in order to make a robust assessment to the monopyly and also to clarify the generic delimitation. Futhermore, increasing sampling of taxa in a phylogenetic analysis will result in more accurate estimates of overall phylogeny by dispersing homoplasy across the tree and eventually reducing the effect long branch attraction (Pollock et al., 2002; Heath et al., 2008).

Regression analyses indicated the presence of a strong linear positive relationship between the number of sampled taxa in this study against total estimated diversity by order and family level with an R^2 of 95% and 96%, respectively (Figure 2.3 and Figure 2.4). The directions of the residuals (positive and negative) indicate whether a family or order are over or under-sampled (Table 2.4 and Table 2.5). Compared with the 39% overall density of sampling, there are several lineages at the family level that are over-presented such as Pteridaceae, Osmundaceae and Cystopteridaceae while Lomariopsidaceae and Anemiaceacea are under-sampled (Figure 2.4; Table 2.5).

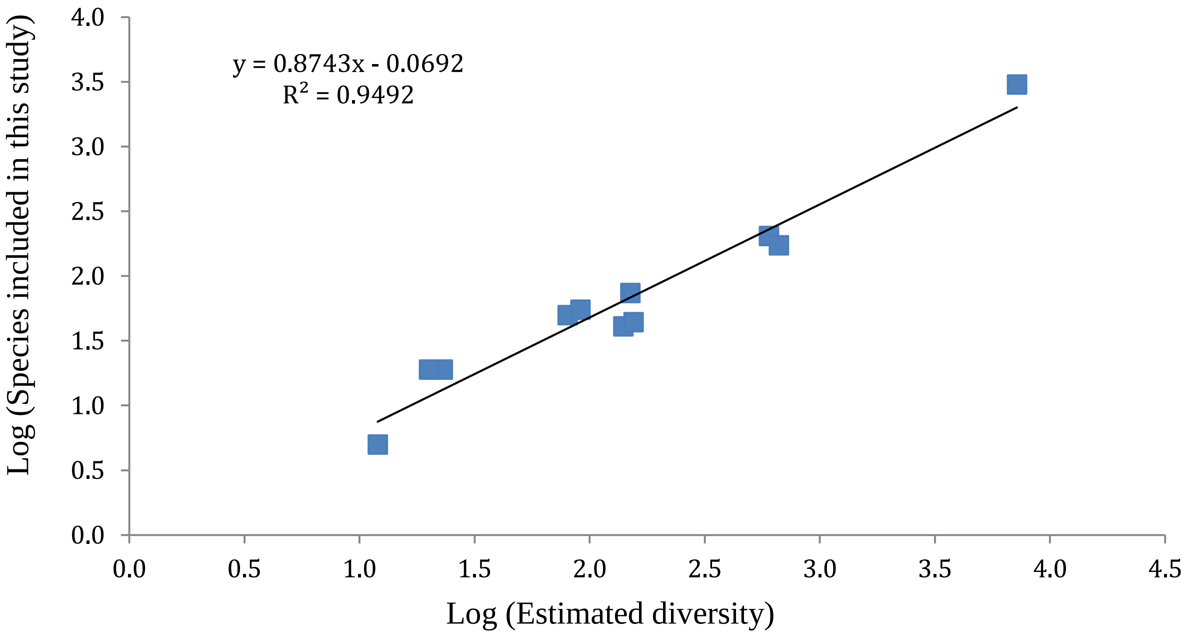


Figure 2.3 Relationship between the total estimated diversity at order level and the species in this study.

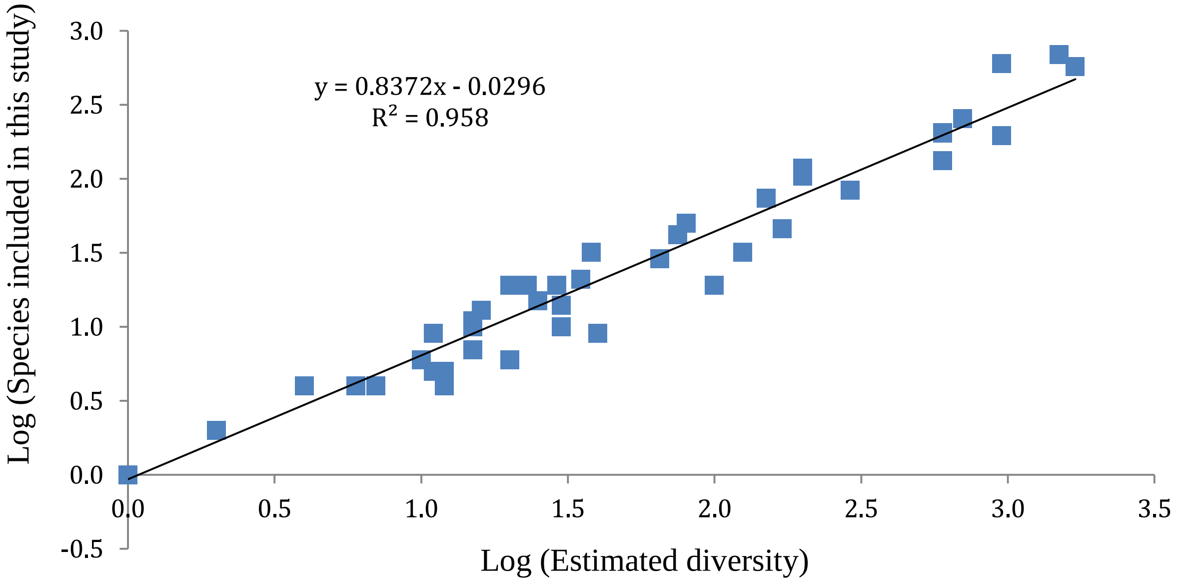


Figure 2.4 Relationship between the total estimated diversity at family level and the species in this study.

It is necessary to sample all ca. 287 accepted genera to move towards a phylogeny of ferns fully sampled at species level. In order to achieve this goal, a list of accepted names of fern genera was compiled based on Christenhusz et al. (2011) and was updated to take account of subsequent taxonomic generic changes. This study indicated that there are only three small genera and six monotypic genera from six families representing 15 species for which no nucleotide sequence data is available from any genomic region up to November 2015 (Table 2.6). Among these, generic level sampling gaps are mostly in Polypodiaceae where three monospecific genera remain unsampled. The missing genera are mainly from tropical regions, notably South East Asia. There are two genera, *Nephopteris* and *Oenotrichia* for which sequence data have been added to Genbank during preparation of this chapter which are not included in the phylogeny. One genus, *Arthrobotrya* for which sequence data is available in Genbank was not included in the phylogeny as it only represented by a region, *rps4-trnS*.

Table 2.6 Accepted names of fern genera based on Christenhusz et al. (2011) for which there is no sequence data deposited in Genbank (November 2015). Their geographic distribution and the number of species is indicated.

Family	Accepted name (Generic)	Number of species in the genus and its geographical distribution
Dryopteridaceae	*Dryopolystichum*	1sp; New Guinea to Solomons
	Stenolepia	2 sp; Malesia
Lindsaeaceae	*Xyropteris*	1 sp; Borneo and Sumatera
Lomariopsidaceae	*Thysanosoria*	1sp; Indonesia
Tectariaceae	*Aenigmopteris*	5 sp; Malaysia, Indonesia, Philippines and New Guinea
Pteridaceae	*Tryonella*	2 sp; Brazil
Polypodiaceae	*Luisma*	1sp; Columbia
	Paraselliguea	1 sp; Borneo
	Podosorus	1 sp; Philippines

One factor that might negatively influence the assemblage of fern nucleotide data in Genbank is authors reporting on DNA sequences yet intentionally failing to deposit their sequences in Genbank (Noor et al., 2006). Another possible explanation might be unsuccessful molecular work (due to difficulties of extracting and sequencing) or problems in collecting species or identifying them. These finding suggest that sampling from existing herbarium and targeted field work for underpressented species or missing species in unsampled genera, especially in Southeast Asia, a priority region, need to be done in order to achieve the most comprehensive of fern phylogeny in the future.

2.4 Conclusion

With the advent large sequence databases such as Genbank as a rich repository of phylogenetically relevant information at the molecular level, many attempts are being made to reconstruct large phylogenetic trees that may include from hundreds to thousands of sequences. Generally, four primary factors that will affect the accuracy of molecular phylogenetic estimates; i) selection of appropriate target gene for analysis; ii) collection of enough sequence data; iii) the use of accurate of analytical methods and iv) adequate taxon sampling for the group interest (Judd et al., 1999).

The utilities of molecular data over the past decades have provided a better understanding of fern classification (Christenhusz et al., 2011). Lehtonen (2011) generated fern megaphylogeny based on four genes and 2656 fern taxa, inferring the first supermatrix-based fern pylogeny. Nevertheless, the taxonomy and nomenclature in the previous fern megaphylogeny are outdated to some degree and several types or errors exist in the phylogeny. Hence, this present study here provides the most comprehensive fern phylogeny to date, by including more than one thousand more fern taxa than previously, thus representing over one-third of the extant global fern diversity. The taxonomy and nomenclature in this fern megaphylogeny also was updated according to the recent revision of the fern classification from the current publications and has a potential to be used in addressing other evolutionary research, such as ecological, biogeographical and community phylogenetic questions particularly those seeking for a pattern of phylogenetic properties (e.g. phylogenetic relatedness). In the context of this thesis, the phylogeny will be used to better understand the selection and use of ferns in herbal medicine systems.

However, in terms of taxonomic understanding for fern classification, some genera need still need to be the focus of further work for their generic redefinition, such as pteridoid genera and several larger families in eupolypods. Several internal nodes also were not well supported, as expected, given several known issues. Molecular phylogenetic data sets have been increasing and there has been a growing effort to use as much available sequence data as possible to construct very large trees. Furthermore several program such as MUSCLE (Edgar, 2004) together with phylogenetic analysis programs such as RAxML (Stamatakis et al., 2008) have made it possible to assemble and analyze larger data sets than ever before. A more stable tree topology may be achieved by overcoming uneven sampling of lineages and loci. In conclusion, the megaphylogeny of ferns presented here provided a much wider view for future studies. Inclusion of missing lineages, increasing number of loci with addition of nuclear markers and and taxon sampling are critically needed, specifically those poorly sampled in hyperdiverse families in order to refine generic delimitation. Adding fuller sampling of extinct clades may hold greatest promise for resolving the overall pattern of fern phylogeny (Rothwell & Nixon, 2006). Greater taxonomic completion of the matrix and increasing numbers of genes not only improve the tree topology but also enable moving even closer to a full understanding of fern evolution and diversification.

Chapter 3. Medicinal ferns: compiling a database of ethnomedicinal use

3.1 Introduction

Since ethnobotanical data are scattered in various publications and in different languages with limited accessibility, initiatives are needed to collect data into a centralized database for easy reference (Gaikwad et al., 2011). A database of this kind will eventually facilitate further studies by other researchers. Many researchers around the world have been involved in compiling traditional knowledge of medicinal plants, and many studies have been published in the form of dissertations or theses, in journals, proceedings, articles, books or monographs and technical reports (Mat-Salleh & Latiff, 2002). There is no recent, comprehensive synthesis of ferns used medicinally.

Ferns (monilophytes) are found in most terrestrial habitats, but most species are found in the wet tropics (Benniamin, 2011; Chin, 2005; Winter & Amoroso, 2003). Almost all parts of ferns, including the stems, rhizomes, leaves, young fronds and shoots can be used in some way, for example as a source of food and beverages, fertilizer, for ornament, material for crafts or building and not least as medicines (Chin, 2005; Mannan et al., 2008; Srivastava, 2007; Winter & Amoroso, 2003). Ferns may be particularly important in the Chinese system of medicine (Kimura & Noro, 1965) and other indigenous system of medicine (Pan et al., 2014). A number of fern species have been reported in traditional pharmacopoeias and are continuously used in herbal medicine in many countries (Puri, 1970). A critical review may reveal more medicinal applications of ferns than expected, including documented uses going back more than

2000 years (Chin, 2005; Benniamin, 2011). A recent study conducted in Brazil showed that low recording of ferns as medicine can be attributed to inadequate data collection, but also to the perception that these plants are inferior to flowering plants in their therapeutic value. However, when appropriate data collection methods are used, the proportion ferns used medicinally is as high as the proportion flowering plants used medicinally (dos Santos Reinaldo et al., 2015).

Medicinal plants comprise a rich source of compounds with a variety of pharmacological activities. As many ferns have been used as folk medicine, some of them have become the focus of research concerning their bioactive constituents. Numerous compounds especially phenol and phenolic glycosides such as tannins, coumarins and their glycosides, quinones, flavonoids and related compounds, alkaloids, terpenoids and steroids have been isolated from fern species, suggesting they are an extremely rich resource for the discovery of novel drugs (Winter & Amoroso, 2003).

3.1.1 Objectives

To date, all information regarding the medicinal uses of ferns is scattered in various publications, including digital resources. The first objective of this project is to compile data on medicinal use of fern species throughout the globe into a single comprehensive literature source. This ‘Medicinal Ferns Database’ will facilitate access to data, and can be used for further analysis. By gathering a large quantity of information, therapeutic potential of ferns can be assessed and at the same time gaps in our knowledge can be highlighted.

Secondly, pharmacological and biological activities (e.g., anthelmintic activity, antitumor activity, antimicrobial, anti-inflammatory activity and antioxidative activity) will be compiled for all medicinal species in this study. This database might therefore be used to evaluate the likely efficacy of these traditional medicines.

3.2 Materials and methods

3.2.1 Database structure

A database for medicinal uses of ferns data has been constructed using Microsoft Access 2007, since it is the most economic relational database solution suitable for small databases or academic purposes. Eventhough the older version of Microsoft Access was used, there is no limit in term of the data storage as well as the input functionality as the data entry forms and queries functionality in this study are quite basic. Twenty three data fields excluding the identification key have been defined and all these fields have been organized into eight main interrelated tables (Figure 3.1).

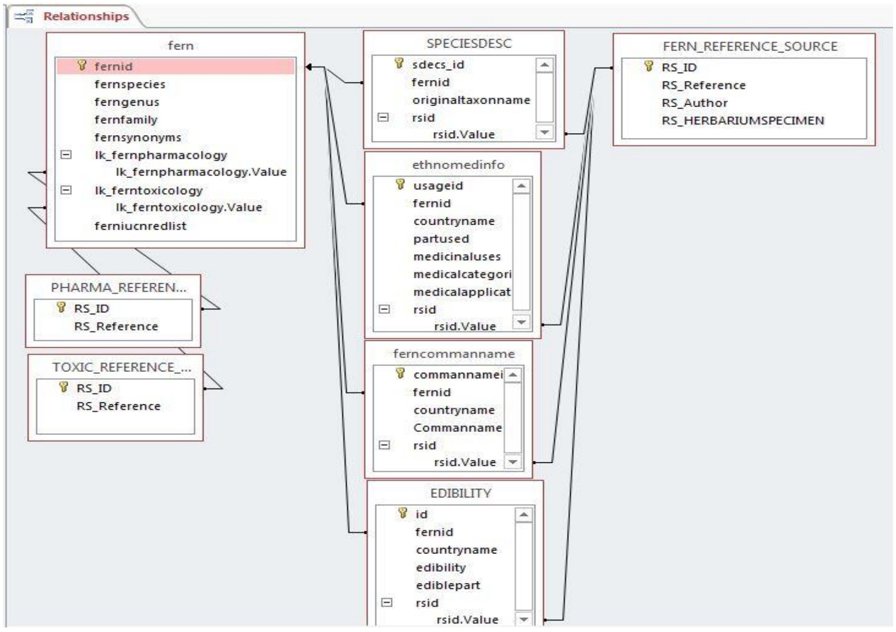
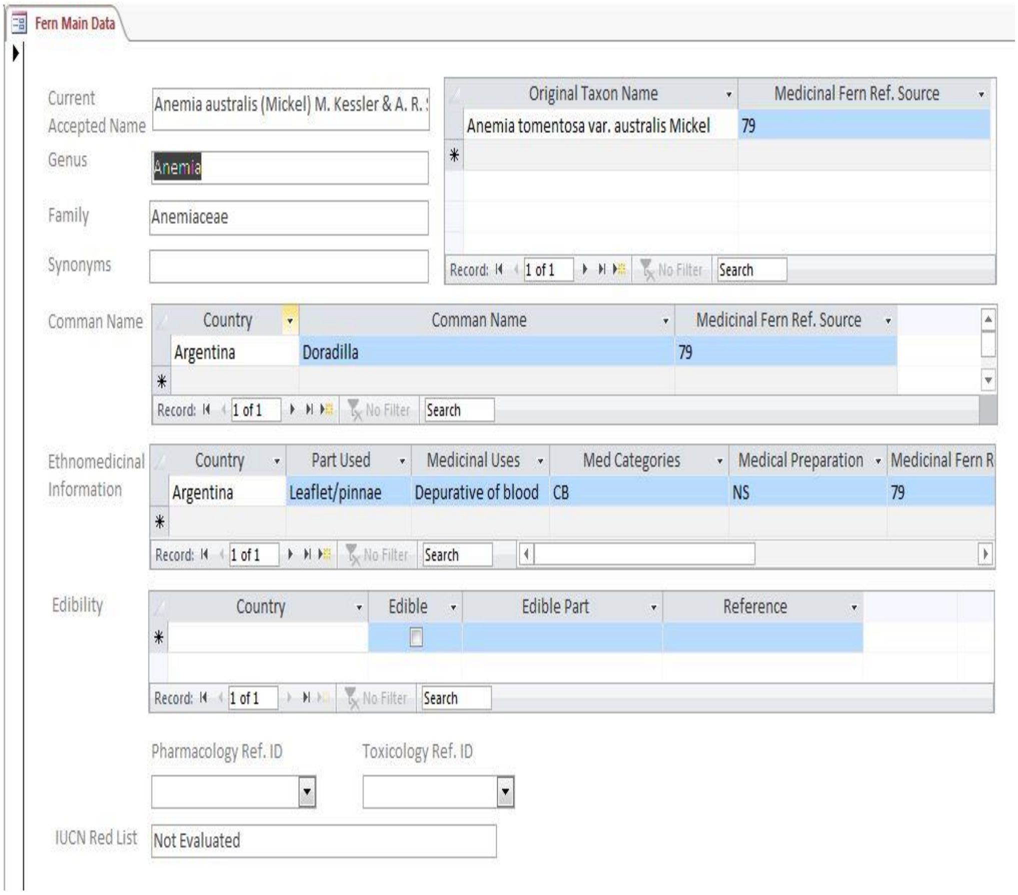


Figure 3.1 Database structure showing the relationship within the database.

The database is being maintained directly using the Microsoft Access 2007 application. This includes the development and maintenance activities and data entry. Four main interfaces were created to enable data entry into the database, which are referred to as forms in Microsoft Access terminology. The four main forms are shown in the screen shots in Figure 3.2 and Figure 3.3.



Current Accepted Name: Anemia australis (Mickel) M. Kessler & A. R.

Genus: Anemia

Family: Anemiaceae

Synonyms:

Original Taxon Name	Medicinal Fern Ref. Source
Anemia tomentosa var. australis Mickel	79

Comman Name

Country	Comman Name	Medicinal Fern Ref. Source
Argentina	Doradilla	79

Ethnomedicinal Information

Country	Part Used	Medicinal Uses	Med Categories	Medical Preparation	Medicinal Fern R
Argentina	Leaflet/pinnae	Depurative of blood	CB	NS	79

Edibility

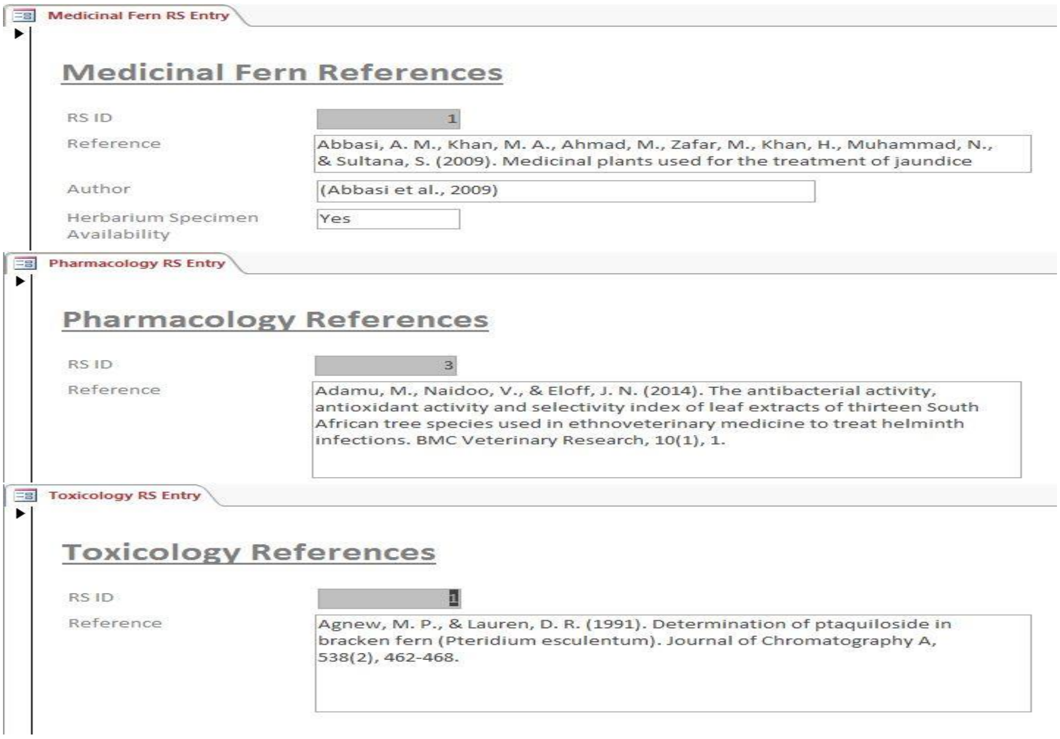
Country	Edible	Edible Part	Reference
	☐		

Pharmacology Ref. ID:

Toxicology Ref. ID:

IUCN Red List: Not Evaluated

Figure 3.2 Main form for fern data entry with search and navigation features.



Medicinal Fern References

RS ID	1
Reference	Abbasi, A. M., Khan, M. A., Ahmad, M., Zafar, M., Khan, H., Muhammad, N., & Sultana, S. (2009). Medicinal plants used for the treatment of jaundice
Author	(Abbasi et al., 2009)
Herbarium Specimen Availability	Yes

Pharmacology References

RS ID	3
Reference	Adamu, M., Naidoo, V., & Eloff, J. N. (2014). The antibacterial activity, antioxidant activity and selectivity index of leaf extracts of thirteen South African tree species used in ethnoveterinary medicine to treat helminth infections. BMC Veterinary Research, 10(1), 1.

Toxicology References

RS ID	1
Reference	Agnew, M. P., & Lauren, D. R. (1991). Determination of ptaquiloside in bracken fern (Pteridium esculentum). Journal of Chromatography A, 538(2), 462-468.

Figure 3.3 Form for data entry for references. These are the sources of each reference type in the database.

3.2.2 Sourcing data

Ethnobotanical field surveys focussed on ferns are scarce, so in addition to the 21 publications focussed on ethnopteridology we identified, use reports were sourced from general surveys. Journals, books, reports from national, regional and international organizations, and online databases were scrutinized and the information related to ferns extracted. An extensive search for literature/articles including information about ethnomedicinal uses of plants was made using Google Scholar, PUBMED and ISI Web of Science. The key words “ethnobotany”, “ethnobotanical”, “ethnomedicinal”, “ethnopharmacological”, “medicinal” and “medicinal plant” were used independently or in combination with the term “ferns” or “pteridophytes”. All the sources, including books, were examined for information about the ferns used medicinally. For each source it was recorded whether the findings were substantiated by citing herbarium specimens. Whether vernacular names or specified uses (therapeutic application, plant part used, mode of application) were provided was recorded for each source.

From the sources identified, use reports were compiled, where a use report is the species' scientific name and all the associated data originating from one publication for that name (one row in Appendix 3.1). The associated information included therapeutic application, plant part used, mode of preparation and application and vernacular name. For each report, the country/region where the report originated was recorded; reports were assigned to botanical continents following Brummitt et al. (2001).

The three additional fields for the species with documented ethnomedicinal use were as follows. The IUCN Red List 2015 was used to record the conservation status of all the medicinal ferns recorded here. The pharmacological and toxicological studies of these species also were recorded (Appendix 3.2). To document these properties in the database, a critical analysis of existing pharmacological literature was made, by searching for the accepted name and most common synonyms along with the following terms; "pharmacological", "anthelmintic activity", "antitumor activity", "antimicrobial", "anti-inflammatory activity", "antiviral activity", "antioxidative activity" and "bioactivity" and "toxicological" in combination with species name or with the term "ferns" or "pteridophytes".

3.2.3 Standardizing data

The nomenclatural history of ferns has been unstable, so the scientific names in the literature were often not those currently accepted. For this study, family and generic classification followed Christenhusz et al. (2011) with some minor changes based on more recent studies (Christenhusz & Schneider, 2011; Rothfels et al., 2012; Liu et al., 2013). When a species name in a publication was considered a synonym of a currently accepted name, we use the current accepted name to resolve synonymy and correct spelling errors. All names, including the authors' names used here have been checked against Tropicos (2016) and Catalogue of Life (2016). Appendix 3.1 reports both names as used in the source publication and the currently accepted name.

Therapeutic applications were recorded as in the source, and then grouped into 14 categories (Heinrich et al., 1998); cardiovascular problems/blood purity (CB), dentistry/mouth (DM), gastro-intestinal (GI), gynaecology/fertility (GF), neurological conditions (NC), ophthalmology (OP), musculo-skeletal (MS), skin conditions/dermatology (SD), otorhinolaryngology (OT), respiratory/pulmonary (RP), urinary conditions (UC), poisonous animal bites (PA), general health problem (GR) and other/unclassified (OU). Table 3.1 shows the original terms from use reports assigned to each category.

Table 3.1 Circumscription of therapeutic application categories. The 14 therapeutic application categories follow Heinrich et al. (1998). The therapeutic applications as described in the source publication are assigned to Heinrich's categories.

Therapeutic application categories	Therapuetic application as described in the source
Cardiovascular problems/blood purity (CB)	Blood purification/purifier/depurative, high/low blood pressure, heart strength, cardio/ heart problem, cardiac infection, cardioac problem, circulatory disorder, hematocarthartic, hypocholesterolemia, chest pain, angina pectoris, spleen treatment, antihemorragic, venous problem, arteriosclerosis, depurative of blood, hyperlipidemia, hypertension.
Dentistry/mouth (DM)	Bleeding gums, toothache/odontalgia, mouth sore, mouth blister, mouth infection, dental neuralgia, Periodontitis/pyorrhea, mouth infection, tongue blemish, tongue ulcer, unhealthy tongue, dentirifice.
Gastro-intestinal (GI)	Stomach problem (stomachache/stomach pain, stomach ulcer/peptic ulcer, stomach cancer, stomach acidity, stomach cramp) gastric,anthelmintic/vermifuge/vermicide, diarrhea,dysentery,emetic/vomiting/nausea, carminative, dyspepsia, indigestion, digestion problem, diabetes, constipation, intestinal problem, jaundice, appendicitis, liver problem, nausea, hepatitis, enteritis, colitis, laxatives/purgative, hepatic disease, aperient, hemorrhoids, pancreas problem, hematemesis.
Gynecology/fertility (GF)	Gynecological problem, women problem, white discharge/leucorrhea, syphilis, gonorrhea, menstrual promoters, menstrual problem, menstrual pain/ dysmenorrhea, uterine contractors, uterus problem, ovarian cyst, emmenagogue, menorrhagia, uterine hemorrhage, female haemorrhage, abortion, delivery pain, male/female fertility, venereal diseases, prostate problem, aphrodisiac, child birth problem, facilitate childbirth, mastitis, contraceptive, postpartum recovery.
Neurological conditions (NC)	Epilepsy, sedative, convulsion, sciatica pain, insomnia, sleep disorder, antidepressant, encephalitis, nervous system problem, spastic, meningitis, mental problem, amnesia, neuralgia.
Ophthalmology (OP)	Eye problem/opthalmic, ophthalmia, sore eyes, indolent ulcer, eye infection, conjunctivitis.
Musculo-skeletal (MS)	Fracture in bone, body pain,waist pain, joint pain, muscle pain, muscle problem, rheumatism, sprain cramps, abdominal pain, knee aching, bone injuries/problem, arthritis, backache, lumbago, gout, arthralgia, rickets.
Skin conditions/dermatology (SD)	Any skin disease/problem, skin wound and cut, swelling, skin irritation, skin rashes, skin itching, skin ulcer, astringent, dandruff, scalp, hair growth, hair problem, hair loss, boils, eczema, emmolient, scabies, seborrheic dermatitis, leprosy, burn and scalds, bruise, carbuncle, leucoderma, scald, abscess, nail separation.
Poisonous animal bites (PA)	Scorpion-sting, snakebite, insect bite, honeybee sting.
Otorhinolaryngology (OT)	Catarrh, ear infection/problem, nose problem, nosebleed, sinusitis, throat infection, deafness, pharyngitis, tinnitus, laryngopharyngitis, nasopharyngeal infection.
Respiratory/pulmonary (RP)	Respiratory disorders, lung problem, cough (antitussive), cold, bronchitis problem, asthma, expectorant, tuberculosis, phthisis, haemoptysis, pneumonia.
Urinary conditions (UC)	Kidney problem/disorders, kidney inflammation, kidney swelling, kidney stones, diuretic, urinary problem/ cystitis, urethritis, urolithiasis, urinary calculus, renal problem, renal calculus, bladder complaints, hematuria, nephritis.
General health problem (GR)	Fever, anodyne, health tonic (for body strength), headache, antioxidant, antibacterial, antiviral, antifungal, febrifuge, sickness, food poisoning, general pain, fatigue.
Other/unclassified (OU)	Anti-inflammatory, illness, allergic, poisoning, ulcer (not specified), human ailments, tumor, cancer, allergic, medicinal properties.

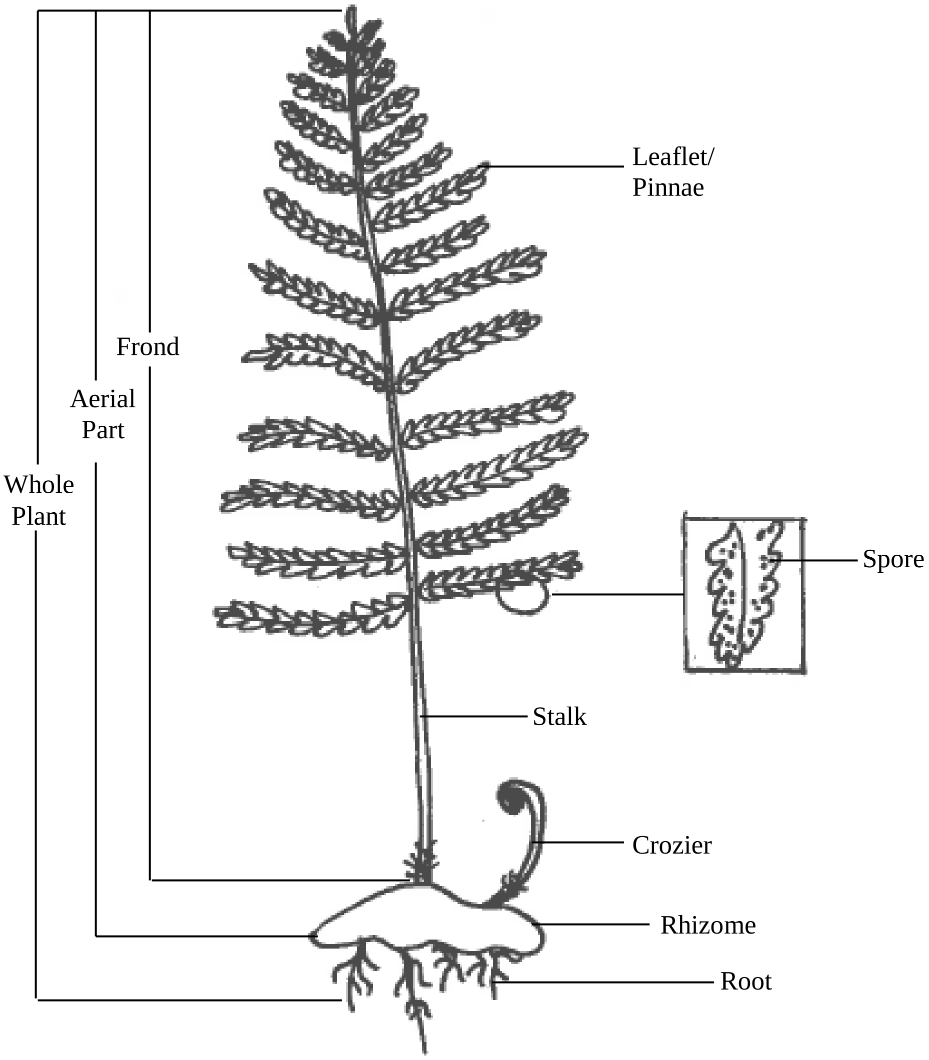


Figure 3.4 Parts of a fern. The terminology used here for plant part categories is illustrated.

As the nomenclature for the parts of ferns is sometimes confused, plant parts used were recorded exactly as in the source, then standardized (Figure 3.4; Table 3.2). Modes of preparation and application as reported in the literature were also standardized (Table 3.3).

Table 3.2 Plant part use categories. Each plant part as reported in the source literature is assigned to one of nine categories.

Plant part categories	Part used as reported in the source
Aerial part	Aerial part, frond and rhizome, frond and stem
Frond	Frond, leaf and stem,
Leaflet/pinnae	Leaf, pinna, pinnule, leaf and spore
Whole plant	Whole plant, all parts of the plant
Stalk	Stem, stipe, hard part, leaf stalk, nodal lump, petiole, pith, rachis
Spore	Sorus, seed, spore, inflorescence, cone, strobilus, spike
Rhizome or root	Rhizome, root, tuber
Crozier	Crozier, bud, shoot, sprout
Other	Sap, slimy tissue, gum, pulp, scale.

Table 3.3 Categories of modes of preparation and application. Each mode as reported in the source literature is assigned to one of 11 categories.

Modes of application categories	Preparation or application reports in literatures
Decoction	Decoction, extraction, boiling
Infusion	Tea, tisane, soaked with water, drink /drink with other herbs, mixed with cow milk
Tincture	Tincture, add with wine, soaked with wine
Maceration	Maceration, macerated in olive oil
Paste	Paste, applied on skin/head/hair/teeth/gum, poultice, pounded, pressed on the body, crushed (external application), powdered, powder mixed water, oil or other, cataplasm, grinding, mashed, pulverized, milling, blended, external application
Juice	Filteration, juice, squeezed
Bath/Wash	Skin wash, steam bath, scrub, bath, hand wash, body wash, gargle
Chew/Eat	Eaten as vegetables or mixed with sugar, onion, garlic, herbs, coconut, betel, porridge, fried with butter, toasted, pill, soup, chewed, cooked, grilled, oral application
Smoke	Smoke, smoke inhalation, put on fire
Oil	Oil, ointment, oleolite, lotion, burn in oil
Others	Ash, sap, latex excretion, heated

3.2.4 Summarising data

The full data set, describing all use reports and including reference to pharmacological and toxicological studies, is presented in Appendix 3.1 and Appendix 3.2. The reports compiled were explored in several ways to identify the most frequently reported families, genera, species and uses, the spatial distribution of fern use, the conservation status of the species used and the availability of pharmacological data for each species.

3.3. Data Summary

3.3.1 Use of ferns documented in literature

In total, 3220 use-reports were compiled. We sourced our use reports from 258 publications comprising 239 journal articles and 16 books, and from three online databases. Three books and 18 other publications (8% of published sources) reported only ferns (Appendix 3.1). Most of the publications (98%) included information about therapeutic applications, but plant parts used (91%) and mode of application (76%) were less often detailed. Across all sources, 75% cited herbarium specimens and 84% gave local names. The three databases were Ngā Tipu Whakaoranga: Maori Plant Use Database (2016) , Plants For a Future (2012) and Raintree-Tropical Plant Database (1996) ; two are global databases, and one, the Maori Plant Use Database, had a restricted geographic focus. Of the 16 books, 11 presented regional accounts and five were multi-countries. Most of the 3220 use reports compiled here came from journal articles (2059; 64%), followed by 856 (27%) from books and the remaining 305 (9%) from online databases.

Reports varied in their information content, sometimes depending on whether they were primary or secondary sources. All of the databases and books were secondary sources, as were 12% of the journal articles. Overall, the percentage of reports providing other associated information were as follows: 54% local names; 99% therapeutic applications; 56% mode of preparation/application; 75% plant parts used; 94% locality of use. We found usage the reports with localities were, in general, derived from ethnobotanical field studies that recorded the site of data collection. Secondary sources rarely report locality data and for 5% of use reports not even the country where use takes place is recorded. For example, some data extracted from PROSEA recorded use as taking place in “South East Asia”. Of the 3220 reports, just 46% cited herbarium specimens. The collection of voucher specimens is generally thought to be crucial for correct identification of a species. Incorrect identification devalues a database, with negative implications for analyses using those data. Some metanalyses will only include primary data including a methodology for data collection and an explicit statement that voucher specimens were compared to herbarium material or examined by experts (de Medeiros et al., 2013). Despite the paucity of vouchered specimens, we show that our large dataset reveals patterns in the use of ferns, providing novel insight into fern use globally.

3.3.2 Distribution of fern use records

Table 3.4 shows the continental distribution of the medicinal fern reports, species and sources. Asia-Tropical includes more than three times as many reports as the next most report-rich continent, Southern America. These spatial patterns are also found when countries from which use reports originate are considered. Use record originates from 84 countries, with most records from India (936 use reports and 132 species) followed by Malaysia (219 use reports and 50 species) and China (193 use reports and 77 species).

Table 3.4 Reviewed literatures based on continents.

Continents	**Number of use-reports**	**Number of species**	**Number of Sources**
Asia-Tropical	1643	204	95
Southern America	400	118	49
Asia-Temperate	339	100	38
Europe	298	34	57
Africa	177	57	29
Pacific	93	38	9
Australasia	52	25	4
Northern America	40	18	12

dos Santos Reinaldo et al. (2015) suggested ferns were poorly represented in ethnobotanical surveys because they are unrecorded by existing methods of survey, and because they are less commonly used, at least in Brazil. Without studies comparable to Dos santos Reinaldo et al.'s in different countries, it is impossible from the literature survey to explain the strong spatial patterns in fern use recovered here. We cannot account for differences in field data collection methods, for biases in reporting of fern use and for biases in our survey methods. Ferns comprise approximately 9600 species worldwide (Smith et al., 2006) with almost half of the number are known from South-East Asia (Winter & Amoroso, 2003). Furthermore, fern communities are highly rich in wet tropical regions especially in South-East Asia and neotropical region (Mehltreter et al., 2010). Thus, it is not surprisingly when ferns in this study are more apparent in these regions as proportion of ferns species are rich in these two continents.

Overall, ferns are genuinely well-represented in ethnomedicinal plant selections in India, China and Malaysia, as compared to other regions. A possible explanation for this might be that some Asian countries are rich with fern species especially in China that comprised of 2600 fern species (Lu, 2007) while India with more than 1200 species (Dixit, 1984) and Malaysia with more than 1000 species of ferns (Parris & Latiff, 1997). These total number of species represent more than 50% of the global ferns species which might explains why the number of medicinal ferns is high in these area. Furthermore, the use of herbal medicine is quite popular in India and China where Ayurvedic medicine and Chinese herbal medicine are originated which also include ferns in their therapies (Nadkarni, 1996; Ching, 2007). Beside that, China, India and Malaysia are among top five countries using plants for medicinal purposes (Schippmann et al., 2002). The ethnobotanists especially in India are also interested in ferns, for instance, 57% of papers that are only specific on ferns are originated from here. These factors might be the reason behind the inflation of reporting of fern use in Asia-Tropical, or maybe exist simply because of the greater importance of ferns in this region.

3.3.3 Taxonomic diversity

A total of 454 taxa (442 species, seven subspecies, five varieties) belonging to 121 genera in 36 families were recorded as having medicinal uses in different parts of the world. A detailed overview of all species with associated information included therapeutic application, plant part used, mode of preparation and application, vernacular name, continent for each record and IUCN Red list status are given in Appendix 3.1. The name listed in Appendix 3.1 has been updated according to the current accepted nomenclature and are arranged in alphabetic order according to family. This total amount contributed to about 5% of total estimated of extant fern diversity (Smith et al., 2006). Considering there are 9000 species of ferns wordwide, there is a possibility that a lot of traditional knowledge of medicinal fern species has not yet been really explored. On the other hand, the 454 medicinal taxa in this study demonstrate that we have good source of promising plants that should be studied in detail.

In term of conservation, two species in this study; *Blechnum eburneum* Christ. is listed as vulnerable (VU) and *Culcita macrocarpa* C. Presl as near threatened (NT). It is not possible to estimate the real threat for all these medicinal species as there is still many medicinal species lacking a global assessment. Aside from overexploitation, introduction of invasive species and urban development, by far, forest fragmentation is the most important threat to fern communities (Paciencia & Prado, 2005). Deforestation has negative impact since trees provide shade for understory ferns and are hosts for epiphytic species (Mehltreter et al., 2010). According to Sodhi et al. (2004), the rapid rate of deforestation in Southeast Asia, the highest in the tropics, would result loss of 42% of its biodiversity by 2100. This current rate of habitat fragmentation and deforestation would lead to a worrying situation due to the high diverse fern species in tropical region (Winter & Amoroso, 2003; Ranker & Haufler, 2008). Various types of action can be taken to the conservation and sustainable use of medicinal plants such as protection of species by conservation of their natural habitat (*in situ*) or by cultivation of individual species outside form their natural habitats as in botanical garden or horticultural institutions (Mehltreter et al., 2010). At the same time, public awareness with economic incentives are crucial for conservation efforts especially in hot spot regions.

The 20 most commonly medicinally-used families and genera are presented in Figure 3.5. Of a total 36 families-used, Pteridaceae has the highest number of medicinal taxa (92 taxa; 20%), followed by Polypodiaceae (91; 20%), Dryopteridaceae (47; 10%), Aspleniaceae (30; 7%), Thelypteridaceae (21; 5%) and Cyatheaceae (20; 4%). These six families together represent 66% of the medicinal taxa, while the remaining 34% of the taxa belonged to 30 families that are represented by less than 15 taxa. It seems that Pteridaceae and Polypodiaceae contribute the highest number of medicinal species. The Polypodiaceae and Pteridaceae are among of the most diverse group of extant ferns that include approximately 1200 spp. and 950 spp., respectively, with a nearly worldwide distribution (Smith et al., 2006). The genera with the highest number of medicinal taxa are *Adiantum* (32) followed by *Asplenium* (30), *Dryopteris* (24) and *Pteris* (19) while the other genera were represented with less than 15 taxa. *Adiantum* and *Asplenium* not only have cosmopolitan distribution (Bir, 1963; Boonkerd & Pollawatn, 2013) but also have long been used medicinally. For instance, out of 34 *Adiantum* species found in China ("Flora of China:Volume 2," 1994), half of this number have been used in Traditional Chinese Medicine to cure human ailments (Wu, 1990). The species most frequently cited in this survey are *Adiantum capillus veneris* L. (272 use-reports in 66 publications), *Equisetum arvense* L. (113 use reports in 42 publications), *Asplenium ceterach* L. (49 use reports in 27 publications), *Equisetum ramosissimum* ssp. *ramosissimum* Desf. (66 use reports in 22 publications), while *Adiantum philippense* L., *Asplenium trichomanes* ssp. *trichomanes* L., *Lygodium flexuosum* (L.) Sw., and *Pteridium aquilinum* (L.) Kuhn, each have more than 40 use reports in 21 publications respectively.

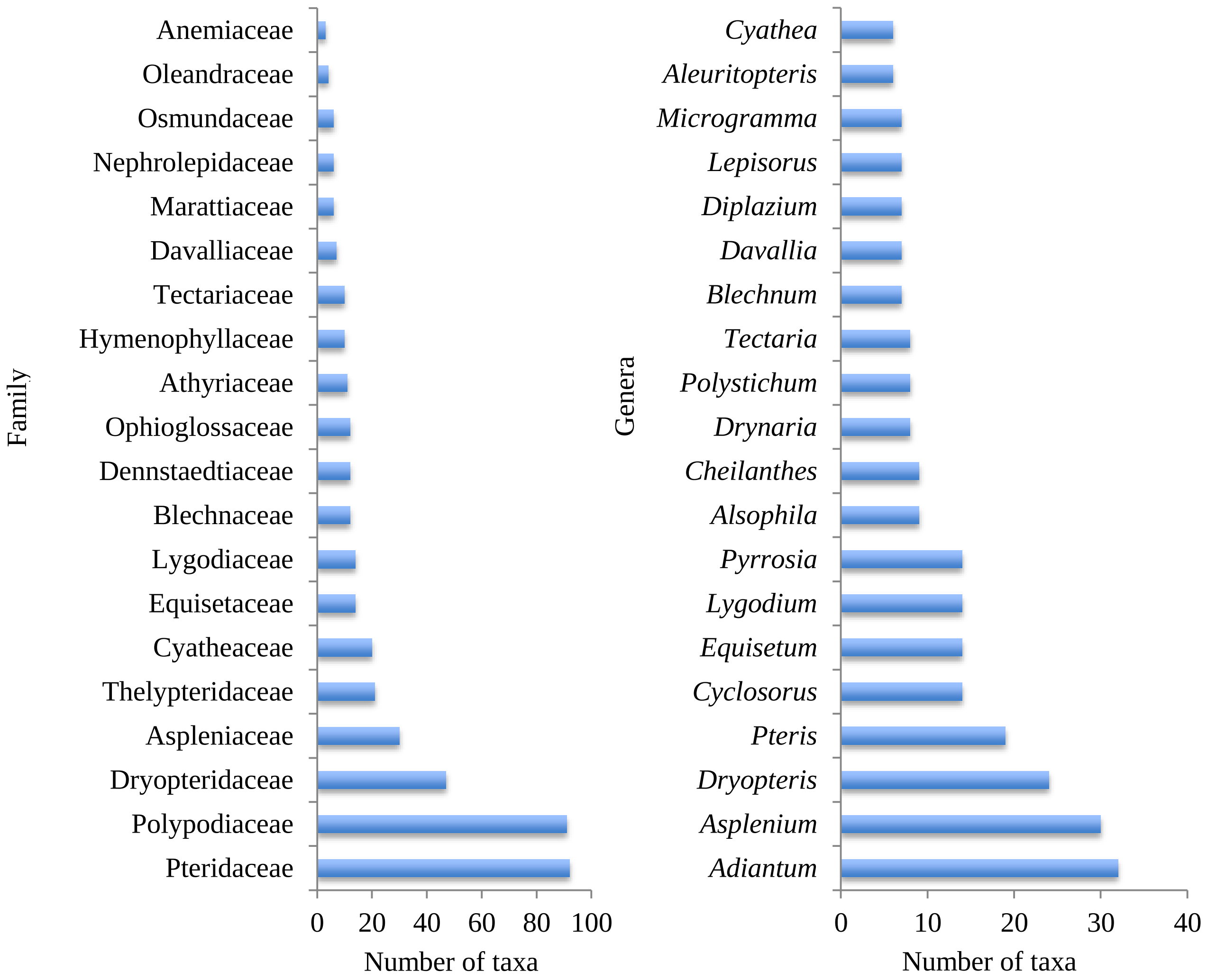


Figure 3.5 Most commonly used families (left) and genera (right) reported in the reviewed literatures with number of medicinal taxa.

Many medicinal plants are cultivated close to home/in homegardens, which serve as ‘medicine cabinets’ (Agelet et al., 2000; Ssegawa & Kasenene, 2007). Several genera in the most used families such as *Adiantum*, *Pteris*, *Drynaria* and *Platycerium*, are widely cultivated in many homegardens (Winter & Amoroso, 2003; Chin, 2005; Mannan et al., 2008).

The criteria used when selecting plants for traditional medicine can include morphological characters such as shape and colour (Bennett, 2007), or organoleptic properties including taste and smell (Ankli et al., 1999; Leonti et al., 2002; Molares & Ladio, 2009). Examples of morphological and organoleptic selection are apparent in the ferns. For example, the hair-like, delicate stalk of *Adiantum* (Maidenhair) was thought useful for the treatment of hair problems (May, 1978). Indigenous women of the Guarani of Misiones (Argentina) believe they will have a large family by eating ferns of *Pecluma* and *Pleopeltis* that are characterized by their prolific production of small fronds (Keller et al., 2011). Several species of *Polypodium* (Polypodiaceae) also have a liquorice flavour and contains small amounts of ostadin, a steroid saponin 3000 times as sweet as sucrose (Prance & Keller, 2015).

3.3.4 How ferns are used

Of the 3220 use reports, there were about 500 that described the therapeutic application of the species used. Figure 3.6 summarises the therapeutic applications recorded. Gastro-intestinal ailments, mainly stomach ailments and pains, including other diseases that related with stomach, small or large intestine and accessory organs of digestions, were most frequently reported (206 taxa , 45% of the total taxa). The high frequency of fern use for treating gastro-intestinal ailments suggests ferns may not be used differently to medicinal plants in general. Many studies of whole medicinal floras also report gastro-intestinal applications as the most frequent therapeutic application (Heinrich et al., 1998; Novais et al., 2004; Benítez et al., 2010). Poor sanitation amongst some poor communities in the tropics reliant on medicinal plants might explain the high frequency of gastro-intestinal problems reported. Skin and dermatological problems is the second most frequently reported therapeutic application, treated with 177 taxa (39% of all taxa used) followed by gynecology/fertility problems (141 taxa; 31%), respiratory/pulmonary illness (90 taxa; 20%), urinary conditions (82 taxa; 18%), general health (120 taxa; 26%) and musculo-skeletal (111 taxa; 24%). The reporting of all other therapeutic applications is much less, with fewer than 110 therapeutic applications and 67 species used.

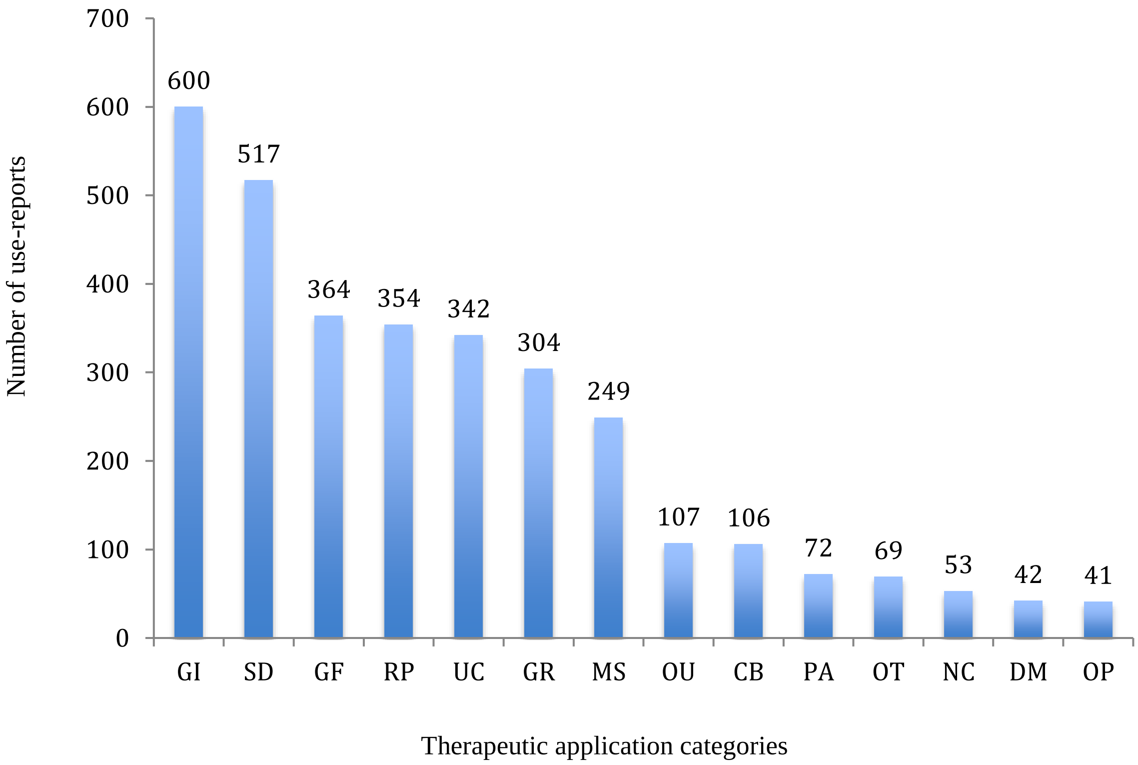


Figure 3.6 Summary of species use-reports by therapeutic application. Categories; gastro-intestinal (GI), skin conditions/dermatology (SD), gynaecology/fertility (GF), respiratory/pulmonary (RP), urinary conditions (UC), general health problem (GR), musculo-skeletal (MS), other/unclassified (OU), cardiovascular problems/blood purity (CB), poisonous animal bites (PA), otorhinolaryngology (OT), neurological conditions (NC), dentistry/mouth (DM) and ophthalmology (OP).

Many species are reported to have multiple uses. Analysis of the data also revealed that half of the total medicinal species are used to treat many different medical problems and have many therapeutic applications. Two hundred and sixty one taxa (58%) are applied to more than one category of while the other cited medicinal species (193 taxa, 42%) were used to treat only single therapeutic application categories.

The plant parts used for medicinal purposes and method of preparation and application are shown in Figure 3.7 (a) and (b). The part used and method of preparation and application were less frequently specified in the literature, with 25% and 44% of use reports.

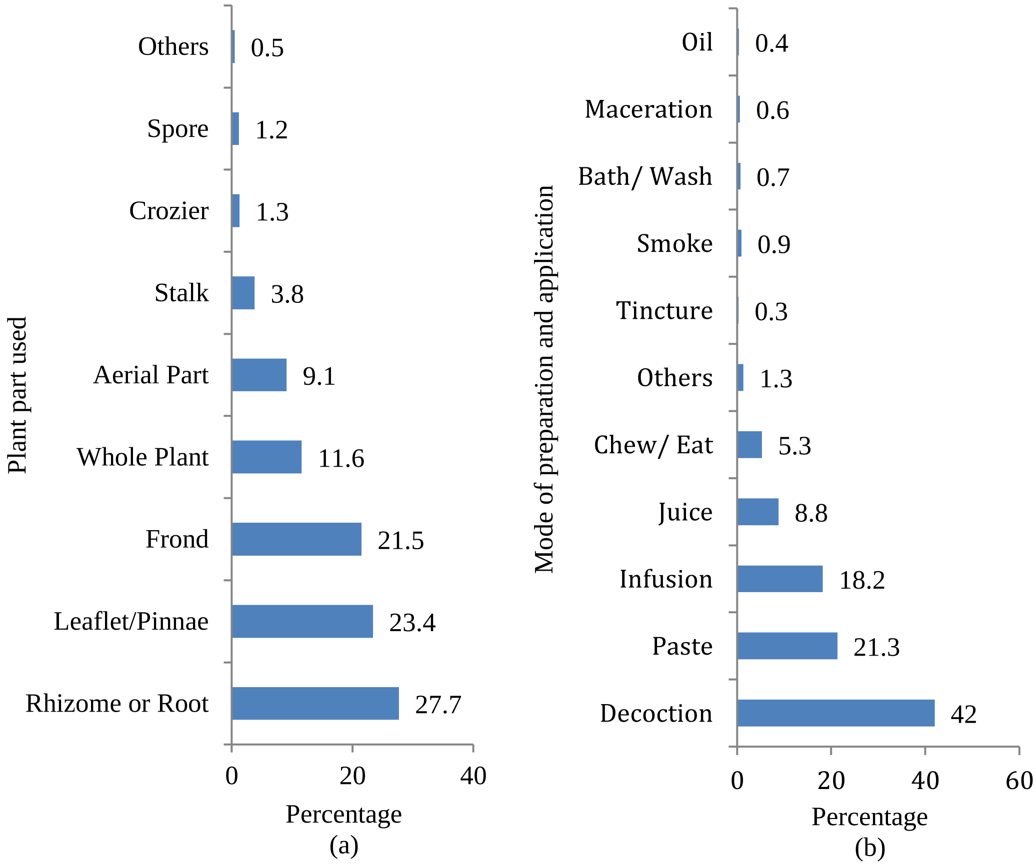


Figure 3.7 Fern use: Plant part used and mode of preparation and application. (a) Percentage of plant parts used of total used-reports of medical remedies, (b) Percentages of modes of preparation and applications of total used-reports of medical remedies.

All parts of the fern sporophyte are used medicinally, but the rhizome or root are most frequently used (689 reports for 184 taxa). Leaflets/pinnae (581 reports for 151 taxa), fronds (533 reports for 168 taxa), the whole plant (287 reports for 95 taxa), aerial parts (226 reports for 42 taxa) and the stalk (94 reports for 43 taxa) also find significant use. The croziers and spores are less frequently mentioned (each fewer than 32 reports, 1.3% or less of the reports). Four use reports referred to sap, four to scales, two to gum, and one each for slimy tissue and pulp. Field surveys of ethnomedicinal plant use refer to the use of fruits, seeds, wood bark or flowers, structures not present in ferns. However, many general surveys report the preferential use of rhizomes, roots or leafy parts (Di Stasi et al., 2002; Giday et al., 2003; Shrestha & Dhillion, 2003; Okello & Ssegawa, 2007; Teklehaymanot & Giday, 2007). It is possible that, in ferns as in plants generally, roots are significant sources of bioactive chemicals and this underlies their selection and use (Robinson, 1974). Since ferns in tropical rainforests are mostly moderately sized with short, erect fronds (Page, 1979), they are easily collected and stored.

Of the 11 categories of preparation and application, decoction is the most frequently reported (Figure 3.7b). The mode of preparation and application of medicinal plants could vary both according to the cultures using them and the plant group being used; it is beyond the scope of this study to assess whether decoction is used for preparing ferns more or less than for medicinal plants in general. The high frequency of decoction may be a result of the frequent of rhizome/root parts. Decoction may be popularised because of the belief that the longer the plant is boiled the higher the efficacy of the remedy (Speck, 1941), though in some situations the cost of preparing a decoction may be limiting (Daswani et al., 2011). Pastes, infusions and juices are the next most important forms of use, and chewing or eating ferns for medicinal purposes is also common. The use of ashes, latex, smoke, body/washes, maceration, tincture or oil are rare, each accounting for less than 1% of reported preparations/applications. Most ailments and pains were usually treated with a single plant. In 25 cases, mixtures of two or more different plant species are used to prepare a remedy for ailments. For example, in India, fresh rhizome of *Alsophila gigantea* is mixed with *Piper nigrum* together with cows' milk (Singh & Singh, 2012). Since the majority of our reports are from Asia, where written systems that emphasise multi-herbal mixtures originated, we might expect more reports of mixtures including ferns in our data set. Ethnobotanical studies are rarely focused on herbal mixtures, so the frequency of fern use of mixtures could be under-reported.

3.3.5 Ferns in pharmacology and toxicology

The review of the pharmacological and toxicological properties of medicinally-used ferns and their phytochemistry highlights the tension between therapeutic and deleterious effects of medicinal plants exemplified in the ferns. Out of 454 taxa in this study, 189 (42%) had pharmacological properties reported in the literature and 81 taxa (18%) had been the focus of a toxicological study (Appendix 3.2).

This finding, while preliminary, suggests that there are still many medicinal ferns species not been investigated regarding their biological activities and only little information on toxicological impacts to human healths have been reported. Other studies have shown that ferns are rarely the focus of such research (Santos et al., 2010). However, the majority of the medicinal species that find the highest number of use reports are rather well known in terms of their bioactivity, with at least two studies, or as many as 30 in *Pteridium aquilinum* (Table 3.5). Notably, the toxic nature of *Pteridium aquilinum* has been well-investigated, but for other species pharmacological activity has been the focus. The scale of the public health problem associated with toxic properties might be considerable and further research is needed (Chapter 5).

Table 3.5 Most frequently cited medicinal species showing the numbers of reports of their pharmacological and toxicological properties.

Species	No. of use reports	Pharmacological studies	Toxicological studies
Adiantum capillus-veneris	272	16	0
Equisetum arvense	113	12	0
Lygodium flexuosum	82	8	0
Equisetum ramosissimum ssp. *ramosissimum*	66	4	0
Adiantum philippense	62	10	0
Pteridium aquilinum	58	4	26
Asplenium ceterach	49	2	0
Asplenium trichomanes ssp. *trichomanes*	41	2	0

Drynaria roosii Nakaike, known as ‘Gu Sui Bu’ in China, received most attention in pharmacological experimental studies with 20 papers found, more than any other species, although we found only four studies reporting its traditional use (Table 3.6). This might reflect the large number of pharmacological studies of plants in the Chinese Pharmacopeia, but also the relative lack of studies of Chinese traditional use in the English language, abstracted scientific literature. *Drynaria roosii* is frequently used clinically to treat bone fractures, arthritis, osteoporosis and rheumatism in China (Li et al., 2006) and in the Chinese Pharmacopoeia, it is cited for treating kidney problems in Traditional Chinese Medicine (Chinese Pharmacopoeia Commission, 2005). Its potential in treating bone fractures, bone reconstructions and deafness has been demonstrated; several studies report flavonoid and phenylpropanoid compounds such as as epicatechin, naringin and neoeriocitrin have osteoprotective effects (Wang et al., 2008; Wang et al., 2010; Huang et al., 2012; Wong et al., 2013).

Table 3.6 Medicinal species that are most frequently studied for their pharmacological properties with the numbers of use-reports and numbers of publications.

Species	Pharmacological studies	No of use-reports	No. of publications
Drynaria roosii	20	12	4
Adiantum capillus-veneris	16	272	66
Diplazium esculentum	13	23	12
Drynaria quercifolia	13	65	19
Blechnum orientale	13	42	16
Adiantum philippense	10	62	21
Equisetum arvense	12	113	42

Adiantum capillus-veneris L. was also well studied pharmacologically, with 16 papers found (Table 3.6). The literature reveals the presence of flavonoids such as astragalin (kaemp- ferol 3-glucoside), isoquercitrin (quercetin 3-glucoside), kaempferol 3-glucuronide, querciturone (quercetin 3-glucuronide), nicotiflorin (kaempferol 3-rhamnoglucoside) and rutin (quercetin 3-rhamno glucoside) (Akabori & Hasegawa, 1969; Pourmorad et al., 2006), different classes of triterpenoids (Nakane et al., 1999; Reddy et al., 2001; Nakane et al., 2002), phenyl propanoids (Imperato, 1982b, 1982a), steroids (Marino et al., 1989), alicyclic acids (Minamikawa & Yoshida, 1972) and lipids (Sato & Furuya, 1983). Extracts of this species have also been reported to possess many pharmacological activities, such as antiurolithic (Ahmed et al., 2013), antimicrobial (Banerjee & Sen, 1980; Mahran et al., 1990; Singh et al., 2008; Parihar et al., 2010; Alipour & Khanmohammadi, 2011), antifungal (Guha et al., 2005) and antioxidant (Pourmorad et al., 2006) activities which could justify its traditional use in 13 therapeutic application categories. For example, alcohol extracts of the rhizome have been shown to be antimicrobial when it inhibited the proliferation and metabolism activity of rifampicin-resistant pulmonary tuberculosis cells (Tan & Xiang, 2002) which could explain its traditional use in the treatment of respiratory problems (Perry & Metzger, 1980; Manandhar, 2002; Winter & Amoroso, 2003; Talip et al., 2013) and in urinary infections (Pieroni et al., 2002; Winter & Amoroso, 2003; Hanlidou et al., 2004; Everest & Ozturk, 2005; Hamayun et al., 2006; González-Tejero et al., 2008; Lee et al., 2008; Upreti et al., 2009; Altundag & Ozturk, 2011; Mati & de Boer, 2011; Cornara et al., 2014).

In addition to *Drynaria roosii* and *Adiantum capillus-veneris*, other medicinal species have been the focus of pharmacological investigations (Table 3.6, Appendix 3.2). To a certain extent, the data from pharmacological investigations validated the applications of these medicinal species in traditional medicines and provided the association between the ethnomedicinal uses and bioactivity evaluations. For instance, the species used for diarrhea and urinary tract infection have antimicrobial activities (*Equisetum arvense*; Radulović et al., 2006), species used for the treatment of skin diseases have antidermatophytic activities (*Drynaria quercifolia*; Nejad & Deokule, 2009) and species used for wound healing have antioxidant activities (*Blechnum orientale*; Lai et al., 2010). A review by Zhu et al. (2011) revealed no approved drugs from fern species, and no fern bioactivities undergoing clinical drug trials. The only species in the preclinical trial drugs list is *Thelypteris torresiana* (Thelypteridaceae), which is being tested for anticancer activity (Zhu et al., 2011). Protoapigenone, a natural flavonoid, isolated from this species exhibited significant cytotoxic activity against several types of human cancer cells using both in vitro and in vivo models (Lin et al., 2005; Chang et al., 2008a; 2008b; Chiu et al., 2009; Hunyadi et al., 2011; Chen et al., 2013) which demonstrated a prospective future of anticancer drug development. Although there is comparatively little data in the literature on the pharmacological investigations of ferns that are used in traditional medicine (Appendix 3.2), numerous compounds especially flavonoids, steroids, alkaloids and terpenoids were isolated from ferns and some of these compounds have become the focus of intensive research concerning their pharmacological properties due to the potential of bioactive agents (Tomsik, 2014; Cao et al., 2017). These pharmacological data suggest there maybe more leads amongst the ferns.

3.4. Conclusion

Compilation and scrutiny of the ‘Medicinal Ferns Database’ has shown the diversity of medicinal ferns throughout the globe and has revealed the many fern species used medicinally in various cultures worldwide. In conclusion, more than 400 fern species are found to treat different ailments by people throughout the globe and some may appear to have the potential for new drug discovery. As ferns comprise about 9,000 to 12,000 species (Smith et al., 2006; Lehtonen, 2011; Christenhusz & Chase, 2014), only a small proportion (5%) are known to have medicinal use as opposed to between 12% of medicinal species of flowering plants (Schippmann et al., 2002). This study shows 58% and 82% of medicinal ferns species have not been the focus of pharmacological and toxicological effects respectively (Appendix 3.2).

A lot of online databases are found to be directing their focus to specialized content such as traditional medicine systems, regional uses, disease-specific and phytochemical information and most of the existing independent databases are variable in their content with regional or cultural focus. Ningthoujam et al. (2012) found specialized databases have some advantages as compared to comprehensive ones. The United Nations Industrial Development Organization (UNIDO) recommended that a comprehensive medicinal plant database should contain information in terms of botanical aspects along with other information such as ethnopharmacological, chemical, agrotechnical, chemotaxonomic, market and other relevant aspects (Ningthoujam et al., 2012). With the exception of large, publically funded projects, the comprehensive approach seems to be impractical as the data collection from a wide geographical area in multiple disciplines into a single database might create the risk of possible inconsistencies. However, to avoid unnecessary duplication of research or inconsistencies, a comprehensive database can be achieved by optimization of data and development of data standards for better management and sharing networking (Gaikwad et al., 2011; Ningthoujam et al., 2012). For example, the using of multiple terms such as; rhizomes, rootstock and rizoma in various literature that carry the same meaning or referring to the same part of the plant can be confusing to the public users, thus a standard term must be used throughout the study to avoid such confusion. Hence, continuing updates to include more information such as chemical compound data or biogeography of fern species in the database will be developed in the future and the potential to be made as public or online database will be explored. Data collection is not only crucial in conserving traditional knowledge, but also contributes towards evaluation of the likely efficacy or safety of these traditional medicines.

Chapter 4. The limits of phylogenetic predictivity in bioprospecting: phylogenies do not reveal lineages rich in medicinally-used ferns

4.1 Introduction

The use of plants in the treatment and prevention of disease has been practiced for thousands of years in many countries, for example China (Chang et al., 1986) and India (Kappor, 1990). It is estimated that about 53,000 medicinal species (Schippmann et al., 2002) are used to provide healthcare for 80% of the world population especially in developing countries (Hamann, 1991; Van Wyk & Wink, 2004; Gurib-Fakim, 2006). Given the extensive traditional knowledge of these plants' medicinal use, it may be possible to make a tremendous contribution to bioprospecting by targeting traditionally-used medicinal plants for drug discovery. However, only a small proportion of plants have been investigated and screened for their biological activity (Gurib-Fakim, 2006).

Screening plants as candidates for drug discovery programs can be either through "random" collection or through investigating the leads of prior ethno-pharmacological knowledge (Lewis & Elvin-Lewis, 1995; Fabricant & Farnsworth, 2001). However, the progress of focusing drug discovery by ethnobotanical data is not consistent, and the popularity of this approach has fluctuated (Saslis-Lagoudakis et al., 2012). This approach also has been critized because traditionally-used medicinal plants may be used predominantly ritually and not for bioactivity. Therefore, they would not yield interesting chemicals that are efficacious, thus resulting in disappointing outcomes (Firn, 2003; Saslis-Lagoudakis et al., 2012). In the meantime, given the alarming rate of biodiversity loss, with recent estimations indicating more than 20% of the world's plants threatened with extinction (Brummitt & Bachman, 2010), faster and better systematic approaches to find new-plant derived drugs are more necessary than ever before. Some of the species that are threatened with extinction may well include plants with significant medicinal value or might yield novel bioactive compounds with the potential therapeutic use. The early identification of these valuable species can help researchers find the same compound in other plants.

In recent years, new methodological approaches have renewed interest in bioprospecting, specifically by investigating the phylogenetic patterns of plant therapeutic uses or bioactivities across medicinal floras. This approach correlates phylogenetic analyses based on DNA sequence information from plants with medicinal data or chemical properties by examining the relatedness between the particular group of species (eg. medicinal species) on the phylogeny. In the earliest study in 2006, Lukhoba et al. found an interesting trend whereby most traditionally-used medicinal species of *Plectranthus* (Lamiaceae) were found within the same large phylogenetic clade. One year later, using a more quantitative method, a study by Forest et al. (2007) reported that the medicinal plants in the Cape of South Africa were significantly phylogenetically clustered. In an analysis aimed at identifying target species for drug discovery, the distribution of alkaloids with specific activity was found significantly constrained by phylogeny when Rønsted et al. (2008) succesfully mapped bioactivity on to phylogenetic trees within a plant genus, *Narcissus*. A similar situation also was observed by Zhu et al. (2011) when they revealed that nature-derived drugs were derived mostly from drug-productive families and tended to be clustered rather than scattered randomly in the phylogenetic tree. Since then, there is increasing evidence of phylogenetic clustering of traditionally-used or bioactive species. In 2011, Saslis-Lagoudakis et al. investigated the pattern of ethnomedicinal usage in *Pterocarpus* (Leguminosae) and detected significant clustering of species in certain usage categories on the phylogeny. Another study conducted by Saslis-Lagoudakis et al. (2012) also revealed strong phylogenetic signal in traditionally-used medicinal plants. Medicinal plants were overpresented in certain branches of the phylogenetic tree when traditionally used species from three different regions, Nepal, New Zealand and the Cape of South Africa, were investigated. Interestingly, the ‘hottest’ branches contained more than 60% more traditionally used plants than expected. Most recently, Ernst et al. (2016) found that the plants used medicinally in the *Euphorbia* are significantly phylogenetically clustered, as were traditional uses inferred to be anti-inflammatory applications, though traditional therepautic applications were not.

Considering all of this evidence, it seems that there is a correlation between species’ evolutionary history and the diversity of services (eg. medicinal uses and chemical properties). An evolutionary predictive tool for prioritizing specific lineages to be investigated is underpinned by the observation that phylogenetically closely related species generally share similar biochemical properties. However, studies to date are relatively few, so it is difficult to generalise about expectations at different hierarchical levels and in different plant groups (Table 4.1). Most of the previous studies have been carried out on Angiosperms, no studies have been focused on other groups and this approach has never been applied to the ferns.

Table 4.1 Phylogenetic studies of traditional use of plants for medicine. Level of sampling indicates hierarchical level of which examplars were sampled, for example, one exemplar per genus was sampled by Forest et al. (2007).

Study	**Hierarchical level of phylogeny reconstruction**	**Taxonomic level of sampling**	**Spatial scale**
Plectranthus: A review of ethnobotanical uses [1]	Genus (*Plectranthus*)	Species	Global distribution of genus in Tropical Africa, Asia and Australia
Preserving the evolutionary potential of floras in biodiversity hotspots [2]	Angiosperm	Genus	South Africa
The use of phylogeny to interpret cross-cultural patterns in plant use and guide medicinal plant discovery: an example from *Pterocarpus* (Leguminosae) [3]	Genus (*Pterocarpus*)	Species	Global distribution of genus in three regions, Neotropics, tropical Africa and Indomalaya
Phylogenies reveal predictive power of traditional medicine in bioprospecting [4]	Angiosperm	Genus	Across three regions, Nepal, New Zealand and the Cape of South Africa
Phylogenetic exploration of commonly used medicinal plants in South Africa [5]	Angiosperm	Genus	South Africa
Evolutionary prediction of medicinal properties in the genus *Euphorbia* L. [6]	Genus (*Euphorbia*)	Species	Global distribution

*Lukhoba et al., 2006 [1], Forest et al., 2007 [2], Saslis-Lagoudakis et al., 2011[3], Saslis-Lagoudakis et al., 2012 [4], Yessoufou et al., 2015 [5], [6]Ernst et al. (2016)

Studies of medicinal uses of ferns are available in different inhabited continents especially in Asia-Tropical region (Chapter 3). Different fern species throughout the globe have been or are presently utilized to treat various ailments as explained in Chapter 3. Ferns are numerous, they do find medicinal use traditionally and so they are potentially valuable. The phylogeny (Chapter 2) and the database of fern medicinal use (Chapter 3) represent an opportunity to advance medicinal fern research by investigating evolutionary patterns.

4.1.1 Objectives

The primary objective of this chapter is to explore phylogenetic methods as applied to medicinal ferns. By using the robust megaphylogeny of the ferns (Chapter 2) and the global database of medicinal ferns use (Chapter 3), the use of ferns is investigated at a range of spatial scales, from global, to continental and regional/national scales. In this chapter, I will subsample the available data to encompass the range of different hierarchical levels of phylogeny reconstruction and sampling, and also investigate spatial scales so that the pattern revealed for ferns can be compared to all patterns revealed for Angiosperms to date. Patterns amongst fern species that are used medicinally will be investigated with the expectation that lineages rich in medicinal ferns will be discovered which eventually might provide a framework for further prediction. With an increasing number of studies revealing lineages rich in medicinally-used flowering plants, this phylogenetically-guided approach will be used on ferns for the first time.

4.2 Materials and methods

Nine analyses were carried out (Table 4.2). The ethnomedicinal dataset and phylogeny were prepared for these analyses as follows.

Table 4.2 Taxonomic scope and level of sampling for nine analyses performed. Ethnomedicinal information and phylogeny were processed to represent different hierarchical levels and spatial scales.

No	Taxonomic scope of sampling	Hierarchical level of sampling	Geographical extent of sampling
1.	All ferns	Species	Global
2.	Family (Pteridaceae)	Species	Global
3.	Genus (*Adiantum*)	Species	Global
4.	Eusporangiate ferns (Early diverging species)	Species	Global
5.	All ferns	Generic	Nepal
6.	All ferns	Generic	New Zealand
7.	All ferns	Generic	South Arica
8.	All ferns	Generic	Asia-Tropical
9.	All ferns	Generic	Global

4.2.1 Ethnomedicinal data

Information on the medicinal uses of ferns for each species throughout the globe was collected and recorded as in Chapter 3 (Appendix 3.1). Overall, a total of 454 taxa (442 species, seven subspecies, five varieties) belonging to 121 genera in 36 families were recorded as having medicinal uses in different parts of the world.

Asia-Tropical was selected for continental level study since this continent is rich in medicinal fern species as reported in Chapter 3. Three different regions/nations; Nepal, New Zealand and South Africa were chosen in parallel with studies by Saslis-Lagoudakis et al. (2012).

Therapeutic applications for all studies were grouped into 14 categories (Chapter 3). These are; cardiovascular problems/blood purity (CB), dentistry/mouth (DM), gastro-intestinal (GI), gynaecology/fertility (GF), neurological conditions (NC), ophthalmology (OP), musculo-skeletal (MS), skin conditions/dermatology (SD), otorhinolaryngology (OT), respiratory/pulmonary (RP), urinary conditions (UC), poisonous animal bites (PA), general health problem (GR) and other/unclassified (OU).

4.2.2 Tree preparation

The phylogeny of ferns reconstructed at the species level (Chapter 2) was the basis of the phylogenies for this study. Nine new phylogenies were reconstructed to carry out analyses as outlined in Table 4.2. For analysis 1, the species level tree previously generated (Chapter 2) has been modified by adding manually 55 fern taxa for which genetic data was unavailable. Their placement in polytomies on the phylogeny was based on their taxonomic affinities (Appendix 4.1). Mesquite (Maddison & Maddison, 2001) was used to manipulate the phylogenies. Overall, sampling reached a total of 3768 of fern taxa representing approximately 40% of the global fern diversity.
This most inclusive species-level tree was pruned to make generic level trees. One exemplar species from each genus was selected at the global, continental, and regional/national levels. The distribution of genera included in the continental and regional/national levels studies were checked againt the Global Biodiversity Information Facility (GBIF; www.gbif.org). Thus, 185 out of 271 genera were selected for Asia-Tropical, 94 genera for Nepal, 66 genera for New Zealand and 90 genera for South Africa. In addition, due to synonymy problems and changes in fern nomenclature, additional sources were also used to confirm the generic checklist for each country selected; Nepal (Fraser-Jenkins et al., 2015), New Zealand (Brownsey & Smith-

Dodsworth, 1989) South Africa (Roux, 2001). Phylogenies were pruned in the R environment version 3.3.1 (https://www.r-project.org/). All the scripts are available in the Appendix 4.2. Prior to analysies, phylogenies were further pruned to remove outgroup taxa and the phylogenis were also unrooted.

4.2.3 Phylogenetic analyses and manipulations

Metrics from phylogenetic community ecology were used to compare the ‘communities of taxa’ in this study. Generally, in phylogenetic community ecology, ‘communities of taxa’ are grouped by ecological zone or geographical zone, however ‘communities of taxa’ here were compiled based on the group of species traditionally-used for medicine and by therapeutic application categories.

The ‘comstruct’ command in Phylocom v4.1 (Webb et al., 2008) was used to assess the significance of phylogenetic signal/structures for a ‘community of taxa’, which are a subset of the taxa in the phylogeny. It measures whether a group of species are significantly clustered on the phylogeny. In this analysis, the significance of phylogenetic signal for a group of species on the phylogeny is measured by calculating the mean phylogenetic distance (MPD) and mean nearest phylogenetic taxon distance (MNTD). MPD measures the phylogenetic distance between every pair of individuals drawn from the sample and compares the average. Mean nearest phylogenetic taxon distance (MNTD) measures the average distance from each individual in the sample to its closest relative in the sample (Figure 4.1).

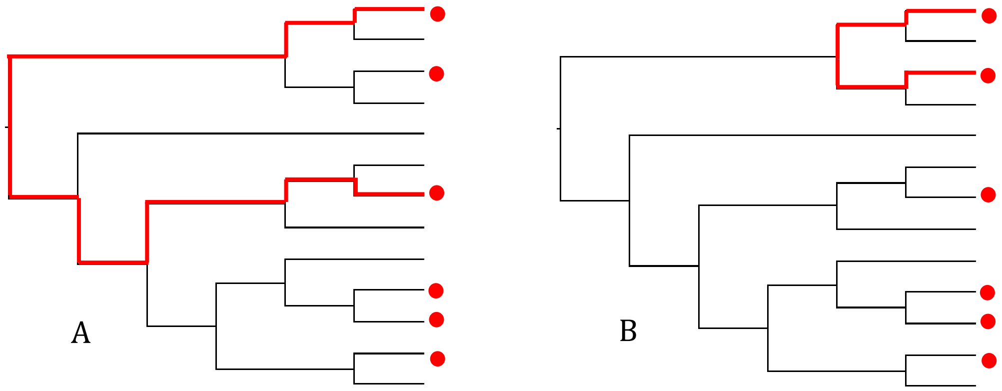


Figure 4.1 Red lines indicate measured distances between taxa in this study. A: MPD measures the mean of the distances (length along phylogeny) between all possible pairs of sample taxa. B : MNTD measures the mean of the distances between each sample taxon and its most closely related sample taxon.

These average distances were compared to MPD/MNTD values for randomly generated samples to provide p values for the significance of phylogenetic signal for the given sample. The p values were calculated by dividing the number of randomly generated samples that were more clustered on the phylogeny than the observed sample (rankLow) by the number of runs (1,000). The Net Relatedness Index (NRI) quantifies the overall clustering of taxa on a tree, and the Nearest Taxon Index (NTI) quantifies the extent of terminal clustering on a tree. Both were calculated for each dataset. Positive values (NRI/NTI > 0) indicate under-dispersed communities (phylogenetic clustering) whereas negative values (NRI/NTI < 0) indicate over-dispersed communities (phylogenetic evenness). By using this method, the phylogenetic signal/structure for all samples can be assessed to provide an estimation of the relation between the taxa used medicinally. The rationale of this method is shown in Figure 4.2.

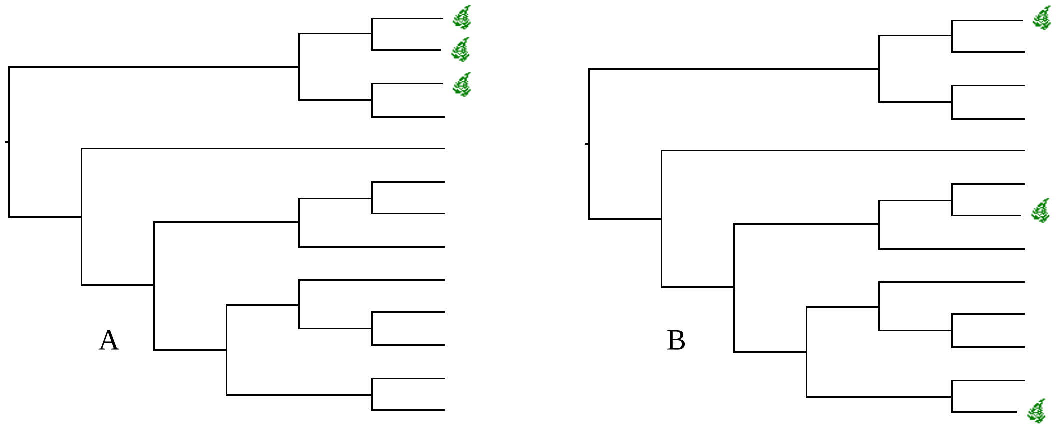


Figure 4.2 Two different scenarios (A and B) for the distribution of traditionally-used medicinal species of ferns on a hypothetical phylogeny. Three traditionally-used medicinal taxa are indicated with a frond symbol under scenarioA and B. The phylogenetic signal (phylogenetic clustering) in scenario A is strong as the phylogenetic distance within the medicinal taxa is small while there is a signal of phylogenetic evenness (overdispersal) in scenario B as the phylogenetic distance within the medicinal taxa is large.

4.3 Results

Investigation of the phylogenetic distribution of all taxonomic levels of medicinal use of ferns including their categories of uses with the ‘comstruct’ tool, are summarised in Table 4.3. Details of the results are presented in Appendix 4.3.

Table 4.3 Phylogenetic structure indicated by NRI and NTI values for medicinally-used medicinal ferns across different hierarchical levels and spatial scales, and 14 categories of use. Green cells indicate significant overdispersion, green cells with (-) indicate nonsignificant overdispersion whereas red cells with (+) indicate nonsignificant clustering.

	NRI									**NTI**								
	Species level (global)	Pteridaceae	*Adiantum*	Eu-sporangiate	Nepal	New Zealand	South Africa	Asia-Tropical	Genus level (global)	Species level (global)	Pteridaceae	*Adiantum*	Eu-sporangiate	Nepal	New Zealand	South Africa	Asia-Tropical	Genus level (global)
Medicinal ferns overall	🟩	🟩	🟩	🟩	🟩	+	-	🟩	-	🟩	🟩	🟩	-	-	+	-	🟩	🟩
Cardiovascular problems/blood purity	🟩	-	+	+	NA	NA	NA	🟩	-	-	+	+	+	NA	NA	NA	🟩	-
Dentistry/mouth	🟩	-	+	+	NA	NA	NA	🟩	🟩	-	+	+	-	NA	NA	NA	+	🟩
Gynaecology /fertility	🟩	-	🟩	+	-	NA	NA	🟩	-	🟩	-	-	-	-	NA	NA	-	-
Gastro-intestinal	🟩	-	-	-	-	+	-	🟩	🟩	🟩	-	🟩	-	-	-	-	-	🟩
General health problem	🟩	-	-	-	🟩	+	+	🟩	🟩	-	🟩	🟩	-	-	+	+	-	🟩
Musculo-skeletal	🟩	-	+	+	-	NA	NA	-	-	🟩	-	+	-	-	NA	NA	🟩	🟩
Neurological conditions	🟩	+	+	-	NA	NA	+	🟩	🟩	🟩	+	+	-	NA	NA	+	🟩	🟩
Ophthalmology	🟩	+	-	+	NA	+	NA	🟩	🟩	-	+	-	-	NA	+	NA	🟩	-
Otorhinolaryn-gology	🟩	-	-	-	NA	NA	NA	-	-	-	-	-	+	NA	NA	NA	-	-
Other /unclassified	🟩	-	-	+	NA	NA	NA	🟩	-	🟩	-	+	-	NA	NA	NA	-	-
Poisonous animal bites	-	-	+	NA	NA	NA	NA	+	-	-	-	+	NA	NA	NA	NA	+	-
Respiratory /pulmonary	🟩	+	-	-	-	NA	+	🟩	🟩	🟩	+	-	🟩	-	NA	+	-	🟩
Skin conditions /dermatology	🟩	-	-	-	+	+	NA	-	-	🟩	-	-	-	+	+	NA	-	-
Urinary conditions	🟩	-	-	+	-	NA	NA	-	-	-	-	+	-	-	NA	NA	🟩	-
	Species				Genus					Species				Genus				

Overall, the NRI metric identified that ferns that are used medicinally were not phylogenetically clustered at any spatial or taxonomic scale. In other words, fern species that are used medicinally are not found in specific lineages, but are distributed throughout the phylogeny, suggesting an overall phylogenetic overdispersion of medicinally-used fern species. None of the 14 therapeutic application categories of use showed significant clustering. At all hierarchical levels, only phylogenetic distribution of medicinal uses at the species level (global) including eight categories of uses; cardiovascular problems/blood purity (CB), gastro-intestinal (GI), ophthalmology (OP), skin conditions/dermatology (SD), respiratory/pulmonary (RP), urinary conditions (UC), general health problem (GR) and other/unclassified (OU) were found to be strongly significantly overdispersed ($P < 0.001$). The phylogenetic tree with strong significant overdispersal of medicinally-used fern species is presented in Figure 4.3. In summary, 36 out of 107 samples were shown to have significantly overdispersed phylogenetic distributions. A similar pattern was also found for all medicinally-used fern and categories of use when the phylogenetic structures were analysed using the NTI metric. Out of 107 samples, 27 showed significantly overdispersed distributions.

There were some positive NRI and NTI values in almost majority samples in New Zealand samples and when the taxonomic scope of sampling narrowed down to certain therapeutic categories in Pteridaceae, *Adiantum*, eusporangiate lineage, Nepal, South Africa and Asia Tropical. However, none of this clustering was significant. Overall, these results indicate that medicinally-used fern species are overdispersed at deep and shallow phylogenetic levels.

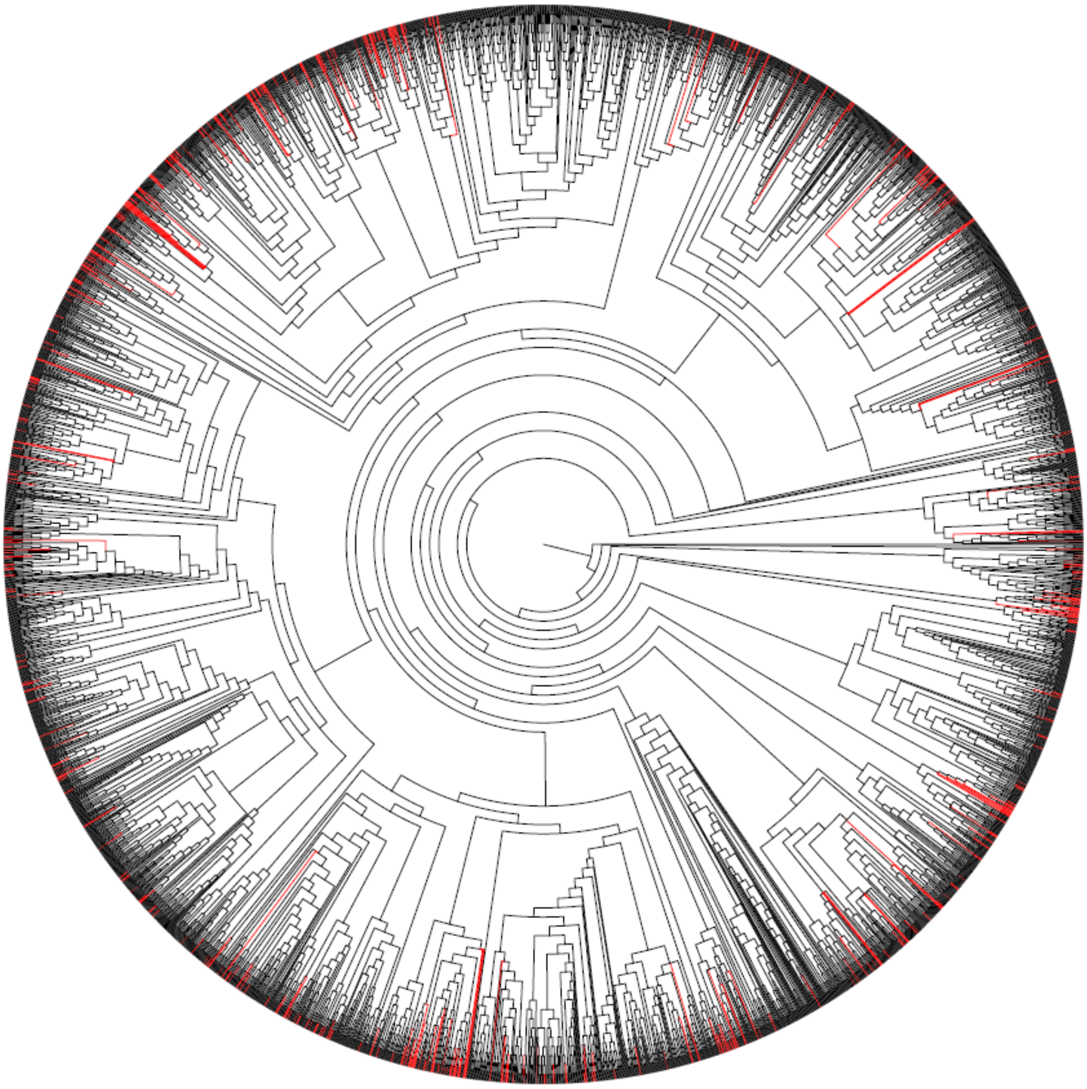


Figure 4.3 Fern phylogeny based on maximum likelihood analysis at the species level (global) indicating the distribution of medicinally-used fern species. Fern species that are used medicinally are indicated by red branches that show significant overdispersion (P<0.01).

4.4 Discussion

In recent years, phylogenetic comparative methods have emerged as promising tools for exploring correlations between phylogenetic diversity and useful attributes of medicinal plants (Lukhoba et al., 2006; Forest et al., 2007; Saslis-Lagoudakis et al., 2011; Saslis-Lagoudakis et al., 2012; Yessoufou et al., 2015). Here, these analyses are applied to a previously unstudied group, the ferns. Surprisingly, despite the increased number of studies suggesting lineages rich in flowering plants used for medicinal purposes, the findings of this study showed that no such lineages can be identified for ferns. Indeed, the findings suggest a different pattern, one in which of medicinally used fern taxa are dispersed across all spatial and taxonomic scales. This pattern of overdispersion, whereby co-occuring species are phylogenetically more distantly related than expected by chance (Emerson & Gillespie, 2008), is seen both in deep-evolutionary lineages, as revealed by NRI, and at shallow levels, as revealed by NTI. The results thus indicate that medicinally-used fern taxa around the globe are spread along the phylogeny.

This is the first time any global species-level phylogeny has been the focus of such study. Contrary to expectations, it is somewhat surprising when medicinally-used ferns overall are significantly phylogenetically overdispersed at deep and shallow phylogenetic levels. As this finding is not directly comparable to previous studies, patterns of the distribution of traditionally-used medicinal ferns were further investigated at genus level as in Lukhoba et al., (*Plectranthus*, 2006); Saslis-Lagoudakis et al., (*Pterocarpus*, 2011); Ernst et al., (*Euphorbia*, 2016). In addition, phylogenetic signal was also tested by investigating the species level sampling for one family. Pteridaceae was selected to represent the family level and *Adiantum* for the genus level. These two groups were selected in these analyses since they contribute a high number of medicinally-used species (Chapter 3) and are diverse groups of extant ferns with more than 40% and 60% of their taxa sampled in the phylogeny respectively (Chapter 2). Nevertheless, there is still no evidence for phylogenetic clustering, instead both groups demonstrate the same overdispersed patterns. Phylogenetic patterns at species level also were further explored for early diverging, eusporangiate ferns, and the finding here is also of overdispersal of distribution of medicinal use. The results here are contrary to previous studies with species-level sampling at generic level, which found clustered medicinal usage (Lukhoba et al., 2006; Saslis-Lagoudakis et al., 2011; Ernst et al., 2016). In some cases, phylogenetic clustering were observed in *Adiantum* samples when the usage was organised into categories, however, none of this clustering was significant. It could be argued that the results for *Adiantum* sample might not be directly comparable to previous studies of genus level in terms of sample density or deficiencies of use-reports data. Ernst et al. (2016) have 560 species in the phylogeny representing 65 species with genus of which 12% are medicinal. Saslis-Lagoudakis et al. (2012) sampled 19 of the 25 species in the genus for phylogeny reconstruction and 76% were used medicinally. However, even though figures for *Adiantum* are comparable (32 of the 73 species) in the genus were represented, and more than 40% were used medicinally, a different pattern is emerging in ferns at this level.

Generic-level phylogenetic studies of the medicinally-used plants in whole Angiosperm floras have also been given considerable attention by researchers. Thus, in this study, the predictive power of phylogeny in traditionally-used fern species was further explored at generic level. Following Saslis-Lagoudakis et al. (2012)’s study, phylogenetic signal in the medicinal use of ferns at generic level for the same regions; Nepal, New Zealand and South Africa were tested. Traditionally-used fern species overall, especially in Nepal and South Africa, were overdispersed. All samples in New Zealand were found clustered based on NRI, nevertheless these findings were not significant. An investigation was made at the continental scale, for the Asia-Tropical region, because here 40% of fern genera are used medicinally. Interestingly, we consistently demonstrate phylogenetic overdispersion in this area. Finally, patterns of use at the global scale for medicinal ferns using a generic level phylogeny were explored; consistent with other scales, the global generic-level study showed overdispersion in distribution of medicinal use.

The comparison of previous studies of the Angiosperms (Lukhoba et al., 2006; Forest et al., 2007; Saslis-Lagoudakis et al., 2011; Saslis-Lagoudakis et al., 2012; Yessoufou et al., 2015; Ernst et al., 2016) to the findings presented here have unequivocally demonstrated that ethnomedicinally-used ferns are not phylogenetically clustered in the same way as flowering plants are. Indeed, they are overdispersed. These results may be explained by the fact that ferns, which do not flower and lack seeds, are fundamentally different from Angiosperms (Chin, 2005) and suggest that the selection criteria for ferns and flowering plants have differed throughout their history of medicinal use. Thus, the finding presented here lead us to think that ferns and flowering plants overall are fundamentally different in terms of how they are selected for traditional medicinal use.

Despite the increasing number of documented phylogenetic clustering patterns in Angiosperms, there is one other recent and as yet unpublished study which provides a rather different view suggestive of overdispersal at the family level. Estevao N. F. Souza (in prep.) revealed that traditionally-used plants of family Leguminosae in Brazil are significantly overdispersed across the phylogeny. It seems that when a family in

Angiosperms is considered in isolation, it shows overdispersed genera with medicinal use, comparable to the whole ferns. In this broader perspective, it seems as if ferns may be selected in the same way as genera of an Angiosperm family.

Organoleptic properties seem to be one of the important criteria in the selection of plants as medicine (Leonti et al., 2002). Indigenous people in many cultures around the globe use smell and taste properties to characterize medicinal plants since medicinal plants tend to have distinctive taste or odor (Ankli et al., 1999; Leonti et al., 2002; Molares & Ladio, 2009). For instance, astringent and bitter taste properties were most frequently used in the treatment of gastrointestinal disorders such diarrhea or for expelling internal parasites (Leonti et al., 2002). Such attributes are directly associated with the presence of certain secondary plant metabolites (eg. bitter taste is often due to alkaloids). Thus, medicinal properties are not randomly distributed in Angiosperm phylogeny as closely related species share biochemistry (Fairbrothers et al., 1975; Rønsted et al., 2008; Rønsted et al., 2012). Whether organoleptic diversity of ferns is equivalent to organoleptic diversity in Angiosperms is unclear. The sweet and bitter tastes were quite often described in ferns (Mabberley, 2008; Keller et al., 2011; Harmaja, 2012), without mentioning other tastes commonly attributed to Angiosperms, which are more diversified in taste properties (eg. bitter, astringent, sweet, tasteless, spicy, acid (Ankli et al., 1999; Leonti et al., 2002)). Whether ferns are biochemically less diverse is also unclear. The insect-herbivory that drives chemical diversification in flowering plants is rather well known (Becerra et al., 2009; Agrawal & Weber, 2015). Cyanogen glycosides, phenoloids and ecdysteroids have been detected in several fern species as protection agents against herbivorous insects (Lafont et al., 2011; Vetter, 2011). However, ferns are sometimes argued to have fewer herbivorous pests because they lack flowers and fruit, therefore, they have a relatively limited chemical defence repertoire compared to flowering plants (Cooper-Driver, 1978; Auerbach & Hendrix, 1980; Cooper-Driver, 1985; Markham et al., 2006). A study by Rathinasabapathi et al. (2007) revealed that arsenic accumulation in *Pteris vittata* is an elemental defence against insect herbivory. Alternatives modes of defences against herbivores would account for low phytochemical variation across the ferns, as many ferns are arsenic accumulator (Ma et al., 2001; Francesconi et al., 2002; Zhao et al., 2002; Meharg, 2003; Du et al., 2005; Srivastava et al., 2006; Van et al., 2006; Rathinasabapathi et al., 2007; Zhang et al., 2008). Phylogenetic overdispersal in traditionally-used species in *Adiantum* may partly explained by the limited variation in chemical compounds as at least some species of *Adiantum* also able to accumulate arsenic (Srivastava et al., 2010).

The Doctrine of Signatures concept has also been applied to the ferns even though they lack flowers and seeds. For instance,indigenous women of the Guarani of Misiones (Argentina) believe they will have a large family by eating ferns of the genera *Pleopeltis* and *Pecluma* that are characterized by their prolific production of small fronds (Keller et al., 2011) whereas *Adiantum* species are used to treat hair weakness or baldness due to the similarity between the fern leaf and human hair (May, 1978; Lev, 2002; Dafni & Lev, 2003). Therefore, leaflet (pinnae) characteristics, especially the leaflets of bipinnate or tripinnate leaves, appear to play a role for selection in fern species. The morphology of leaf pinnation in ferns is homoplasious, occurring across distantly related lineages due to the convergent or parallel evolution during long evolutionary history of ferns (Little & Barrington, 2003; Ranker et al., 2004; Rothfels et al., 2008). The great morphological homoplasy within fern lineages may contribute to the pattern of overdispersal of traditionally used fern species in the phylogeny. The

Doctrine of Signatures could drive fern selection and use, however, compared to flowering plants, ferns have fewer morphological and colour characteristics.

Ecology also seems to play a role in species selection. For instance, weedy plants are more likely to be used in indigenous pharmacopoeias (Stepp & Moerman, 2001; Gazzaneo et al., 2005; Giday et al., 2009). Furthermore, families that are rich in weeds such as mints are more likely to be found in home gardens and the phylogenetic relatedness of traditionally-used plants might reflected their 'weediness'. In contrast, for fern species, the epiphytic habit might be associated with selection for medicine. *Drynaria roosii*, *Drynaria coronans*, *Davallia divaricata* and *Davallia solida* which are called as 'Gusuibu' in the Chinese traditional medicine system are examples of the important medicinal ferns which have long been used in the treatment of bone injuries (Chang et al., 2007). Interestingly, all of these species are epiphytic (Benzing, 2008). Considering that about one third of leptosporangiate species grow as epiphytes on Angiosperm trees and the diversification of of epiphytic ferns is not limited to only one clade but evolved several times in the ferns in many families (Schuettpelz & Pryer, 2009), which may contribute to the pattern of overdispersal of traditionally used fern species in the phylogeny at species level.

Generic complexes are suites of closely related plants interchangeably used for a particular purpose (Martin, 1995). They are common in medicinal use of flowering plants (Berlin et al, 1973; Pardo-de Santayana & Morales, 2010). It may be that turn-over in species in a generic complex reflects turn over in space of availability of species. This could be named phylogenetic replacement, and this phenomenon would explain the shallow phylogenetic structure revealed in several studies. It could conceivably be hypothesised that widely distributed species may be contributing to a failure to recover shallow phylogenetic clustering in ferns overall. Fern species are overall more widespread than flowering plant species, so that actually the same fern can be used in many different places, so there is no turn-over in space. In Angiosperms, the pattern may be one of overdispersed clusters, but the clusters are sufficiently rich in species for the single statistic that reduces the complexity of relationships across the entire phylogeny (Borregaard et al., 2014) to a single figure reflecting clustering overall. If the "clusters at the tips" in Angiosperms are single or few species in the ferns, then the single statistic will record an overall pattern of overdispersal. Just as community ecology needs more sophisticated metrics to tease apart the complex patterns of clade over-representation, methodological refinement is needed to exploit phylogenetic predictivity more widely in bioprospecting.

4.5 Conclusions

Ferns comprise ca. 9000 species (Smith et al., 2006), and account for about 3% of globally-known vascular plants (Christenhusz & Byng, 2016) and could be an important source of new medicines if continued commitment is devoted to the search for new medicinal plants. Whilst all the previous studies were focusing on Angisoperms for ethnomedicinal patterns, investigation on the generality of phylogenetic patterns by exploring evolutionary relationships between fern species that are used medicinally was shown here for the first time.

Evidence for phylogenetic overdispersion was found consistently at every taxonomic level and spatial scale investigated. This results are not encouraging to identify potentially bioactive fern lineages according to the bioprospecting approach described in Saslis-Lagoudakis et al. (2012). Here, I have shown that the ethnomedicinal selection of ferns is not comparable to selection of flowering plants. Neither the MPD nor the MNTD have shown significant clustering at any taxonomic hierarchical level. Instead, a pattern of significant overdispersal of traditionally used fern species is observed. This finding suggests that, overall, fern diversity is better sampled by traditional medicine than angiosperm diversity, where medicinal plants are found on selected lineages (Lukhoba et al., 2006; Saslis-Lagoudakis et al. 2011, 2012).

Chapter 5. Phylogenetic prediction of public health risk associated with medicinal fern consumption

5.1 Introduction

The use of plants in the treatment and prevention of disease has been practiced for thousands of years. The continued use of medicinal plants in traditional cultures may be because of their perceived magical or symbolic importance. There may be no scientific proof of efficacy, but beliefs are passed verbally from one generation to the next generations by elders and shamans, and plant use is therefore considered to be safe and effective (Van Wyk & Wink, 2004; Chin, 2005; Van Wyk & Wink, 2015). Although many plants that are used medicinally are often perceived ‘natural’ and are believed to be safe to use, their ‘naturalness’ does not guarantee that they are harmless. Intensive research concerning phytochemical and pharmacological activities focused on many medicinal plants has shown some constituents of medicinal plants are potentially toxic, mutagenic, carcinogenic, clastogenic and teratogenic (Akintonwa et al., 2009; Kuete, 2014; Van Wyk & Wink, 2015; see Box 1 for definitions). This raises concerns about their safety and has implications for their use as medicine.

Box 1: Definitions

Precise definition * of terms used to describe specific forms of toxicity used in this chapter:

Toxic - a poisonous susbtance that can damage an organism.
Mutagenic - having the ability to cause a permanent change in an organism's genes.
Carcinogenic - used to refer to a substance that causes cancer.
Clastogenic - capable of causing breakage of chromosomes.
Teratogenic - used to refer to a substances that causes malformation of an embryo.

* Source of definitions of each term adapted from http://dictionary.cambridge.org

Several fern species that are used medicinally are known to damage animal and human health. It has been demonstrated that some fern species contain a variety of toxic secondary metabolites, of which the best known is Ptaquiloside (PTA). PTA is mutagenic, clastogenic and carcinogenic (Van der Hoeven et al., 1983; Niwa et al., 1983(a); Tomšík, 2014). This compound was first isolated in 1983 and was determined to be a norsesquiterpene glucoside of the illudane type containing a reactive cyclopropane ring, and readily soluble in water (Niwa et al., 1983(b)) (Figure 5.1). This molecule produces pterosin B under acidic conditions and an unstable dienone under alkaline conditions which is believed to be the ultimate carcinogen (Smith & Seawright, 1995; Vetter, 2009, 2011). Consumption of contaminated meat or milk from cattle grazing on plants that contain PTA and spore inhalation, as well as direct consumption of PTA-containing species as a food or herbal remedy are the potential sources of exposure to this poisonous and carcinogenic constituent (Alonso-Amelot, 1996; Simán et al., 2000; Alonso-Amelot & Avendaño, 2002).

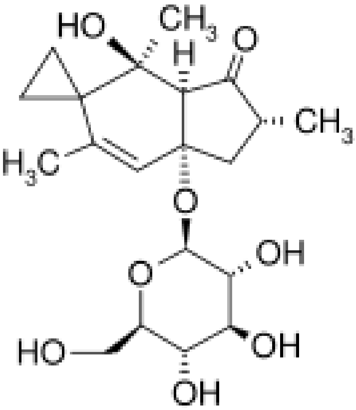


Figure 5.1 The chemical structure of ptaquiloside (PTA) (Image adapted from Wikipedia)

Heavy metal contamination in soil is an another toxicological risk to human health which has been continued to gain global attention. Several heavy metals are carcinogenic to humans. Exposure is via contaminated soil, the food chain (soil-plant-human or soil-plant-animal-human), or contaminated ground water (McLaughlin et al., 2000). Some heavy metals such as iron (Fe), zinc (Zn), chromium (Cr), copper (Cu), manganese (Mn) and Nickel (Ni) are essential to human health in small amounts (Chen et al., 1995), however, some others, such as lead (Pb), cadmium (Cd), chromium (Cr) and arsenic (As) become toxic in excess of certain concentrations (Singh & Kalamdhad, 2011).

Many species of plants are able to to absorb heavy metals from soils and accumulate them. A plant that is capable of taking up large quantities of contaminants from soils, so the level of heavy metals in the plant is higher than in the soils, is known as a hyperaccumulator. More precisely, a hyperaccumulator is defined as a plant that able to accumulate more than 100 mg/kg^{-1} Cd, 1,000 mg/kg^{-1} As/Co/Cu/Pb/Ni or 10,000 mg/kg^{-1} Mn/Zn in its above-ground parts (Brooks, 1998). To date, more than 400 Angiosperm species have been identified as heavy metal hyperaccumulators of As, Cd, Co, Cu, Mn, Ni, Pb, Sb, Se, Tl and Zn (Rascio & Navari-Izzo, 2011) of which about three-quarters are nickel (Ni) hyperaccumulators (Baker et al., 1999). The majority of plants that hyperaccumulate arsenic are ferns, and arsenic hyperaccumulation was first reported in *Pteris vittata* (Ma et al., 2001). Long term exposure to arsenic causes health problems, and arsenic poisoning (arsenicosis) is a major health problems in some countries especially in Asia (Tseng, 1999). Atherosclerosis, cancers in various organs, especially the skin, lung, bladder, liver, skin lesions, neurological effects, cardiovascular disease, pulmonary disease and diabetes mellitus have been well documented to be associated with arsenic exposure in some epidemiological studies (Rahman et al., 1999; Tseng, 1999; Smith et al., 2000). Mining activity is one of the important sources of exposure to arsenic or other heavy metals and abandoned mining areas are often colonized by natural vegetation or replanted. If the species are hyperaccumulators used as foods or medicine, this causes a serious health risk, especially for those people living on contaminated land and relying more on natural resources in their daily life.

5.1.1 Objectives

Several species in different families of ferns are toxic or carcinogenic because of PTA or arsenic hyperaccumulation. However, not all ferns contain PTA or are arsenic hyperaccumulators. Thus, the purpose of this chapter is to review the literature on the toxicological properties, either PTA or arsenic accumulation, in ferns, especially in medicinally-used species, and also to explore the phylogenetic distribution of these toxic elements using the robust megaphylogeny of the ferns (Chapter 2).

To understand evolution of toxic compounds, PTA and arsenic hyperaccumulation in fern species, it is necessary to know not only the character states in extant ferns but also in their ancestors. Ancestral state reconstruction is increasingly popular; by optimising the character states of living organisms onto phylogenies, it serves as a method for testing hypotheses about evolution of any character traits (Pagel et al., 2004; Goldberg & Igić, 2008; Litsios & Salamin, 2012). For example, if all members of a monophyletic group have PTA, it can be concluded that their last common ancestor most probably contained PTA. More importantly in this study, if there are missing data, the ancestral occurrence of PTA could be used to predict which ferns had these properties. With more than 9000 species of fern species, of which more than 400 species are used medicinally

(Chapter 3), predicting or recognised which clades or groups of ferns contain PTA or hyperaccumulate arsenic has important implications for health.

5.2 Materials and methods

5.2.1 Sourcing data describing toxicity

Toxicological literature for all fern species, including non-medicinal and medicinal species was made using Google Scholar with the following terms; “Ptaquiloside” and “arsenic accumulation” in combination with the term “ ferns” and “pteridophytes”.

5.2.2 Sourcing data describing use as food and medicine

All the species that have been screened for their PTA and arsenic accumulation were then matched with the medicinal use that have been recorded in Chapter 3. At the same time, searches were made for PTA-containing and arsenic hyperaccumulating species, to see whether they have been recorded as having food use. For the subset of the medicinal species and species that are used as food that were identified as toxic, the plant parts eaten or used were recorded.

5.2.3 Predicting the taxonomic distribution of toxicological and carcinogenical species

Initially, predictions for the species used were made based on their taxonomic distribution, but an explicit phylogenetic approach was also taken. The taxonomic approach was made by identifying all the species that are used medicinally which are known to contain PTA or arsenic hyperaccumulation. All the use reports and number of references for these species that cited their medicinal use were then counted, and the number of their congeners and confamilial species that were not screened for PTA or arsenic hyperaccumulation were calculated and recorded.

The maximum likelihood (ML) phylogeny of ferns reconstructed at the species level (Chapter 2) was used in this chapter. This phylogenetic tree represents a total of 3719 of fern taxa, including the outgroup was used for mapping the distribution of PTA compound and arsenic hyperaccumulator. Two maximum likelihood ancestral reconstructions, one of PTA and the other of arsenic hyperaccumulation, were undertaken using Mesquite (Maddison & Maddison, 2001). The characters of PTA and arsenic hyperaccumulation were coded as presence or absence characters and missing data were coded as “?”. The “Trace Character Over Tree” option was employed to reconstruct the ancestral state. The tree was then visualized as ‘Balls and Sticks” representation. In the case of arsenic hyperaccumulation, the investigation was limited to family Pteridaceae, since literature survey found there are no arsenic hyperaccumulators outside of this family.

5.3 Results and discussions

5.3.1 Known toxic species and their medicinal and food use

Studies of the toxicology of ferns have been published. The possibility of using ferns in phytoremediation has also resulted in several studies of heavy metal hyperaccumulation. This chapter reviews the toxicological and carcinogenical studies in ferns and distribution of arsenic hyperaccumulators. In general, assessments of the toxicity and carcinogenicity of fern species focusing on arsenic PTA and arsenic accumulation were very few, with only 1.5% of the global fern diversity investigated (Appendix 5.1 and Appendix 5.2).

Out of 58 species that have been investigated for the PTA, 41 have been recognized to contain a potent carcinogenic compound, ptaquiloside (PTA) or its analogue, pterosin B with 23 out of these having medicinal use and some of them serving as food in many countries (Table 5.1).

Table 5.1 List of toxic and carcinogenic species used for food or medicine. P indicates a species with PTA and A indicates an arsenic hyperaccumulating species. Country indicate the countries for which there are use reports either as a food (F) or medicine (M).

Species	**Toxin**	**Country and Use for food (F) and medicine (M)**	**Part used for food (F) or medicine (M)**
Aleuritopteris farinosa (Cheilanthes farinosa)	P	India (M), Ethiopia (M)	Rhizome (M), Frond (M)
Cibotium barometz	P	China (F), India (M), Malaysia (M), Japan (M), Philippines (M), Vietnam (M)	Rhizome (F, M), Frond (M)
Cyclosorus aridus (=Christella arida)	P	India (M), Papua New Guinea (M)	Leaves (M)
Cyclosorus dentatus (=Christella dentata)	P	DR Congo (M), Africa (F)	Young leaves (F), Whole plant (M)
Diplazium esculentum (=Callipteris esculenta)	P	Malaysia (F,M), Indonesia (M), China (M), Taiwan (M), Philippines (F), India (F,M), Bangladesh (M), Nepal (F, M), Nigeria (F), Africa (M)	Frond (F, M), Rhizome (M), Young leaves (F,M)
Dryopteris cochleata	P	Nepal (F), India (M)	Young leaves (F), Frond (M), Whole Plant (M)
Dryopteris juxtaposita	P	Pakistan (M)	Frond (M)
Histiopteris incisa	P	DR Congo (F), New Zealand (M)	Leaves (F), Frond (M)
Hypodematium crenatum	P	India (M)	Rhizome (M), Leaves (M)
Hypolepis punctata	P	India (M), China (M)	Frond (M), Leaves (M)
Hypolepis tenuifolia	P	Taiwan (M)	Frond (M)
Myriopteris myriophylla (=Cheilanthes myriophylla)	P	Peru (M)	Leaves (M)
Onychium japonicum	P	India (M), Taiwan (M), China (M)	Leaves (M), Spore (M
Pityrogramma calomelanos	P and A	Malaysia (M), India (M), Philippines (M), South Africa(M), Bangladesh (M), Nepal (M), Peru (M), Indonesia (M)	Frond (M), Rhizome (M)
Polystichum squarrosum	P	Nepal (F), India (M)	Rhizome (M), Young leaves (F)
Pteridium aquilinum	P	Japan (F), Korea (F), China (F,M), Angola (F),Cameroon (F), Dr Congo (F), Gabon (F), Madagascar (F), Nigeria (F), South Africa (F,M), Greece (M), India (M), Europe (M), Thailand (M), Papua New Guinea (M), Italy (M), Bosnia (M), Kosovo (M), Mexico (M), Portugal (M)	Frond (F, M), Crozier (F, M), Crozier (F,M), Aerial Part (M), Leaves (F,M)
Pteridium aquilinum var. *arachnoideum (=Pteridium arachnoideum)*	P	Argentina (M)	Frond (M)
Pteridium aquilinum var. *latiusculum*	P	Japan (F), China (F, M), Korea (F)	Frond (F), Rhizome (F), Crozier (M)
Pteridium esculentum	P	New Zealand (F, M), Australia (M)	Frond (M), Rhizome (F, M), Crozier (M)
Pteridium revolutum (=Pteridium aquilinum subsp. *wightianum)*	P	China (F), India (M)	Frond (F), Rhizome (F, M)
Pteris aspericaulis	P and A	Nepal (M)	Rhizome (M)
Pteris biaurita	P and A	Nepal (F,M), India (M)	Frond (M), Young Leave (F), Young leaves (F)
Pteris cretica	P and A	India (M), China (M), Pakistan (M)	Frond (M), Leaves (M), Whole Plant (M)
Pteris longifolia	A	China (M)	-
Pteris multifida	A	India, China (M), Vietnam (M)	Rhizome (M), Frond (M)
Pteris quadriaurita	P and A	Nepal (M), Africa (M), India (M)	Rhizomes (M), Leaves (M)
Pteris wallichiana	P	China (F, M), India (M), Papua New Guinea (M)	Frond (M), Leaves (F, M)
Pteris vittata	A	India (M)	Rhizome (M), Frond (M)

The majority of PTA-containing species are members of the genus *Pteridium*. PTA has been recorded in seven families (Athyriaeae, Cibotiaceae, Dennstaedtiaceae, Dryopteridaceae, Hypodematiaceae, Pteridaceae and Thelypteridaceae) but most of the investigated species that contain this carcinogenic compound are belong to the Dennstaedtiaceae or Pteridaceae (Appendix 5.1).

On the other hand, a range of fern species accumulate arsenic from the soil (Appendix 5.2). Of the 102 species identified as able to accumulate arsenic, 62% are found to have medicinal use and some of them are used as a vegetable. Several studies indicating species were hyperaccumulators were conducted at arsenic-contaminated sites (Ma et al., 2001; Visoottiviseth et al., 2002; Van et al., 2006). However, Zhao et al. (2002) found the same species exhibited hyperaccumulation from uncontaminated soils. It seems that the hyperaccumulating trait in ferns is constitutive, regardless of whether species grow in contaminated soils or not (Zhao et al., 2002; Wang et al., 2007; Bundschuh et al., 2014). Since the distribution of arsenic in soils may vary with soil type (Smith et al., 1998), only hyperaccumulator species are further discussed in detail in this study. Collection of the hyperacumulating species in any environment, not only polluted environments, poses potential risk of toxicity as this metal can affect biological activities as a carcinogen and mutagen. Arsenic hyperaccumulator species are limited to the family Pteridaceae. Almost all the hyperaccumulator species are placed in genus *Pteris*, however, some of the *Pteris* species also are not hyperaccumulators. Of 12 hyperaccumulating species, eight are used medicinally in different cultures (Table 5.1).

Twenty-six species are identified and still being used in traditional medicine in different cultures or customarily eaten in local areas of many countries, despite the fact that they contain PTA or are able to accumulate arsenic in large amounts (Table 5.1). Notably, five species; *Pityrogramma calamelonaos, Pteris aspericaulis, Pteris biaurita, Pteris cretica* and *Pteris quadriaurita* which belong to Pteridaceae not only contain PTA but are also arsenic hyperaccumulators. Even more alarming, all these five species have significant medicinal use despite known toxicological properties.

In those species studied to date, the toxic compound, either PTA or arsenic, can be found in all parts of plants especially in croziers and fronds (Rasmussen et al., 2003; Srivastava et al., 2006). Rasmussen et al. (2003) revealed that the highest concentrations of PTA in ferns were found in fronds while Ma et al. (2001) showed fronds accumulate more arsenic than other parts. Rhizomes and fronds are the plant parts most often served as a vegetable or used in traditional medicine (Table 5.1). Ferns are widespread, found in many enviroments, including in alkaline soils where the arsenic is more extractable and can therefore accumulate to increase risk among consumers.

Despite the reports of toxic compounds, the majority of species with known toxicity remain importance in ethnopharmacopeias. For instance, *Aleuritopteris farinosa* (*Cheilanthes farinosa*) which has been reported to contain PTA, also has been used in folk medicine in India to treat stomach ache and menstrual problems (Jain et al., 2005; Mannan et al., 2008; Benniamin, 2011). This species is rich in flavonoids (Erdtman et al., 1966) and has been shown to pose strong anti-inflammatory and anti-nociceptive properties due to the presence of active ingredients such as rutin, cinnamic acids, caffeic acid and its quinic acid derivative, chlorogenic acid (Yonathan et al., 2006). Nevertheless, the use of whole plant extracts from ferns as food or herbal remedies should be approached with caution and future research also needs to clarify which constituents could be separated for use.

Pteridium aquilinum, including subspecies and varieties, is a notable species in this survey, exemplifying significant medicinal and food use despite known toxicological properties (Table 5.1). The detrimental effects of this invasive species on livestock have been appreciated since the 18th century and it has since been shown to be a cause of thiamine deficiency, bladder tumours, and acute haemorrhagic disease in horses, pigs and in cattle and sheep respectively (Fenwick, 1989; Shahin et al., 1999). The presence of PTA, a reactive norsesquiterpenoid glucoside, has been demonstrated to contribute to the toxicity of bracken (Van der Hoeven et al., 1983; Yamada et al., 2007). The carcinogenic properties of PTA are not only responsible for animal disease, but believed to constitute a risk to human health either through environmental exposure or consumption. A close association was found between the consumption of bracken and human gastric cancer (Alonso-Amelot & Avendaño, 2002). More than 20 toxicological studies of *P. aquilinum* were identified in this survey, so the toxicity of *P. aquilinum* is well known. Despite a long history of research establishing a link between human cancers and consumption (Kamon & Hirayama, 1975; Marliere et al., 1999), many reports of medicinal use in this study are fairly contemporary; 8 of the 58 use reports were less than 5 years old, and 50 less than 10 years old (Chapter 3). Arguably, processing methods that detoxify the plants might be used; or, since carcinogens are slow-acting, the link between consumption and negative health effects may not be made. PTA is water soluble and decomposes under acidic or alkaline conditions (Hirono & Weisburger, 1986). In order to remove the bitter taste for food use, *P. aquilinum* is soaked in water for a few hours prior to cooking, greatly reducing the level of PTA. However, we find 17 reports of decoction of *P. aquilinum* used medicinally (Chapter 3), where decoction might be expected to maximize expose to PTA. Just as use of *Aristolochia indica* is associated with health risk, and has been the focus of ethnobotanical and metabolomic research to assess public health risks (Michl et al., 2013), more research on *P. aquilinum* including subspecies and varieties should be also prioritized. If the risk of exposure to PTA is proven, public health interventions are needed.

Diplazium esculentum is one of the examples of an edible fern that is popular and commonly consumed in Asia (Table 5.1). The young frond of this species was found to contain PTA (Somvanshi et al., 2006) and an earlier study by Kumar (2004) discovered that this species caused mild pathologic effects in rats and induced mortality in guinea pigs. In a more recent study, Roy and Chaudhuri (2015) discovered this species may also induce immune dysfunction by altering cytokine balance in mice. However, a study by Junejo et al. (2015) found that the leaf extract of this species is non-toxic in their acute oral toxicity test and safe to be used for medicinal purposes. Even though the findings from previous studies are contradictory, further studies are still needed, so the public health risk associated with eating this fern as a vegetable can be determined.

Another notable species, *Pteris vittata*, is well-studied due to its remarkable capacity for arsenic hyperaccumulation. Even though this species has received extensive attention since discovery as the first arsenic hyperaccumulator in ferns (Ma et al. 2001), it is still used as traditional medicine. More than ten use reports in seven different studies, particularly in India, have been recorded (Chapter 3) clearly showing that *Pteris vittata* has been extensively used to treat several ailments. Abou-Arab and Abou Donia (2000) investigated the heavy metal levels during processing of Egyptian medicinal plants and found that boiling the plant in water leads to the extraction of higher amounts of the metal from the plant than immersing it in the hot water. One unanticipated finding here is that remedies using *Pteris vittata* are mostly prepared as decoctions. This processing method might concentrates heavy metals and might ultimately lead to the death of the people using this remedy.

Even though this study emphasizes hyperaccumulator species, non-hyperaccummulator species should never be overlooked as many fern species that able to accumulate arsenic also are used as traditional medicine and as a vegetable. Exposure to arsenic even at low concentration may also cause a variety of human health problems if the comsumption was on long term basis (Kaltreider et al., 2001).

With only 1.5% of all fern species screened so far, and only 1% of the species with medicinal use investigated, further screening is urgently needed. Until resources are directed to this end, it is necessary to predict the extent of the problem. In terms of predicting the public health risk from a species that is not screened yet, three approaches were taken here; 1) Taxonomic distribution of known toxic species used medicinally. 2) Using phylogeny and ancestral reconstruction to explore and trace evolution of toxicity in ferns and 3) Mapping the distribution of toxic species at family level using phylogeny.

5.3.2 Predicting health risk using taxonomic distribution

As several species in the fern group have been shown to have toxic and carcinogenic properties, their close relatives might be also have the potential to have these toxic properties. Thus, understanding the taxonomic distribution of these species could provide a basis to estimate risk and at the same time to help to identify which groups should be the focus of further screening. Table 5.2 shows that many species in the same genera and families as PTA-containing species are used medicinally, but have not been screened for PTA.

Table 5.2 Many PTA-containing species are used medicinally. Many of their congeners and confamilial species are used, but not screened for PTA. If PTA follows a taxonomic distribution, the exposure of PTA through medicinal fern use could be more significant than suggested by the known use of 23 PTA-containing species.

Medicinal genera known to contain PTA	**Species, and number of use reports (number of publications source reports drawn)**	**Number of congeneric species with medicinal use for which PTA status is unknown**
Athyriaceae (10 species with medicinal use and unknown PTA status)		
Diplazium	*D. esculentum* 23(12)	6
Cibotaceae (no confamilial with medicinal use)		
Cibotium	*C. barometz* 24(9)	0
Dennstaedtiaceae (4 species with medicinal use and unknown PTA status)		
Pteridium	*P. aquilinum* 58(21)	0
	P. aquilinum var. *arachnoideum* 1(1)	
	P. aquilinum var. *latiusculum* 2(1)	
	P. esculentum 15 (4)	
	P. revolutum 6(1)	
Histiopteris	*H. incisa* 1(1)	0
Hypolepis	*H. punctata* 3(2)	1
	H. tenuifolia 1(1)	
Dryopteridaceae (44 species with medicinal use and unknown PTA status)		
Dryopteris	*D. cochleata* 20(7);	22
	D. juxtaposita 1(1)	
Polystichum	*P. squarrosum* 1(1)	7
Hypodematiaceae (no species with medicinal use and unknown PTA status)		
Hypodematium	*H. crenatum* 5(3)	0
Pteridaceae (83 species with medicinal use and unknown PTA status)		
Aleuritopteris	*A. farinosa* 10 (5)	5
Myriopteris	*M. myriophylla* 1(1)	1
Onychium	*O. japonicum* 3(2)	1
Pityrogramma	*P. calomelanos* 42(13)	1
Pteris	*P. aspericaulis* 4 (1)	12
	P. biaurita 4 (3)	
	P. cretica 5(5)	
	P. quadriaurita 5(5)	
	P. wallichiana 7(4)	
Thelypteridaceae (19 species with medicinal use and unknown PTA status)		
Cyclosorus	*C. dentatus* 3(1)	12
	C. aridus 3(2)	

Meanwhile Table 5.3 shows that many species in Pteridaeae, particularly in genus *Pteris* are used medicinally, but not been screened for arsenic hyperaccumulation. If arsenic hyperccumulation follows a taxonomic distribution, the exposure of arsenic hyperaccumulation through medicinal fern use could be more significant than suggested by the known use of 8 arsenic hyperaccumulating species.

Table 5.3 List of hyperaccumulating species that are used medicinally.

Medicinal genera known as arsenic hyperaccumulators	**Species, and number of use reports (number of publications source reports drawn)**	**Number of congeneric species with medicinal use for which status arsenic hyperaccumulator is unknown**
Pteridaceae (78 species with medicinal use and unknown hyperaccumulating status)		
Pityrogramma	*P. calomelanos* 42(13)	1
Pteris	*P. aspericaulis* 4 (1)	
	P. biaurita 4 (3)	
	P. cretica 5(5)	
	P. longifolia 1(1)	10
	P. quadriaurita 5 (5)	
	P. multifida 13(5)	
	P. vittata 13(7)	

Overall, Tables 5.2 and 5.3 show that of the 14 genera known to include species PTA or hyperaccumulating arsenic, three genera, *Dryopteris*, *Pteris* and *Cyclosorus* include more than ten species that are toxic and used medicinally. These must be priority genera for screening, and their use raises concern. Other genera of concern (≥ five medicinal species in a genus know to include toxic species) are *Diplazium*, *Polystichum* and *Aleuritopteris*. The exposure of PTA or arsenic hyperacumulator through the medicinal fern use could be more significant if unscreened medicinal species have the same toxic compound. For instance, more than 75 species that are used medicinally in family Pteridaceae and more than 40 medicinal species in Dryopteridaceae have been not screened yet for their toxicological and carcinogenical properties.

There are limitations to the taxonomic approach taken here. One is that considering genera and family units, and what is known about them in isolation, means information we have on how genera or families are related is not taken into account. Another limitation is that it is difficult to take into account information about the absence of toxicity. The phylogenetic approach explicitly considers relatedness which is usable for making predictions and eventually able to identify species that do not contain PTA or arsenic hyperaccumulation.

5.3.3 Predicting health risk using phylogenetic approach

Firstly, distribution of toxic properties was investigated using ancestral reconstruction. Trait evolution can be examined by using ancestral reconstruction by mapping particular traits onto a molecular phylogeny (Simpson, 2010). As several fern species have been identified in different genera and families, this approached was used to predict and investigate any patterns of distribution of PTA-containing or arsenic hyperaccumulating species in related families that are not uncovered by taxonomic approach.

Our analyses showed almost all of the ancestral character states of deep nodes representing the most recent common ancestor three or more species are equivocal for PTA and arsenic hyperaccumulation. A possible explanation for these results may be the high proportion of missing and limited data. Therefore, further work to include more screening of fern species for these toxic properties would very valuable.

Since all arsenic hyperaccumulator species belong to family Pteridaceae, thus, it would interesting to explore the phylogenetic prediction between the hyperaccumulating and non-hyperaccumulating species in this family. We predict that more arsenic hyperaccumulating species will be found, however, equivocal nodes seem the norm in this phylogeny too, since only a small proportion of species have been tested for arsenic accumulation to date (Appendix 5.3).

Overall, based on the ancestral reconstruction approach, most of the traits were insufficiently known to predict clades with toxic properties. However, the data tentatively suggest these traits might not be conserved through fern evolution. Further investigation for many species of ferns is needed in order to point clearly to where we should look for more toxic species. Furthermore, further screening can be focused on the close relatives PTA containing or hyperaccumulating species.

Because ancestral reconstruction is influenced by the large proportion of missing data, we also mapped presence of toxic properties onto the phylogeny at family level. The phylogenetic distribution of the properties is summarised in Figure 5.2. The phylogenetic relationships within the Polypodiales clade are of broader interest, especially due to the presence of several toxic and carcinogenic species in this clade.

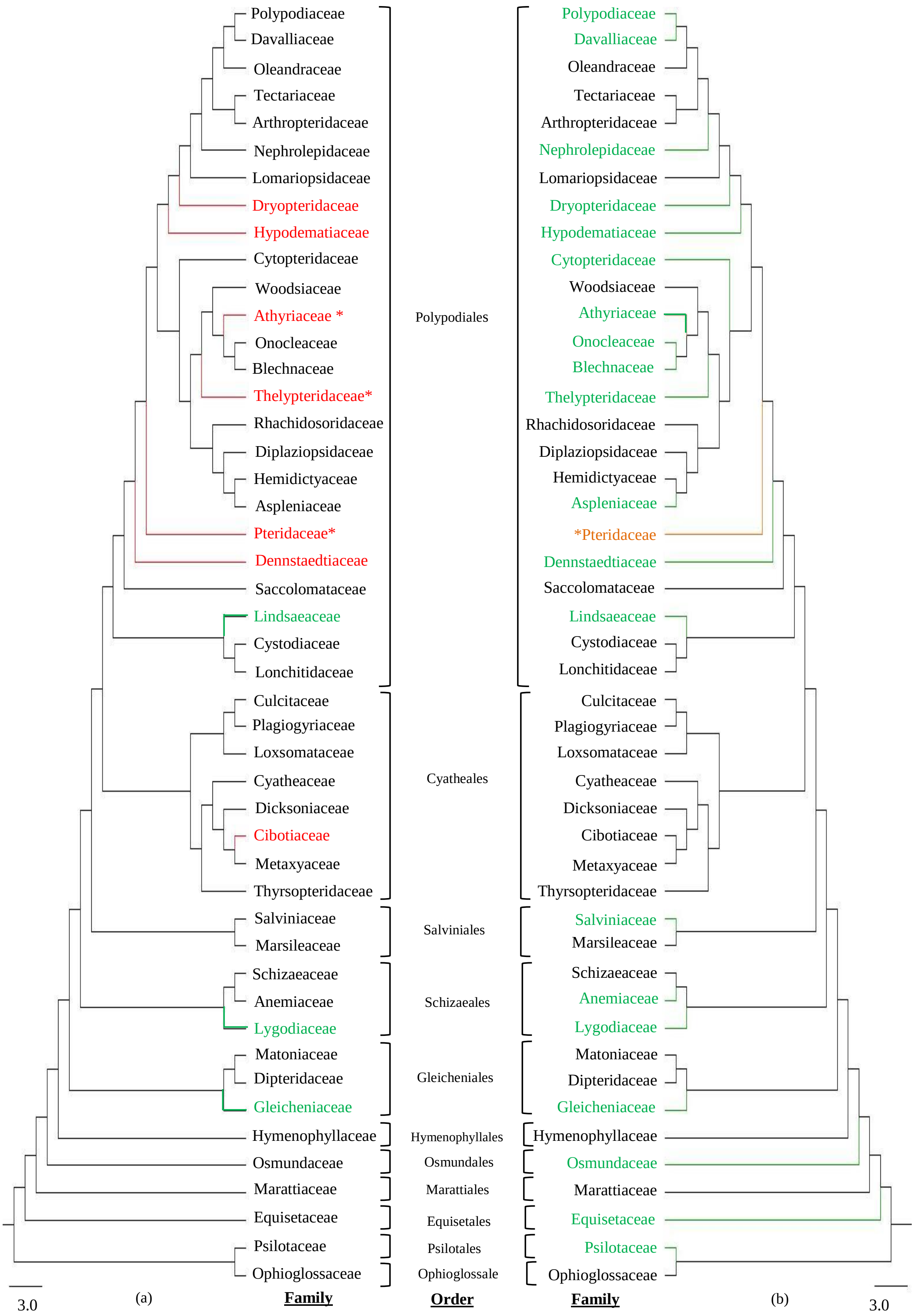


Figure 5.2 Phylogenetic tree for ferns showing the distribution of families posing risk to health. (a) PTA. Families with species containing PTA are shown in red and families in green have been shown to include species that do not contain PTA (b) Arsenic hyperaccumulation. Hyperaccumulating species belong only to the Pteridaceae shown in brown. Several families in green have been shown to include species which do not hyperaccumulate arsenic. * indicate families that include both PTA-containing species and non-PTA-containing species along with hyperaccumulating and non-hyperaccumulating species.

As shown in Figure 5.2 (a), it is apparent that PTA-containing species are all located in the core leptosporangiate ferns (Cyatheales and Polypodiales). Six families containing PTA are concentrated in Polypodiales clade where some of the families are closely related to each other suggesting more hyperaccumulator species might occur in this clade. In contrast, arsenic hyperaccumulator species are more concentrated in the Pteridaceae family within the order Polypodiales, however, other families that are closely related and other families in the same order do not exhibit hyperaccumulation and neither do primitive ferns such as Psilotales and Equisetales (Figure 5.2 (b)). These patterns suggest that these toxic and carcinogenic properties are phylogenetically conserved to some extent in ferns. A similar phylogenetic pattern in heavy metal hyperaccumulation including arsenic is seen in Brassicaceae, (Krämer, 2010). Interestingly, Pteridaceae are unique by having toxic and carcinogenic properties from both PTA and arsenic hyperaccumulation. However, due to time constraints on laboratory work, toxicological screening was not carried out in this study. Therefore, further investigation and experiment on toxicity tests should be prioritized for this family.

Several questions remain unanswered at present. It seems that PTA and arsenic hyperaccumulation arrived relatively late in terms of fern evolution as these characters are not exhibited by some exemplars of primitive ferns. In order to develop full picture of the evolutionary origin of these toxicities in ferns, screening of more primitive species is necessary. Despite toxicity caused by PTA and arsenic hyperaccumulation being absent in earlier-evolving, these linegaes lacking PTA and arsenic hyperaccumulation are not necessarily safe. For instance, *Equisetum* (Horsetail) contains thiaminases, enzymes that can destroy vitamin B_1 and also contain high levels of silicates which can produce digestive problems when used in the long term (Pohl, 1955; Cullina, 2008; Sandhu et al., 2010). This enzyme is also present in bracken and would occur in other fern species.

5.4 Conclusion

The presence of unacceptably toxic compounds in medicinal or edible plants is a concern because of immediate health problems and genetic damage which can be heritable. This study shows a number of ferns have been used in traditional medicine or serve as food in different parts of the globe and many of them have been shown to have to these toxic and carcinogenic properties. Surprisingly, some of them are well known to be toxic. Traditional use despite scientific demonstration of toxicity is known for other plants. For example, *Aristolochia indica* consumption leads to urinary tract cancer and is still widely available in Bangladeshi herbal markets where those buying it are unaware of its toxicity (Michl et al., 2013). Other examples, include *Poliomintha longiflora* (Mexican Oregano) known to contain acutely toxic compounds (Deciga-Campos et al., 2007) and still widely used for culinary and medicinal purposes, while 38 species in Traditional Chinese Medicine (TCM) and numerous indigenous plants in North America have been reported to contain toxic pyrrolizidine alkaloids (PAs) which could be responsible for initiating a range of slowly developing chronic diseases (Roeder, 2002; Roeder et al., 2015). In this context, the risk of these toxic properties in food or herbal remedies for humans must not be ignored. Further assessment into biological and toxicological properties of less well-known species is crucial, in order to assess the real potential of these medicinal species and also to ascertain their safety for human use. Upon confirmation of the species toxicity, awareness needs to be raised not only by scientific communities, but also through intervention by public health authorities**.** The findings here also indicate that studies about the health risks associated with fern species are limited and provides a framework for the further exploration of toxicity in ferns. The scale of the public health problem associated with PTA or arsenic hyperaccumulation might be a considerable and further investigation and experimentation into toxicological properties especially in medicinally-used species are strongly recommended.

Chapter 6. General conclusion and future perspectives

This thesis, “Molecular Systematic Studies of Extant Ferns (Monilophytes) with Emphasis on Medicinal Use of Ferns”, highlights the power of the phylogeny of ferns and meta-analysis of the ethnobotanical data to explore the evolutionary patterns amongst fern species that are used medicinally at different ranges and scales as well as the access of the distribution of toxic in medicinally-used fern species.

6.1 Challenges faced in carrying out this work

The biggest challenges faced in this study was the existence of several names for the same taxon. This occurred in part because of the lack of dissemination of taxonomic information in the past, as a result of limited communication facilities. This problem existed when two or more researchers from different countries were not aware of the same taxon that they described is actually of the same species (Mohamed Said et al., 1999). The phenomenon of synonymy has made it difficult to assemble the uses of particular species. Different names have been used for the same species and thus it has been difficult to collate information about the ethnobotanical uses of ferns. The lack of an up to date, authoritative synonymized checklist is a significant barrier to synthesize knowledge on plant uses from the diverse and fragmented data sources available (Lukhoba et al., 2006). Major challenges in this study began when sequence data were gathered from Genbank to reconstruct the supermatrix. Compilation of a large number of data from Genbank poses a great challenge due to the source organism nomenclature and taxonomic classifications in Genbank being inadequately curated, so changes in fern familial and generic classification are inadequately reflected (Christenhusz et al., 2011; Christenhusz & Chase, 2014). Furthermore, some species name in Genbank were misspelled. In order to validate the current accepted name of the species and also to resolve any related taxonomic problems, the spelling and nomenclature of all species were checked manually following recent studies. Since all the taxonomy and nomenclature for each chapter in this study is linked to each other, standardization of nomenclature for all chapters is very important to provide a consistent view of the data available in a particular field. At the species level, it is desirable to use a single internationally recognized data portal of plant taxonomic information such as TROPICOS (2016), nevertheless recognizing nomenclature can still be imperfect especially where names do not appear in the first source. For instance, *Microsorum grossum* is not listed in TROPICOS (2016), thus the updated nomenclature of this species was checked in other sources such as Catalogue of Life (2016) and World Ferns (Hassler & Schmitt, 2015). Even though the nomenclature updated in this study might not be completely perfect, it has been improved in many aspects from the previous fern megaphylogeny (Lehtonen, 2011). In the face of these challenges, it is important to be able to interact with a taxonomist specialist, but ultimately the ease of the work depends on the quality and completeness of the databases.

We managed to reconstruct the most comprehensive molecular phylogeny for ferns to date, however, we still lack sequence data of many important species, especially those that have been used in traditional medicine, and this may limit its utility in some contexts. Therefore, additional sampling is required for medicinal species that are not available in Genbank. This global phylogenetic study posing other challenges in the data assembly due to the limited field opportunity and the difficulty of getting particular herbarium materials from different part of the globe.

The study is also limited by the small proportion of medicinal data for fern species in ethnobotanical surveys due to lack of ethnobotanical studies focusing on ferns compared to Angiosperms (Benjamin & Manickam, 2007; Rout et al., 2009). This resulted in more time used to identify fern species in general ethnobotanical literature.

There are several challenges in using the community phylogenetic software, Phylocom v4.1, (Webb et al., 2008) which are mostly due to the documentation or user guide not being user friendly. Some commands or features stated in the documentation work only in an Apple IOS environment, but not in Microsoft Windows. Several errors were found during software execution, most which were overcome by changing and structuring input files. Inability to address this software bug deterministically leads to unsafe speculation in software execution.

6.2 How robust is the work

This study uses published data, and as such depends on the availability of quality data. Three elements were the basis of the study described here, the phylogeny, the use database and the database of toxicity. Together, these three shed light on the extent of fern use, patterns of fern use and possible health risks of fern use. However, deficiencies in each of the three elements brought together here should be considered, as possibly limiting the interpretation of the analyses.

Our large-scale fern tree of life demonstrated that it was possible to estimate a well-corroborated, taxon rich phylogeny directly from analyses of a supermatrix. Although not all nodes were well supported and some relationships relatively poorly resolved, this updated fern megaphylogeny could be used as the basis for revised fern taxonomy and able to render a more a clear generic delimitation in several families. With the advent of next-generation sequencing (NGS) technologies in recent years, improvement in high-throughput, time-saving and affordability has helped resolve most of the problematic relationships in flowering plants, thus, resolving remaining problematic deep level questions in ferns in the near future would much easier (Schuster, 2007; Soltis et al., 2010; McCormack et al., 2013). Nevertheless, since the tree in this study has sufficiently dense taxonomic sampling at species level, and I have shown it serves as a valuable framework for comparative research. It may be also useful for other practical applications in many areas of biology (Heath et al., 2008; Lu et al., 2014).

Ethnobotanic data are scattered in various publications in different languages with limited accessibility and application, and some of the medicinal plants databases are written in various languages especially sources from China or other countries that do not converse in English (Ningthoujam et al., 2012). Therefore, there are likely to be some geographical biases in the data collected here. Another issue that might undermix the robustness of the ethnobotanical database is the incorrect identification of species recorded as used. My study revealed 54% of the use data is not associated with herbarium specimens, and field identification by non-experts may be inaccurate. Voucher specimen for herbarium collection are an essential components of any research project by providing valuable tools for assisting researchers in confirming the identity of an unknown plant (Culley, 2013). Therefore, the absence of voucher specimens of a research project, may influence the quality of data analyses as there is no proof of the species identity. Data quality in ethnobotanical meta-analysis is a recognized problem (Albuquerque & Medeiros, 2012). We might expect the identification associated with toxicological studies to be more reliable. However, polymorphism in chemistry is well known, so one weakness of the use of published data is that it might report false negative where the compound tested for is just below recorded threshold.

6.3 Priorities for future work

Extensive literature review in this study indicates that fern species are used in traditional medicine. However, not many ethonobotanical studies include fern species and they have not been recorded as having medicinal uses as frequently as Angiosperms. There are several factors contributing to the low representation of fern species in traditional pharmacopoeias, but fern use is revealed if it is the explicit focus of ethnobotanical survey (dos Santos Reinaldo et al., 2015). Thus, ethnobotanical survey with the most appropriate data collection method focusing on ferns is crucial and should be conducted in the future. This step is important to update the inventory of existing ferns that are used as traditional medicine with respect to the number of ferns yet to be explored. Furthermore, considering the rapid destruction of natural habitats and the ongoing loss of biodiversity, especially in tropic regions (Achard et al., 2002; Sodhi et al., 2004; Hansen et al., 2013), unrecorded species potentially become extinct before their medicinal potential can be sufficiently explored. Ethnobotanical studies have indicated other uses of ferns (Keller et al., 2011), therefore, the approach taken in this thesis (Chapter 4) need not be restricted to the study of medicinal of use but also can be applied to the investigation of patterns of ferns used in food, as pesticide, fertilizer and material for construction.

The wide use of fern species in traditional medicine may contribute to the health of millions of people throughout the globe as well as to livelihoods through trade of herbal products (Hoareau & DaSilva, 1999). However, although some species have proven to be associated with health hazards (Chan, 2003; Haq, 2004; George, 2011), toxicological information on ferns is still limited. Medicinal plants contain a complex mixture of hundreds chemical compounds (Ross, 2007), thus it is difficult to map all the complex interactions and synergies that might be taking place between all the numerous chemicals found in plants. In order to access the real risk and potential of ferns that are used in traditional medicine, further research is needed in preparation of pure compounds (Sasidharan et al., 2011; Duarte & Rai, 2015) as well as the assessment of their toxicity (Coghlan et al., 2012; Ifeoma & Oluwakanyinsola, 2013). Emphasis on extensive screening programmes for biological activities and toxicity not only provide complete understanding of the effects of these plants to human health, but may also contribute to the discovery of novel compounds for pharmaceutical industry (Fabricant & Farnsworth, 2001; Fennell et al., 2004; Balunas & Kinghorn, 2005; Mitra et al., 2016). Awareness in public health education also should be intensified and scientific findings with regards to the possibility of plants containing toxic, heavy metal contamination or undeclared drugs compounds should be publicized (Street et al., 2008). New toxicological data from the toxicological investigation of medicinally-used ferns could provide new evolutionary insights into the phylogenetic distribution of toxic species indicating the extent to which the phylogeny can predict the health risks associated with the current use ferns.

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Appendices

The following Appendices are given on the CD attached at the end of this thesis:

Appendix 2.1

Summary of fern families and genera based on Christenhusz et al. (2011) with changes based on recent studies (Christenhusz & Schneider, 2011; Rothfels et al., 2012b; Liu et al., 2013). Taxonomic changes at generic level since 2011 are listed in Appendix 2.2. The two new families since 2011 are Hemidictyaceae (1 genus; 1 species (Christenhusz & Schneider, 2011)) and Arthropteridaceae (1 genus, 15 species (Liu et al., 2013)).

Appendix 2.2

Taxonomic sampling for fern phylogenic reconstruction for this this study. Species are arranged under families and genera recognized based on based on Christenhusz et al. (2011) with changes based on recent studies (Christenhusz & Schneider, 2011; Rothfels et al., 2012b; Liu et al., 2013). Blank cells referring to the sequence not available; an asterisk (*) indicates newly generated sequences.

Appendix 2.3

Summary of changes in generic level classification since Christenhusz et al. (2011) based on recent studies.

Appendix 2.4

DNA extraction procedure for silica-gel dried material.

Dataset F1

Concatenated supermatrix in Nexus-format.

Dataset F2

The ML tree with bootstrap support values in Nexus-format (file can be opened for example with Fig Tree).

Appendix 3.1

Data set, describing all use reports and associated information including therapeutic application, plant part used, mode of preparation and application and vernacular name.

Appendix 3.2

List of species that are used medicinally with references citing biological or pharmacological activities and toxicological studies.

Appendix 4.1

List of species that were added manually as polytomies on the phylogeny.

Appendix 4.2

R script for species pruning in phylogeny.

Appendix 4.3

Phylogenetic structure of all taxonomic levels of medicinal use of ferns.

Appendix 5.1 and Appendix 5.2

List of species with assessments of the toxicity and carcinogenicity focusing on arsenic PTA and arsenic accumulation.

Appendix 5.3

Ancestral reconstruction for arsenic in Pteridaceae.