博士論文

Phylogenetic studies on *Hymenophyllum* subgenus *Mecodium* C. Presl ex Copel. (Hymenophyllaceae) with special focus on the species boundaries of the *Hymenophyllum polyanthos* (Sw.) Sw. complex

（コケシノブ科*Hymenophyllum*属*Mecodium*亜属におけるホソバコケシノブ種複合体の系統・種分化の研究）

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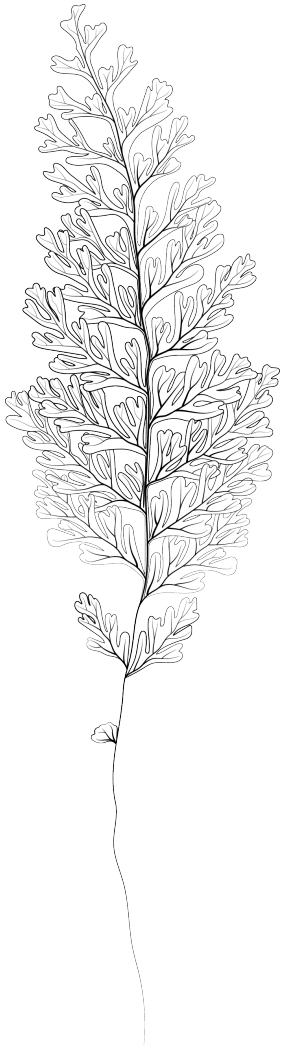

"Ferns delighted me with their curlicues, their croziers, their Victorian quality (not unlike the frilled antimacassars and lacy curtains in our house.) But at a deeper level, they filled me with wonder because they were of such ancient origin... My sense of a prehistoric world, of immense spans of time, was first stimulated by ferns and fossil ferns..."

[Oaxaca Journal – Oliver Sacks]

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General Introduction

Ferns are a traditionally recognized group which circumscription was widely discussed by taxonomists during decades. Recently, the term "ferns and its allies" corresponds to the most applied definition for these plants, representing the divisions Lycopodiophyta (i.e. *Lycopodium*, *Selaginella* and *Isoetes*) and Monilophyta. The monilophytes corresponds to a monophyletic group (Pryer *et al*. 2004) and comprises the whisky ferns (Psilotaceae), the moonworts (Ophioglossaceae), the horsetails (Equisetaceae), the marattioid ferns (Marattiaceae) and the leptosporangiate ferns (Polypodiopsida). Represented by more than 9000 species and more than 260 genera, the leptosporangiate ferns are the most diverse lineage of vascular plants after the angiosperms (Schuettpelz & Pryer 2007).

The leptosporangiate ferns are strongly supported as a monophyletic group by molecular phylogenetic analysis (Pryer *et al*. 2001) and are characterized by ferns which sporangia is originated from a single epidermal cell. The group is diverse in form and habit, and includes several taxonomic lineages within it. Phylogenetic and taxonomic

assessments regarding this group have taken place in the last decades, bringing new insights on the evolutionary history of ferns.

Several efforts towards revealing the relation between the major lineages of lepstoporangiate ferns were made (Hasebe *et al.* 1993, 1994, 1995, Pryer *et al.* 2001, 2004, Schneider *et al*. 2004a, Schuettpelz *et al*. 2006, Schuettpelz & Pryer 2007, Wolf 1997) and resulted in a general phylogenetic framework for the families' limits, which can be refereed to with a considerable accuracy. Nevertheless, infra-familiar categories' taxonomy in ferns still remains as not completely understood, corresponding to a target research topic in the field.

Studies targeting genera and species boundaries within ferns are still in progress, and much is still to be discovered. The main reasons for that vary from technical difficulties to biological reasons. One critical factor is the lack of primers and markers specifically described and developed for ferns' molecular studies. At the moment, the most applied markers in ferns' phylogenetic studies are the *rbcL*, *atpB*, *rps4-trnS* and *matK* (Ebihara *et al*. 2006, Hennequin *et al*. 2006, Kreier & Schneider 2006, Kuo *et al.* 2011, Lehtonen 2011, Pryer *et al*. 2001, Rothfels *et al*. 2012, Sano *et al*.

2000, Schuettpelz & Pryer 2007, Schuettpelz *et al*. 2006, Smith *et al*. 2006, 2008, Schneider *et al*. 2004, 2013). Still, as new investigations targeting different lineages of ferns are made, the applicability and limits of each of these markers become clear. Still, the progress of phylogenetic investigations within fern groups is very important, leading to interesting evolutionary discussions.

This research targets a subcosmopolitan species of filmy ferns called *Hymenophyllum polyanthos,* which belongs to *Hymenophyllum* subgenus *Mecodium*. The biology and distribution, as well some evidences from previous studies, indicate that the species may correspond to a non-monophyletic lineage. Through collection of several specimens of the subgenus *Mecodium*, this research compares DNA sequences of different markers to distributional and morphological traits, aiming to solve the question regarding the monophyly of the complex.

Chapter 1 brings a historical summary of the classification system changes within Hymenophyllaceae, with focus on the generic and subgeneric levels. Taking as a starting point the bigeneric division between *Hymenophyllum* and *Trichomanes*, the chapter gathers and compares different classification systems within these genera,

arriving at the discussions regarding the circumscription of *Mecodium*. This discussion is essential for understanding the questions about the monophyly of *Hymenophyllum polyanthos*, discussed in the next chapter.

Chapter 2 brings, then, the main discussions of the thesis, corresponding to the polyphyly of *H. polyanthos*. The species is subcosmopolitan, distributed through tropical and subtropical regions of the globe. It is included in subgenus *Mecodium*, which was circumscribed recently by Ebihara *et al*. (2006) and Hennequin *et al*. (2006) based on molecular data. At the time of the circumscription, evidences that pointed towards the polyphyly of *H. polyanthos* were also obtained, but further investigations were still necessary to achieve concrete conclusions regarding the species. This chapter focuses on this matter and brings a clarification to the evolutionary history behind the evidences found by Hennequin *et al*. (2006). Based on several molecular markers investigated from different specimens of *Mecodium*, this chapter concludes that *H. polyanthos* corresponds to a polyphyletic species group.

Chapter 3, then, investigates the possible reasons for the polyphyletic condition of *H. polyanthos*. In order to address the occurrence of gene introgressions within the complex, the nuclear marker *LEAFY* is applied on a phylogenetic analysis, and the result is compared to the trees obtained in chapter 2. From this comparison, possible explanations for the diversity within *Hymenophyllum* subgenus *Mecodium* and the *H. polyanthos* complex are explored.

Based on the previous chapters discussions, chapter 4 brings morphological and anatomical comparisons between the samples analyzed, taking as a premise the phylogenetic groups obtained in those chapters. Seven parameters from the leaves and three anatomical parameters from the rhizomes are analytically compared to the phylogenetic groups in this chapter.

Finally, all of the results of the previous chapters are merged in chapter 5 into a novel taxonomic treatment for *Hymenophyllum* subgenus *Mecodium*. As a result, traits of the leaves support the suggestion of two new sections within the subgenus *Mecodium*: section *Cuneatae*, represented by plants from Malesia, Australia, Pacific

islands and South America; and section *Mecodium*, represented by plants from Africa, Asia and Neotropics, including the type clade for *H. polyanthos*.

Chapter 1 – Taxonomic background of *Hymenophyllum* subgenus *Mecodium* C. Presl ex Copel.

INTRODUCTION

The Hymenophyllaceae family is a subcosmopolitan family of ferns represented by about 600 species, and corresponding to one of the largest families of leptosporangiate ferns (Iwatsuki, 1990). Known as the filmy ferns, this is a family of epiphytic saxicolous or terrestrial ferns of small to medium size. The rhizome is creeping, bearing a simplified protostelic vascular system. Leaves' petioles are non-articulated and can be present or not. The lamina can be simple, pinnate, flabellate, digitate, dichotomous or irregularly divided. The blade is composed by one to 4 layers of cells, and stomata or intercellular spaces are not present. Sori are terminal to the veins, solitary and covered by a cup or tube-shaped indusium. Sporangia occur inside of the indusium, attached to receptacles terminal to the leaf veins. Spores are globose-trilete, tetrahedral, bearing chloroplasts and short-lived (Iwatsuki 1990).

The delicate leaves composed by one to few layers of cells are the main distinguishing trait of the family, conferring a filmy appearance to these plants. Usually

occurring in shadowy and moisty environments (such as deep rain forests), these plants are very susceptible to desiccation. As observed by Shreve (1911), due to their physical properties, Hymenophyllaceae individuals are confined to places where specific abiotic conditions are attained: *i.e.* over rocks near waterfalls or by the riverside; over fallen trunks associated to mosses or other bryophytes; and in areas where the temperature and humidity are more or less constant during the day. These conditions are usually achieved in mountainous rain or misty forests, at higher altitudes (over 1200m). Plants that occur at lower altitudes (*e.g.* around 900m) are usually confined to deep forests in ravines, where the humidity and environmental temperature are more constant.

The family Hymenophyllaceae is a major family within the ferns and unique in its appearance. Even restricted to shadowy and moisty areas, the family represents are scattered around the globe, being found not only in tropical, but also in sub temperate areas. The species richness is also notorious, being overcome only by Cyatheaceae (ca. 600+ spp), Dryopteridaceae (ca. 1700 spp), Pteridaceae (ca. 950 spp), Aspleniaceae (ca. 700spp) and Polypodiaceae (ca. 1200 spp) (Smith *& al*. 2006). Base chromosome number also varies within the family, but the lowest counts within ferns (x=11) are

reported here (Smith *& al.*2006). The simplification of leaves and rhizome structure is intriguing, especially due to the restriction of environments in which these plants occur. Nevertheless, asexual gametophytes are reported in regions beyond the distribution range, reaching temperate regions in the North America (Duffy *& al.* 2015, Taylor 1967).

TAXONOMIC CLASSIFICATION OF THE FAMILY

Presl, van de Bosch, Mettenius, Prantl, Christensen, Copeland and Giesenhagen were important scientists that contributed to the taxonomy of Hymenophyllaceae during the 20th century (Morton, 1968). Most of the systems proposed by these scientists divide the species into two genera: *Hymenophyllum* Sm. and *Tricomanes* L.. The main feature distinguishing these two traditional genera is the conformation of the sori sinangium, which is bivalvate in *Hymenophyllum* and cup-shaped in *Trichomanes*.

Copeland system was not very well accepted by other authors of that time, such as Christensen, Alston, Madame Tardieu-Blot, Schelpe, Sledge, Tryon and Holttum (Morton, 1968). The main reason for this was that Copeland proposed the

division of the family in several groups, some of them raised to the category of subgenus and genus later. The circumscription of so many groups was not recognized as practical by Morton and other scientists of the time, specially because Copeland based the description of many of his groups on subjective traits, such as “harsh leaves” or “fronds more divided”. Also, unlikely other taxonomists of that time, Copeland believed that the sori involucre structure was not a reliable character for the classification of the family, although he contradicted himself while applying sori traits into his keys.

According to Morton (1968), the genus delimitation within Hymenophyllaceae is complicated, even for taxonomists and experts on the family. Furthermore, resuming the family to two genera distinguishable by the sori pattern results in several identification mistakes, especially when fertile fronds are not available. The author states that more subtle traits of the rhizome, petiole and lamina are important for the identification of groups within the family.

Clearly against an exaggerated division of fern families into small groups, Morton (1968) expresses that Hymenophyllaceae is very likely to be a monophyletic family. In face of a plant group with such a complicated taxonomic background,

Morton's intention was to gather knowledge on the family acquired over the years into a monography, which resulted in the division of the family into 6 genera.

Years later, Iwatsuki (1984, 1990) found previous classifications either heterogeneous or inconvenient, of hard application when considering a broader range of species. In order to address these inconveniences, Iwatsuki (1984, 1990) designed a system where the family is divided into two subfamilies: subfamily Hymenophylloideae (represented by 8 genera) and subfamily Cardiomanes (monotypic). In contrast with the previous classification systems, Iwatsuki (1984, 1990) system was based on a broader range of traits, taking in consideration aspects of the lamina and venation, and not only traits relative to the sori.

More recently, with the advance of the usage of molecular tools in taxonomic analyses, several studies on the family brought new insights on the circumscriptions of infra-family categories (Dubuisson *& al*. 2003, Ebihara *& al*. 2006, Hennequin *& al*. 2003, 2006). With a molecular approach and aiming to categorize all the species of the family into monophyletic groups, Ebihara *et al*. (2006) proposes a system composed by 9 different genera. In this system, the hymenophylloid ferns are grouped within a single

genus *Hymenophyllum*, while the remaining trichomanod ferns are split into 8 different genera.

HYMENOPHYLLUM SUBGENUS *MECODIUM*

The genus *Hymenophyllum sensu* Ebihara (2006) is represented by long-creeping plants, with glabrous wiry rhizomes and bivalve sori. About 250 different species compose the genus, distributing from tropics to temperate regions and being categorized into 10 different subgenera. A comparison between the categories proposed by Ebihara (2006) and the previous classification systems can be found in table 1.

The subgenus *Mecodium* C. Presl ex Copel. *sensu* Ebihara (2006), one of the most representative subgenus of *Hymenophyllum*, is composed by about 35 species of cosmopolitan distribution and epiphytic habit. Plants of this subgenus are long-creeping, bearing filiform rhizomes, pinnate to tripinnate leaves with entire margins. Hairs and scales are abscent or caducuous, and further morphological specificities are not reported. The base chromosome number for the subgenus is x = 28 and corresponds to a synapomorphy for the group (Hennequin *et al*. 2010). The lectotype for the subgenus is *Hymenophyllum polyanthos* (Sw.) Sw., being selected by Copeland (1937).

Copeland (1937, 1938) circumscribed the subgenus *Mecodium* as a monophyletic group, composed by more than 100 species characterized by the entire, mostly glabrous lamina, and by the bivalve sori, with included receptacles. This circumscription was widely accepted during the 20th century, although some divergences regarding the rank attributed to *Mecodium* were discussed (Hennequin *et al*. 2006). In this period, the works of Morton (1968), Pichi Semrolli (1977) and Iwatsuki (1984, 1990) were very influential for the taxonomy of the group (Table 1). Copeland (1937) had set *Mecodium polyanthos* (= *Hymenophyllum polyanthos*) as the type for *Mecodium*, although in 1947 the author had changed the type to *Mecodium sanguinolentum* Presl ex Copel. Nevertheless, Morton (1968), Pichi Sermolli (1977) and Iwatsuki (1984, 1990) kept *M. polyanthos* as the type for *Mecodium*.

Hennequin *et al.* (2006) tested the 20th century hypothesis of classification for *Mecodium* by sampling several species of *Hymenophyllum* and gathering sequences from plastid markers. The results evidence that the subgenus *Mecodium,* as treated during the 20th, stood as a polyphyletic group, including represents from different subgenera. Furthermore, the clade containing *H. polyanthos* appear in accordance with

the subgenus *Mecodium sensu* Hennequin (2003), although *H. polyanthos* samples do not appear grouped in a monophyletic clade.

Interestingly, *H. polyanthos* represents appear interspersed with other species of *Mecodium* and, ultimately, divided into 2 clades that seem to reflect biogeographic patterns: one clade including species from the Mascarene Islands (*i.e. H. inaequale* (Poir.) Desv. and *H. polyanthos* from La Reunión), Chile (*i.e. H. cuneatum* Kunze) and Australia (*i.e. H. mnioides* Hooker & Baker, *H. ooides* F. Muell & Baker, *H. polyanthos* and *H. rarum* R. Br.); and another clade including species from the Neotropics (*i.e*. *H. apiculatum* Mett. ex Kuhn and *H. polyanthos* from Bolivia) and North Asia (*i.e. H. polyanthos* from Japan, *H. wrightii* Bosch and *H. corrugatum* Christ). At this point, represents of *H. polyanthos* investigated in the analysis appeared more closely related to biogeographically close samples, than to those identified as belonging to the same species, indicating that *H. polyanthos* could correspond to a complex of species with a subcosmopolitan distribution (Fig. 1).

As Hennequin (2006) explains, the non-monophyly of *Mecodium* is not taken as a surprise, since most of the subgenus' description was based on the absence (rather

the presence) of particular traits. Howbeit, the broad investigation undergone by Hennequin *et al*. (2006) has brought evidence to the presence of non-monophyletic groupings inside *Mecodium*, such as *H. polyanthos*, stressing the importance of broad taxonomic sampling. Although *H. polyanthos* specimens were sampled by Hennequin *et al*. (2006), the short coverage of sampling restricts discussions that can lead towards a monophyletic circumscription of the species. To resolve this, a broader sampling of the subgenus is necessary, including both specimens of *H. polyanthos* from several regions of the globe, and also other species included in *Hymenophyllum* subgenus *Mecodium sensu* Ebihara (2006). Like this, both distributional patterns and species limits can be assessed.

Table 1: Comparison between genera and subgenera circumscribed for hymenophylloid species during the last century.

Copeland (1938, 1947)	**Morton (1968)**		**Iwatsuki (1984)**	**Ebihara *et al.* (2006)**
Genus	Genus	subgenus	Genus	Genus
Buesia	*Hymenophyllum*	*Hymenophyllum*	*Hymenophyllum*	*Hymenophyllum*
Hymenophyllum				
Meringium				
Craspedophyllum		*Craspedophyllum*		
Hemicyatheon		*Hemicyatheon*		
Amphipterum		*Mecodium*		
Mecodium				
Apteropteris		*Sphaerocionium*	*Sphaerocionium*	
Leptocionium				
Sphaerocionium				
Cardiomanes	*Cardiomanes*	-	*Cardiomanes*	

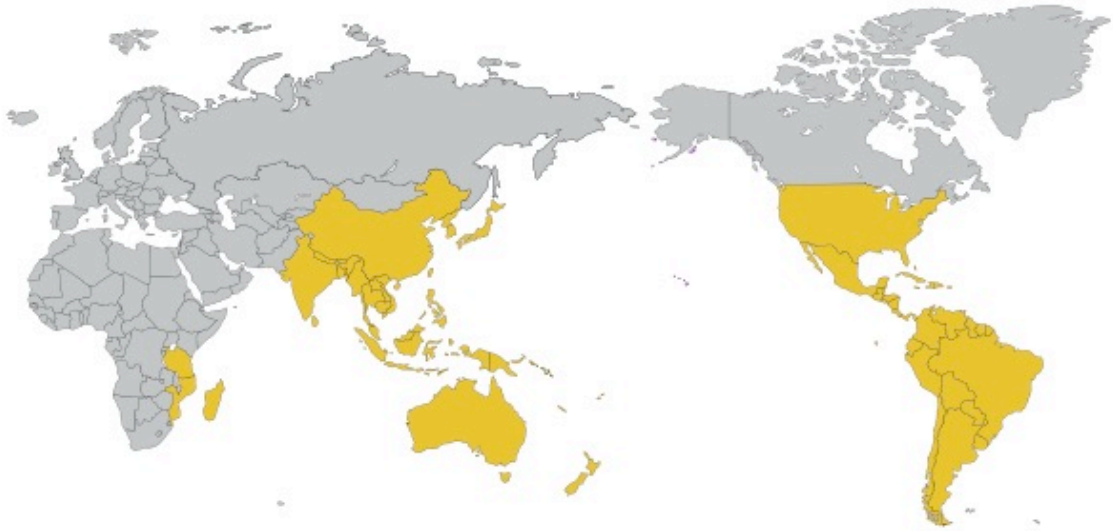

Figure 1: Estimated distribution for *Hymenophyllum polyanthos* (Sw.) Sw. is show by areas highlighted in yellow. Albeit the wide geographical distribution, these plants are usually restricted to more dense and humid forests, at higher altitudes.

Chapter 2 – Molecular plastid markers reveal the actual circumscription of *Hymenophyllum* subgenus *Mecodium* C. Presl ex Copel. and of *Hymenophyllum polyanthos* (Sw.) Sw. (Hymenophyllaceae)

INTRODUCTION

Hymenophyllum polyanthos (Sw.) Sw. corresponds to a subcosmopolitan species of filmy ferns and was first described by Swartz in 1788, under the name *Trichomanes polyanthos* Sw., and later combined to the genus *Hymenophyllum* in 1800 by the same author (Swarts 1788, 1801). The type specimen is from Jamaica and it was described as a plant with 4-pinnatifid and deltoid leaves, pinnae decurrent, apex linear obtuse and sori numerous at the margin of the lamina (Swartz 1801). The epithet "*polyanthos*" (i.e. **Poly**, from the Greek πολύς, meaning "many"; and **Anthos**, from the Greek ἄνθος, meaning "flower") also probably derives from the numerous sori observed on the lamina of this species. Nevertheless, the number of sori on the lamina of these plants is far from being a useful diagnosis trait, since such character is observed in several other species of Hymenophyllaceae.

Not differently from other filmy ferns, the body of *H. polyanthos* is very simple in structure: the rhizome is long creeping, wiry and without any appendices (*e.g.* hairs or scales, that when present are usually caducous); the anatomy of the vascular bundle is of a 'subcolateral' protostele (Ebihara *et al*. 2007), with few exarch xylem cells surrounded by phloem, and a sclerenchyma ring present at the inner part of the cortex; petioles are not articulated to the rhizome and glabrous; lamina also glabrous, ovate to lanceolate, pinnatifid or pinnate; lamina tissue thin, composed of one layer of cells; sori numerous, concentrated on the distal portion of the lamina, composed of bivalve structures originated from terminal veins; sporangia are organized around a receptacle; spores bearing chloroplasts.

Due to the delicate structure of its leaves, the plants of this species are usually found in shadowy and moisty environments, like dense rainy and misty forests and near to rivers and waterfalls (Fig. 2). When occurring in more open areas, hit by direct sunlight and with higher variations in temperature and humidity during the day, *H. polyanthos* usually appears growing over living plants (*e.g.* Bromeliaceae) or fallen trunks, often within populations of mosses (Fig. 2B). As Shreve (1911) states, *H.*

polyanthos is, within the Hymenophyllaceae, one of the species most resistant to desiccation, after the trichome-bearing species. According to the author, as *H. polyanthos*' leaves dry, they curl like a crozier protecting the inner parts from further desiccation (Shreve 1911).

H. polyanthos has a pantropical distribution, occurring in elevated areas of the South and Central America (with few records in North America), South Africa, Asia and Oceania. Taxonomically, the species is grouped in *Hymenophyllum* subgenus *Mecodium sensu* Ebihara *et al*. (2006), despite doubts regarding your monophyly (Hennequin *et al* 2006). As explained in Chapter 1, Mecodium is a name that received several combinations during the last century, being recently revised by Hennequin and collaborators (Ebihara *et al*. 2006, Hennequin *et al*. 2006, 2010).

By comparing data from *rbcL-accD* and *rps4-trnS* plastid markers, Hennequin *et al.* (2006) estimated a tree including represents from several subgenera of *Hymenophyllum*, and aiming to test the monophyly of *Mecodium sensu* Copeland (1938) and Pichi-Sermolli (1977), while comparing to circumscriptions proposed by Morton (1968) and Iwatsuki (1984,1985). As a result, *Mecodium sensu* Copeland

(1938) and Pichi-Sermolli (1977) emerged as a non-monophyletic grouping, with represents scattered through the whole phylogeny.

Ebihara *et al.* (2006) proposed a new circumscription based on these results, delimiting *Mecodium* to the clade indicated as "*H. polyanthos* clade" by Hennequin *et al.* (2006). Like this, *Hymenophyllum* subgenus *Mecodium sensu* Ebihara *et al.* (2006) is circumscribed around more than 35 species, with *H. polyanthos* as the lectotype (selected by Copeland 1937). According to the circumscription of Ebihara *et al.* (2006), *Mecodium* includes epiphytic plants with rhizomes long-creeping, filiform, nearly glabrous; stipes up to 10 cm long; blades pinnate to tripinnatifid, elliptic to subdeltate, 45 cm by 6 cm, margins of segments entire; sori at the tips of ultimate segments, lips bivalve, entire, receptacles included in involucres; chromosome number base *x=28* (Ebihara et al. 2006). Although the subgenus *Mecodium* and other *Hymenophyllum* subgenera were consistently circumscribed by Hennequin et al. (2006) and Ebihara et al. (2006) investigations, the position of *H. polyanthos* samples within the phylogenies indicated that the species might not correspond to a monophyletic grouping.

Species delimitation within *Hymenophyllum* subgenus *Mecodium sensu* Ebihara *et*

***al*. (2006) and research goals**

One of the greatest challenges in the taxonomy and systematics of ferns is to delimit species boundaries. Phenomena like apogamy, apospory and parthenogenesis (although not so common) have been reported in groups of ferns (Steil 1939), as well as hybrids and polyploid lineages (these ones more likely to occur in ferns), indicating that complex evolutionary scenarios are also responsible for the extant diversity of ferns.

Accurate recognition and delimitation of species of the family Hymenophyllaceae are especially complicated, due to the simplicity of the form of these plants, which usually bear one-cell thick leaves and have only a few centimeters of size. The lack of clearly distinguishable taxonomic traits and the difficulties to cultivate and study the biology of these delicate plants leads to the prediction of the existence of many species yet to be discovered. In the case of *H. polyanthos*, the broad geographical distribution (Fig. 1) had led scientists to argue about the existence of different lineages comprised under the same species name, but lack of definitive traits that could delimit such lineages in a category such as ‘species’ has precluded the taxonomy of the group to be further understood.

The goal of this chapter is to explore molecular data evidence that elucidates

the evolutionary relationship between geographically separated populations of *H. polyanthos*, clarifying the phylogenetic status of the species. Due to the confusing taxonomic background of the species and of the subgenus in which it is included, from this point on the subgenus *Mecodium* is going to be treated following Ebihara *et al.* (2006) circumscription, *i.e.* corresponding to *H. polyanthos* and its closely related species, and sharing the basic chromosome number of x=28 (Ebihara *et al.* 2006, Hennequin *et al.* 2006). *H. polyanthos* is going to be referred as a *sensu lato* lineage and a list of all species included in subgenus *Mecodium sensu* Ebihara *et al*. (2006) can be assessed through table 2. In this chapter three main questions will be addressed: 1) If *H. polyanthos* stands as a monophyletic grouping or not; 2) How lineages within the subgenus *Mecodium* are phylogeographycally related; and 3) How many lineages compose *H. polyanthos sensu lato*. Whether H. polyanthos *s.l.* should stand as a unique species lineage or should be divided into different groups corresponds to a question that profoundly affects the taxonomy of the subgenus *Mecodium* and, subsequently, of the family Hymenophyllaceae as well.

MATERIAL AND METHODS

Sampling and DNA extraction -- Samples included specimens recognized as *H. polyanthos* as well other closely related species of subgenus *Mecodium sensu* Ebihara *et al.* (2006). Samples were acquired through both collection trips and donations from collaborators. The main collection trips included visits to the Serra da Bocaina National Park in Sao Paulo, Brazil (22° 43' S, 44° 36' W), to Oku-Tama, Tokyo, Japan (35°N, 139° 7' E) and to Amagi Kogen in Izu, Shizuoka, Japan (34° 51' N, 139° 1' E). The Serra da Bocaina trip was made between 21st-23rd February of 2015, in collaboration with Dr. Jefferson Prado, Dr. Regina Hirai and Danilo S. Gissi, researchers of the Institute of Botany of Sao Paulo. The Serra da Bocaina comprises an area of about 104000 hectares between the states of Sao Paulo, Rio de Janeiro and Minas Gerais. The park area includes formations of Cerrado (Brazilian savanna), rainy forests and pinewoods. It is a part of the Serra do Mar formation, reaching 2000 meters of altitude. The rainy forests in high altitude locations of this area provide a favorable environment for the occurrence of Hymenophyllaceae species.

In Japan, the main collections occurred in two opportunities: in the 22nd May of 2015, to the Oku-Tama area in Tokyo; and in the 4th July of 2015, to the

Amagi-Kogen area in Izu, Shizuoka. In both cases, dense vegetation, high altitude and humidity were conditions of the environment. Additional samples were obtained through small personal trips, as well the donation of collaborators from Brazil, US and Japan. The final dataset contains samples from several regions of the globe, including Central and South America, continental Africa, Asia, Australia and Pacific (appendix 1).

Fresh samples were involved in cloth bags before being placed in plastic bags together with silica gel in order to avoid exaggerated dehydration. Additional plants leaves and stems were pressed between paper towels and left to dry under room conditions for at least three days. After this, plants were organized in vouchers and deposited in the TNS herbarium in Tsukuba Museum of Natural History and at the SP herbarium in the Institute of Botany of Sao Paulo, Brazil (appendix 1).

Samples dried in silica gel as well additional voucher samples acquired from collaborators were used for DNA extraction. About 20mg of leaves were pulverized using TissueLyser II by QIAGEN, followed by DNA extraction using the DNeasy plant mini kit (QIAGEN) standard protocol. Obtained DNA was stocked at -30^{o}C, in

completely dark boxes.

Markers amplification and sequencing -- For the analysis of chloroplast DNA, six different markers were targeted, including the *atpB* coding region, *atpB-rbcL* intergenetic region, *rbcL* coding region, *rbcL-accD* intergenetic spacer, *rps5-trnS* intergenetic spacer and the *matK* coding region (Table 3). The sequencing of most of these markers is novel for the group. Amplification reactions: ***atpB* region**, one cycle of initiation under 95°C for 5 minutes, followed by three folds ten loops of 95°C for 30 sec, 56-59°C grade for 30 sec, and 72°C for 1 minute, and one cycle of 72°C for 10 minutes; ***atpB-rbcL-accD* region**, one cycle of initiation under 94°C for 3 minutes, followed by three folds ten loops of 94°C for 30 sec, 52-54°C grade for 30 sec, and 72°C for 75 seconds, and one cycle of 72°C for 10 minutes; ***rps5-Trns* region**, one cycle of initiation under 94°C for 3 minutes, followed by three folds ten loops of 95°C for 45 sec, 52-57°C grade for 45 sec, and 72°C for 70 seconds, and one cycle of 72°C for 10 minutes; ***matK* region**, one cycle of initiation under 95°C for 5 minutes, followed by three folds ten loops of 95°C for 30 sec, 49-52°C grade for 30 sec, and 72°C for 1 minute, and one cycle of 72°C for 10 minutes. Amplification products were purified with ExoSap-IT

(Affymetrix), following sequencing procedures.

Sequences alignment and phylogenetic analysis -- forward and reverse sequences were assembled using ATGC v. 4.3.5 (Genetyx Corporation) and aligned using MEGA v. 7.0 (Kumar *et al.* 2016). The most suitable evolutionary model for the datasets was calculated using jModelTest v. 2.1.7 (Darriba et al. 2012). As outgroup lineages, sequences of *Hymenophyllum* species belonging to subgenera other than *Mecodium* were used. In total, 83 specimens were analyzed by comparing 4543 bp of the plastid markers.

Bayesian inference analysis was conducted using MrBayes v.3.2.5 (Huelsenbeck & Ronquist 2001, Ronquist & Huelsenbeck 2003). Markov Chain Monte Carlo method (Geyer & Keramidas, 1991) was applied for one million generations, priors were set for equal and the posterior probability was sampled at each 1000 generations, being the first 25% discarded as burn-in. Maximum Likelihood analysis was performed using RAxML (Stamatakis 2006), using the GTRGAMMA model and over a 1000 bootstrap replicates. Finally, trees were edited using FigTree v. 1.4.2 (Rambaut 2012) and Illustrator v. 18.1.1.

Further analysis -- At the moment, a limited variety of markers is sequenced and available in GenBank, mostly corresponding to *rbcL* and *rps4-trnS* sequences obtained by Hennequin *et al*. (2006). Although the restriction of data to these two markers results in a lower resolution of the tree on a subspecific level, an additional analysis (combining the novel data from this research with the GenBank data) is also provided here. The dataset here includes 120 OTUs and 2545 bp.

RESULTS AND DISCUSSION

Plastid markers phylogeny

The resulting phylogenetic tree for the plastid markers is shown in figure 3. Numbers over each node denote the posterior probability for the Bayesian Inference, while numbers underneath each node represent the bootstrap rate for the Maximum Likelihood analysis. The node marked with a star shape is strongly supported by both analyzes and includes *H. polyanthos* and its correlated species samples. For that reason, this clade is understood as the correspondent to *Mecodium* sensu Ebihara *et al.* (2006), ultimately representing the *H. polyanthos* complex.

The *Mecodium* clade divides into two sub clades, herein marked as PSA and AN. Clade PSA is represented by individuals distributed from Asia to South America, passing through the Pacific and can be subdivided into 3 different clades: A South American clade (*H. polyanthos* samples from Brazil and *H. cuneatum* from Chile); a southeast Asia/ Papuasia clade (*H. novoguineense* K. Iwats. from New Guinea and *H. polyanthos* from Indonesia and Malaysia); and a Pacific clade (*H. rarum* from New Zealand and *H. polyanthos* from French Polynesia and Marquesas).

Other samples from the Central America (Costa Rica) and South America (Bolivia) were grouped within clade AN, in a clade identified here as N. The lineages divide here once again into 2 clades, one represented by *H. myriocarpum* Hook., and another composed by represents of *H. undulatum* (Sw.) Sw. and *H. polyanthos*. *H. myriocarpum* and *H. undulatum* are two species distinct by their 2-3 pinnate leaves of relative big dimensions.

The remaining samples in clade AN are distributed in several lineages, but the monophyly of these lineages as one group is not well supported by the plastid dataset applied here (Bayesian Inference posterior probability = 0.84, not supported by the ML

analysis). Nevertheless, these lineages are represented by plants distributed from Tanzania, India and Southeast Asia, including *H. wrightii* Bosch and *H. mikawanum* (Seriz.) Seriz. from Japan, *H. paniculiflorum* C. Presl, *H. corrugatum* and *H. kuhnii* C. Chr., besides other represents of *H. polyanthos*.

The overall topology of the tree suggests the existence of at least two lineages, as shown in figure 4: one clade, represented by plants from Southeast Asia, Papuasia, Pacific islands and South America, will be refereed herein as the "Pacific-South America clade" (PSA), while another, represented by plants from Africa, Asia and Central to South America, will be referred as the "Asia/ Neotropics clade" (AN) (Fig. 3, Fig. 4).

Further analysis

In order to evidence the position of *H. polyanthos* complex samples within the genus *Hymenophyllum* and attempt to improve the sampling here, sequences available in GenBank were added to the analysis. Yet, few pieces of research on the group were performed until now, resulting in that sequences from GenBank are restricted to a number of markers. For that reason, only sequences from *rbcL* and *rps4-trnS* were

included in this analysis, resulting in a lower resolution of the tree.

Figure 5 shows the resulting tree, with the Bayesian inference posterior probabilities and the Maximum Likelihood shown over its respective node. The gray square denotes the *H. polyanthos* complex represents, circumscribing the subgenus *Mecodium* relatively to the other analyzed subgenera. As expected, the subgenus is circumscribed as a monophyletic grouping, supporting previous reports (Ebihara *et al.* 2006, Hennequin *et al.* 2006).

The topology of *Mecodium* tree is similar to the one obtained herein when comparing the whole dataset (Fig. 3). This indicates that even when comparing with other outgroups or when including novel samples, the overall topology of the tree isn't altered. The distinction between the "Pacific/ S. America" and "Asia/ Neotropics" clades is maintained, and there is no contradiction regarding the obtained groups within these clades.

Adding to this, new insights are obtained from this tree containing GenBank data. First, in the "Pacific/ S. America" clade, the PSA group is added with samples from La Reunion, an island from France located east of Madagascar, in the Indian

Ocean. The two samples included here (*i.e.* one *H. polyanthos* sample and one *H. inaequale* sample) appear grouped and may be sister to the S. America clade composed of Brazil and Chile samples. The accurate relative position of these clades cannot be assessed further on in this analysis due to the low support of the acquired nodes. Further analysis including La Reunion samples covering other markers should solve this question. Nevertheless, South America and La Reunion individuals are included in the SSA group.

The "Pacific-South America Clade" (PSA)

This clade is mostly represented by South hemisphere distributed samples, including plants from Chile, Brazil, New Guinea, French Polynesia, Marquesas and New Zealand, with Indonesian and Malaysian samples also included here. The clade is well supported by both ML and Bayesian analysis and is further divided into three clades based on its distribution.

The most basal clade that emerges here is indicated as the PSA1 clade and includes represents from Brazil and Chile (represented by *H. cuneatum*) (Fig. 6). The analysis including GenBank sequences shows that *H. cuneatum* appear clustered with *H.*

inaequale and *H. polyanthos*, both from La Reunion. Hennequin *et al.* (2006) and Larsen (2014) results pointed to a similar tree topology, but the results herein bring more support and resolution to the relation between the taxa, since the dataset applied here is more extensive than the previous works. According to Larsen (2014), subgenus *Mecodium* in the southern part of South America is represented by seven different species (*i.e. H. apiculatum, H. axillare* Sw., *H. cuneatum, H. darwinii* Hook.f. ex Bosch, *H. fendlerianum* J.W. Sturm, *H. polyanthos* and *H. viridissimum* Fée), distinguishable by undulations on the lamina margins and by the shape of the indusia.

Sister to the PSA1 clade, specimens from Southeast Asia and Pacific Islands emerge. The PSA2 clade includes *H. polyanthos* represents from Malaysia and Indonesia, clustered together with *H. novoguineense* from New Guinea, while the PS3 clade is represented herein by French Polynesia and Marquesas H. polyanthos, sister to *H. rarum* from New Zealand (Fig. 6).

The Asia-Neotropics clade (AN)

This clade is represented both by specimens from Southeast Asia and Africa,

as well by specimens from North and Central America. The "Neotropics" (N) clade (Fig. 6) is represented here by *H. myriocarpum, H. undulatum* and *H. polyanthos* from Bolivia and Costa Rica. *H. myriocarpum* and *H. undulatum* are species with a great range of trait variation within individuals according to Stolze & Tryon (1989), occurring from Mexico to South America, including the Amazon region. The addition of Genbank data also included *H. apiculatum* within this clade (Fig. 5).

Taking in consideration the distributional proximity, *H. polyanthos* type (from Jamaica) is also likely to be included here. The clade marked as "type" (T) clade in figure 6, composed solely of *H. polyanthos* samples and sister to *H. undulatum*, is hypothesized as the type clade for *H. polyanthos*. Further confirmation of this hypothesis should be achieved by sampling of Jamaica *H. polyanthos* specimens.

The remaining groups ("A" groups, Fig. 3) include plants from Africa, Indo-China, Southeast Asia and Malesia. *H. kuhnii* from Tanzania and *H. corrugatum* from China appear within these groups, but the resolution at the base of the tree do not allow further conclusions (Fig. 6). Still with a relative position not well estimated by the dataset applied herein, three other clades appear: the "Malaysian" ("M" clade, Fig. 6)

clade, represented by *H. polyanthos* from Malaysia; the "*H. wrightii*" ("W" clade, Fig. 6) clade, represented by *H. wrightii* from Japan; and the "SE Asia – Malesia" clade (SEAM, Fig. 3), composed by several lineages. *H. wrightii* is a small species of *Hymenophyllum* distributed mainly in Japan and Korea, but with some reported occurrences of asexual gametophytes in North America (Duffy et al. 2015, Taylor 1967) and sporophytes in Canada (Iwatsuki 1961).

Finally, the SEAM clade is composed by at least four *H. polyanthos s.l.* clades, plus other related species. The *H. polyanthos s.l.* lineages correspond to the "Japan-Taiwan *H. polyanthos*" (JT) clade, including specimens from Aichi, Kagoshima and Miyazaki prefectures in Japan; the "Malaysia-Indonesia *H. polyanthos*" (MI) clade; the "Indonesia-Buthan-Cambodia *H. polyanthos*" (IBC) clade; and the "Japan *H. polyanthos*" (J1, J2 and J3) clades, including specimens from Nagano, Kanagawa and Tokyo (Fig. 6). Additionally, *H. paniculiflorum*, a compact species of *Hymenophyllum*, appears in a monophyletic clade within here ("Pan group"), closely related to the JT, MI and IB clades. Herein, *H. paniculiflorum* specimens are representative from Japan, Taiwan and Malaysia. Also, *H. mikawanum*, an endemic species to Mikawa, Japan

(Serizawa 1983), appear sister to the J1 clade.

Species boundaries within the complex

The topology of the tree inferred from the analysis of chloroplast markers reflects the diversification history within *Mecodium*, taking as a premise that chloroplasts diversification paths are correlative to the diversification paths of species that carry those chloroplasts. However, being chloroplasts usually unilaterally inherited in plants, the investigation of other markers, such as nuclear ones, is made necessary for unveiling the complete diversification background here.

Based on the plastid data explored in the previous sections, it is evident that *H. polyanthos sensu lato* does not correspond to a monophyletic grouping, hence being pointed as a polyphyletic species here. As explored in Funk & Omland (2003), polyphyletic species are commonly discovered in phylogenetic investigations using molecular markers. The phenomenon, however, can be related to different reasons, including insufficient taxonomic treatment, gene introgression events, or the occurrence of polymorphisms or cryptic species (Funk & Omland 2003).

Constructing a phylogeny based on nuclear markers and comparing it to the

plastid tree on this chapter can address these events. If trees emerge as congruent (*i.e.* bearing the same topology), nuclear markers phylogeny becomes further evidence for the evolutionary background supported by plastid markers. On the other hand, if incongruences emerge, further explanations become necessary, and events like introgressions or hybridizations can be addressed. The next chapter will take these questions as a topic of research, and the nuclear *LEAFY* marker will be the material for further phylogenetic discussions.

CONCLUSIONS

In accordance with previous estimations, but with a novel level of accuracy and detail, the subgenus *Mecodium* (undoubtedly monophyletic as circumscribed by Ebihara *et al.* 2006) is divided into 2 clades: a "Pacific/ S. America" clade, composed by plants that may have evolved in the South America region and then dispersed to the Pacific Islands and to La Reunion in the Indian Ocean; and a "Asia/ Neotropics" clade, composed by plants that evolved from Asia-Malesia regions to the Neotropics, dispersing to the northern part of South America. Furthermore, *H. polyanthos*, the type species for the subgenus, emerges as a polyphyletic species, as expected when taking in

consideration past studies on the genus *Hymenophyllum*. Reasons for this can include taxonomic misleads, but also genetic and evolutionary factors, requiring further research including nuclear markers and also morphological and ecological investigations. Such investigations, as well a novel taxonomical treatment suggestion, will be topics for the following chapters.

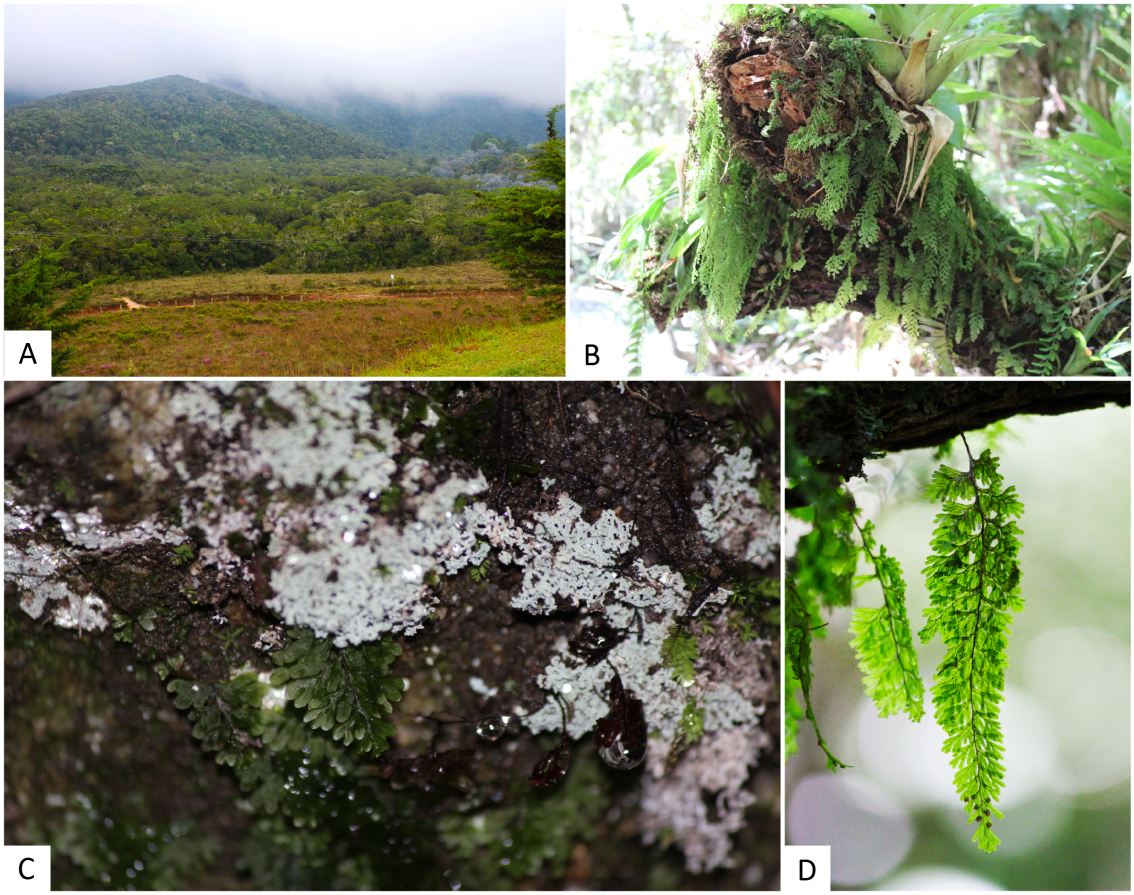

Figure 2: Hymenophyllaceae species usually occurs in humid environments, such as misty or rainy forests. A: misty forests at Serra da Bocaina, in Sao Paulo, Brazil. The mist was common during the mornings, covering the base of the mountains where dense vegetation was observed; B: fallen trunk covered by Bromelliaceae and Hymenophyllaceae speciemens (São Paulo, Brazil); C: *Hymenophyllum paniculiflorum* occurring in the crevice between rocks and together with various lichens (Yamanashi prefecture, Japan); D: *Hymenphyllum polyanthos* pending from a trunk (São Paulo, Brazil).

Table 2: List of species included in subgenus *Mecodium sensu* Ebihara (2006), taken as reference for this investigation.

Species	**Author**	**Original reference**	**Date**	**Locality**
H. abruptum	Hook.	Species Filicum 1: 88, t. 31B	1844	Jamaica
H. apiculatum	Mett. ex Kuhn	Linnaea 35: 391	1868	Venezuela
H. axillare	Sw.	J. Bot. (Schrader) 1800(2): 101	1801	Venezuela
H. brevifrons	Kunze	Bot. Zeitung (Berlin) 5: 185	1847	French Guiana
H. copelandii	C.V. Morton	Contr. U.S. Natl. Herb. 38(4): 173	1968	New Guinea
H. corrugatum	H. Christ	Bull. Herb. Boissier, sér. 2, 3(6): 508–	1903	China
H. cuneatum	Kunze	Analecta Pteridogr. 50	1837	Chile
H. darwinii	Hook.	Ned. Kruidk. Arch. 5(3): 157	1863	Chile
H. fendlerianum	Sturm	Flora Brasiliensis 1(2): 291	1859	Venezuela
H. fumarioides	Bory ex Willd.	Species Plantarum. Editio quarta 526	1810	Madagascar
H. inaequale	(Poir.) Desv.	Mém. Soc. Linn. Paris 6: 335	1827	Madagascar
H. kuhnii	C. Chr.	List Vasc. Pl. Gabon	1988	Madagascar
H. mikawanum	(Seriz.) Seriz.	Journ. Jap. Bot. 58(2): 64	1983	Japan
H. mnioides	Hooker & Baker	Syn. Fil. 57	1867	New Caledonia
H. myriocarpum	Hook.	Sp. Fil. 1: 106, t. 37d	1844	Colombia
H. novoguineense	(Rosenst.) K. Iwats.	Blumea 51(2): 231	2006	New Guinea
H. ooides	F. Muell. & Baker*	J. Bot. 28: 105	1890	New Guinea
H. paniculiflorum	C. Presl	Fl. China - Hymenophyllaceae	1843	China
H. polyanthos	(Sw.) Sw.	J. Bot. (Schrader) 1800(2): 102	1801	Jamaica
H. rarum	R. Br.	Prodr. 159	1810	Tasmania
H. recurvum	Gaudich.	Voy. Uranie, Bot. 376	1829	Hawaii
H. siliquosum	H. Christ	Bull. Herb. Boissier, sér. 2, 4(9): 938	1904	Costa Rica
H. undulatum	Sw.	J. Bot. (Schrader) 1800(2): 101	1801	Jamaica
H. viridissimum	Feé	Crypt. Vasc. Bresil 1. 194 t. 49 f. 3	1869	Brazil
H. wrightii	Bosch.	Ned. Kruidk. Arch. 4: 391	1859	Japan

Table 3: List of primers used for amplification of markers of interest. Following the primer ID, primer sequence, target gene, location of the gene and reference bibliography are presented.

Primer ID	**Sequence (5' -> 3')**	**Target Gene**	**Location**	**Reference**
411R	GAAATTCCAAACGCGAGAGAAC	*atpB*	Chloroplast	Ebihara et al 2003
30F	GTGTTGGATTCAAAGCTGGTG	*rbcL*	Chloroplast	Ebihara et al 2003
1198F	TACAGTTCGGTGGTGGAACC	*rbcL*	Chloroplast	Ebihara et al 2003
132R	TGGAGTCATTCGGAAGGCTGC	*rbcL*	Chloroplast	Ebihara et al 2003
1300R	ACCTTCACGAGCAAGATCACG	*rbcL*	Chloroplast	Ebihara et al 2003
816R	CCATGATCGAATAAAGATTCAGC	*accD*	Chloroplast	Ebihara et al 2003
Rps4F1	GCCGCTAGACAATTAGTCAATC	*rps4-trnS*	Chloroplast	Hennequin et al. 2003
TrnS	TACCGAGGGTTCGAATC	*rps4-trnS*	Chloroplast	Souza-Chies et al. 1997
Rps5	ATGTCCCGTTATCGAGGACCT	*rps5-trnS*	Chloroplast	Nadot et al. 1994
FERmatK dEDR	ATTCATTCRATRTTTTTATTTHTGGARGAYAGATT	*matK*	Chloroplast	L.-Y. et al. 2011
FERmatK rAGK	CGTRTTGTACTYYTRTGTTTRCVAGC	*matK*	Chloroplast	L.-Y. et al. 2011
*atpB*1592R	TGTAACGYTGYAAAGTTTGCTTAA	*atpB*	Chloroplast	Wolf 1997
*atpB*493F	GGATCTTTTGGCYCCGTATCGTCG	*atpB*	Chloroplast	Pryer et al. 2004
HLFY-1Fd	TTGCTACTTCTCTGGAGGGT	*LEAFY*	Nuclear	Ebihara (not published)
HLFY-2Rd	CCTCATTGTCACTACTTGGTTC	*LEAFY*	Nuclear	Ebihara (not published)

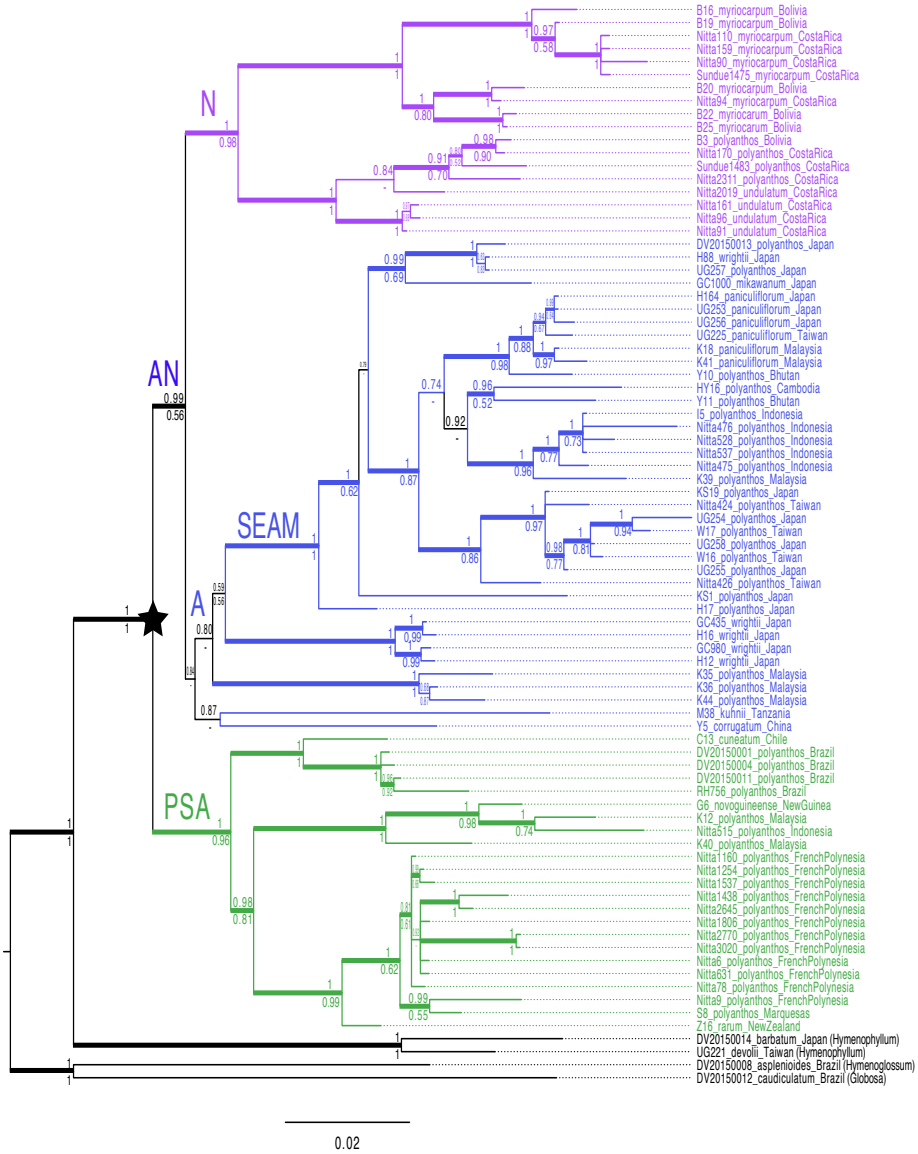

Figure 3: Phylogenetic tree for the subgenus *Mecodium*, focusing on the *Hymenophyllum polyanthos* complex. Sequences of DNA for six different plastid markers were used for this analysis, comprising 4543 bp and 83 OTUs. Values over the branches indicate the Bayesian inference posterior probability, while values below the branches indicate the ML probability. Bold branches indicate branches supported by both analyses. Clades marked in black represent the outgroups, and the star mark indicates the subgenus *Mecodium*. Names over the nodes refer to groups cited in the discussion and colored branches indicate biogeographically related taxa.

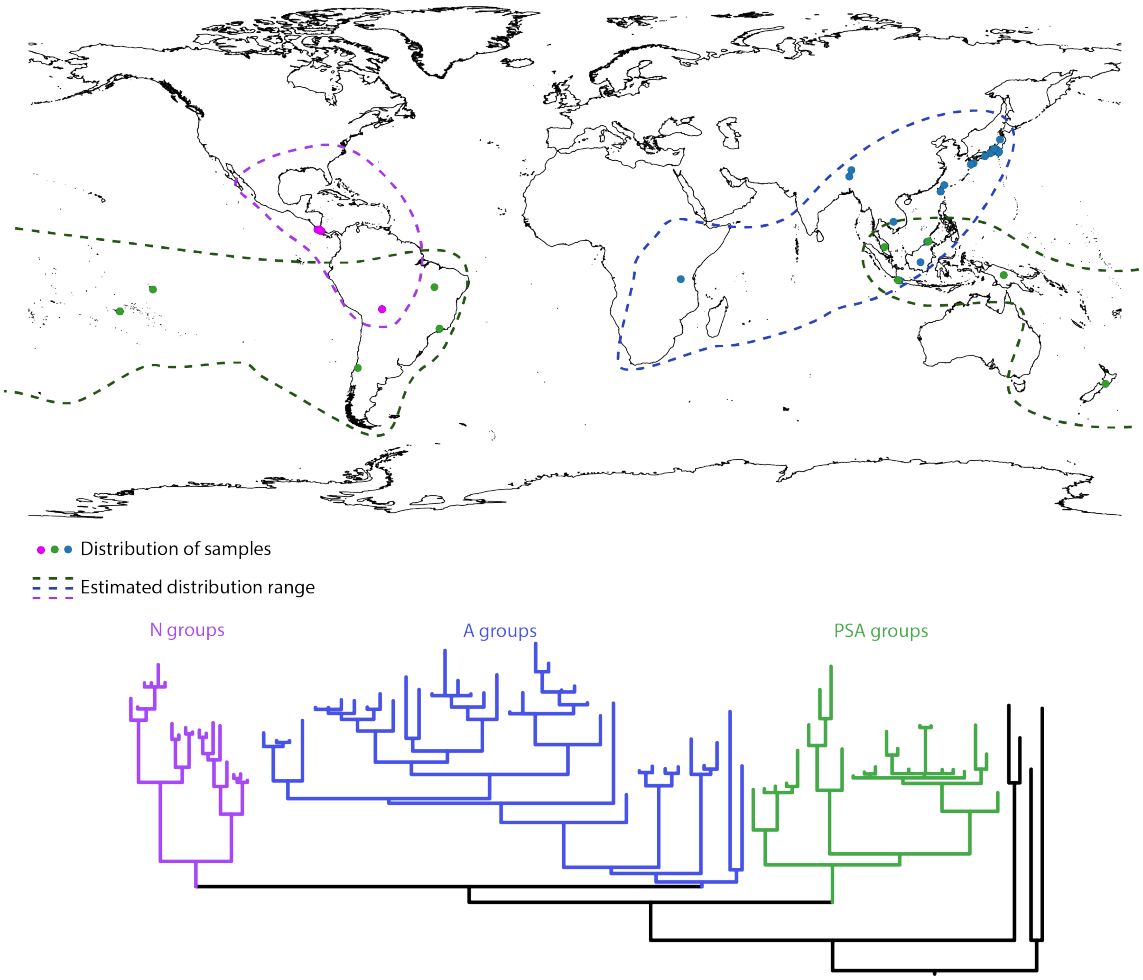

Figure 4: Biogeographic distribution of samples compared to the obtained phylogenetic topology. A simplified tree is shown at the lower part of the figure, representing the topology acquired in figure 3 analyses. Colors are in accordance with the obtained groups (*i.e.* PSA, N and A). The actualsampling distribution is represented by • symbles, while hashed lines delimit the estimated distribution range for each of the groups.

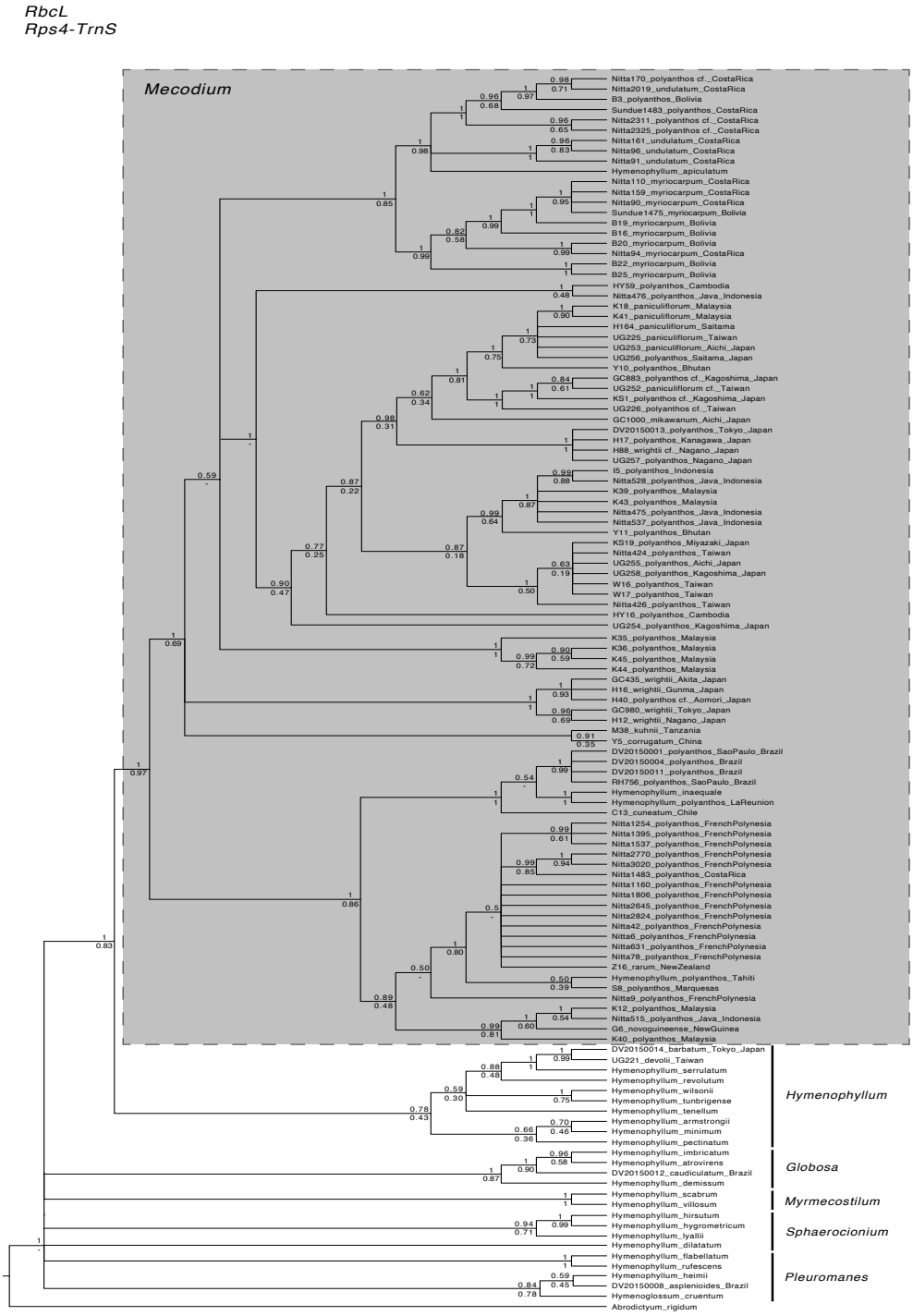

Figure 5: Cladogram for the genus *Hymenophyllum*, acquired by the inclusion of data available at GenBank. Sequences of DNA for *rbcL* and *rps4-trnS* regions are compared for this analysis. Values over the branches indicate respectively the Bayesian inference posterior probability, while values below the branches indicate the ML probability. Subgenera names are indicated on the right side of the figure and the gray box indicates the subgenus *Mecodium*, focus of the analysis.

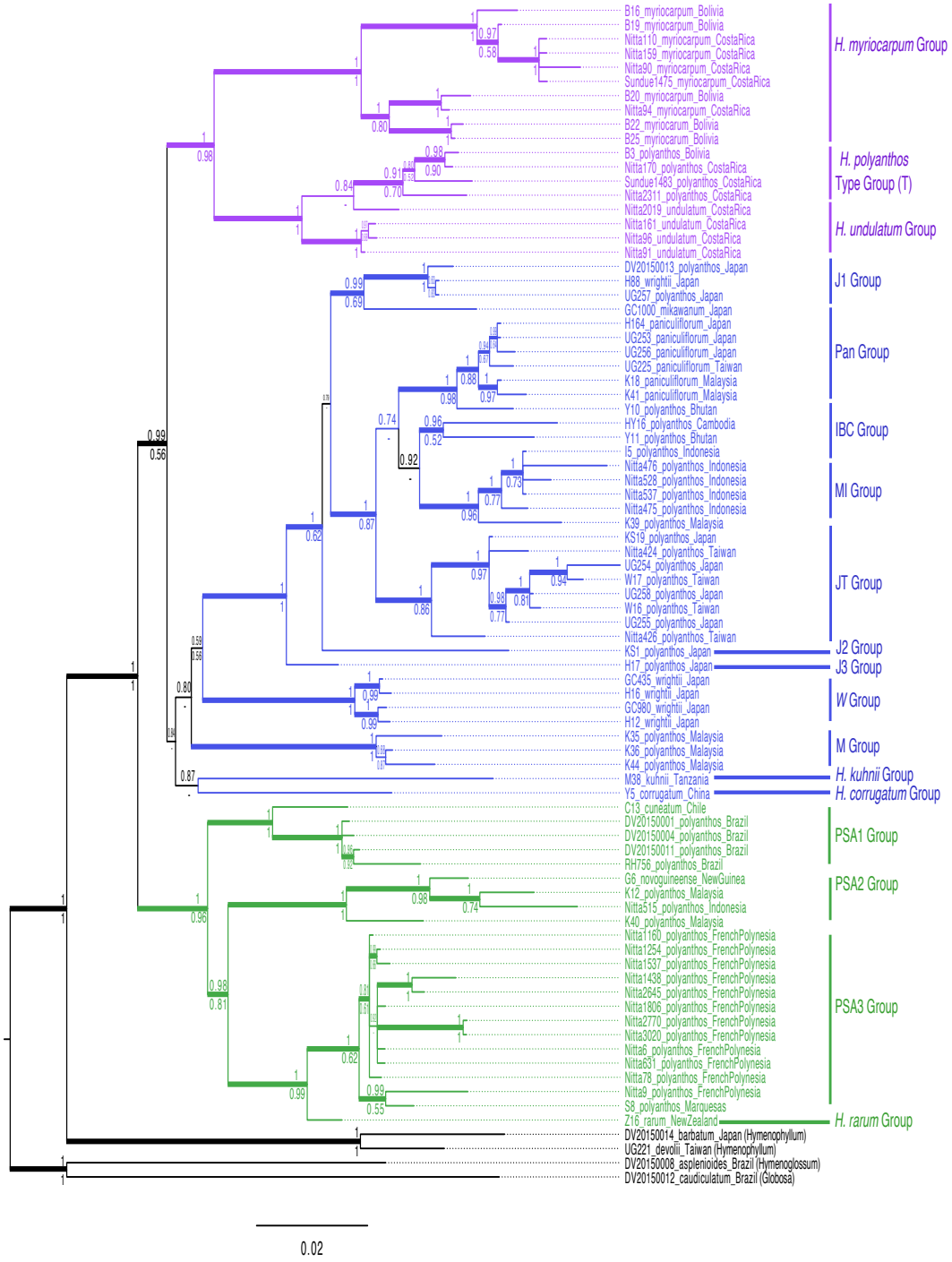

Figure 6: Lineages circumscription range over the phylogenetic tree for the subgenus *Mecodium*, focusing on the *Hymenophyllum polyanthos* complex. The type clade for *Hymenophyllum polyanthos* appear in the "Neotropics clade", highlighted in purple. Groups that need further taxonomical treatment are pointed by names (*i.e.* "A" groups in "Asia clade" and "SSA" groups in "Pacific/ S. America clade").

Chapter 3 – Further insights on the circumscription of *Hymenophyllum polyanthos* (Sw.) Sw. complex (Hymenophyllaceae) based on the nuclear *LEAFY* marker

INTRODUCTION

In addition to phylogenetic analysis using plastid molecular markers, recent studies on plants evolution usually also consider nuclear markers sequences. Although plastid sequences are relatively easy to acquire and provide a straightforward interpretation of the phylogeny, more complex evolutionary backgrounds (*e.g.* hybridization, polyploidization) can only be addressed when considering nuclear markers. One important discussion included in this context refers to the concept of species and how it is applied to different biological studies.

For systematists, the usual premise is that species are monophyletic or, in other words, correspond to lineages that have derivate from a same and exclusive ancestor. In practical ways, when considering species under a systematic scope, it is expected that DNA sequences should be more similar between individuals of the same species, than individuals from different species (Fig. 7A). Based on this idea, systematic taxonomists may circumscribe species, avoiding polyphyletic groupings (herein,

'polyphyletic' is referred as including the concepts of paraphyly and strict-sense polyphyly, Fig. 7B-C). Nevertheless, traditionally recognized species may emerge as polyphyletic groupings when compared to different markers, and reasons for that must be addressed when considering their circumscription.

Polyphyletic species

Funk & Omland (2003) provided a broad revision of the frequency, causes and consequences of the existence of polyphyletic species within animals, by focusing on insights from mitochondrial DNA. While with animals comparisons of nuclear and mitochondrial markers may lead to the insights pointed by Funk & Omland (2003), in plants the comparisons are usually taken between chloroplast and nuclear markers. Chloroplast markers are widely implemented in phylogenetic studies of plants, especially due to their comparatively easy handling. However, chloroplasts are usually unilaterally inherited in plants, hence other sources (such as nuclear markers) also need to be compared.

When doing so, incompatibilities between trees calculated using plastid and nuclear data may emerge and reasons for that are various. As explored by Funk &

Omland (2003), background reasons might include what they calls imperfect taxonomy (*i.e.* occurrence of polymorphisms, geographic variation or cryptic species), interspecific hybridization, or even paralogy (*i.e.* inclusion of paralog sequences in the analysis). By comparing the topology of trees acquired from nuclear and plastid markers, one can address the occurrence of such events. Nevertheless, obtaining nuclear sequences remains a challenge due to several factors such as: variations in the number of gene copies between species; gene recombinations and convertions; and the persistence of divergent alleles in populations of species (Archambault & Bruneau 2004).

Within ferns, Ebihara *et al*. 2005 and Adje *et al*. 2007 addressed the occurrence of reticulate evolution using nuclear markers. Nevertheless, studies in the field have difficulty in being further developed, especially due to the lack of markers available for analysis. While several markers are available for studies with angiosperms, within ferns few markers have been implemented until now (Cheng *et al*. 2012). Between these, investigations on the phylogenetic utility of the *LEAFY* gene have provided useful primers and methods while bringing new insights to the evolution of

ferns' genes.

The *LEAFY* gene is a low-copy nuclear gene associated with floral development (in angiosperms) and vegetative growth, including the development of compound leaves (evidenced in *Pisum sativum* L. by Hofer & Ellis 1998). As a low-copy gene, it is expected that few copies of the gene are present in the genome of plants, but studies show that while single copies are observed in diploid species of angiosperms, two or more copies are present in polyploidy species, and in some gymnosperms (Frohlich & Parker 2000). In ferns, this gene's evolution is still poorly understood.

Hymenophyllum polyanthos

In the previous chapter, the results from the phylogenetic study using chloroplast markers for the *H. polyanthos* complex were presented as evidences for the non-monophyly of it. However, as explained in this chapter, reasons for that can be of various kinds. Occurrences of cryptic lineages, polymorphisms and gene introgressions are hard to address, demanding broader investigations that take into consideration nuclear markers and morphological and/or ecological features.

In this chapter, evidences from *LEAFY* nuclear gene sequences are brought in discussion to attempt addressing the occurrence of gene introgressions in the studied groups. One might infer the occurrence of gene introgressions during the diversification of subgenus *Mecodium* by comparing the topology of trees acquired by both nuclear and plastid markers. Possible topology patterns that can be acquired and their meaning are shown in Fig. 7A) monophyly or congruence between topologies (when the phylogenies present the same topology); B) poplyphyly associated to taxonomical or evolutionary reasons (such as occurrence of cryptic species, polymorphs or introgressive genes); and C) poplyphyly associated with the inclusion of paralog sequences in the analysis. Hypothesizing that no introgressions took part in *H. polyanthos* complex, a pattern like the one shown in Fig. 7A might be expected to be recovered.

MATERIAL AND METHODS

DNA amplification and sequencing -- Several researchers in the last decades have developed primers for amplification of nuclear markers in plants, evidencing the validity of these markers for phylogenetic inferences (Álvares & Wendel 2003, Ferguson & Sang 2001, Hoot & Taylor 2001, Raymond *et al*. 2002, Van den Heede

2003, Ishikawa *et al*. 2002). However, most of these developed primers remain untested in ferns, especially in Hymenophyllaceae. The *LEAFY* marker was recently developed and tested in Davalliaceae ferns by Cheng *et al*. (2012) and in Hymenophyllaceae by Ebihara (unpublished, 2005). Herein, the target region was part of the *LEAFY* intron 1 and exon 2, and the primers applied are included in table 3. Samples applied here are representatives of the same lineages included in the chloroplast markers analysis (appendix 1).

Direct PCR and sequencing resulted in double banding and unclear sequence readings, so cloning using Promega pGEM-T Easy Vector system experiments were performed. The ligation reactions included: 2X Rapid Ligation Buffer (5µl), pGEM-T Easy Vector 50ηg/µl (1µl) and T4 DNA Ligase (1µl), PCR products (1:3 vector to insert ratio), completing with deionized water to a final volume of 10µl.

For the cloning, TOYOBO *Escherichia coli* Competent Quick DH5α cells were used. After incubation of the cells with the vectors, cells were spread over a LB medium containing ampicillin (25µg/µl) and covered by IPTG and X-Gal. Colonies were cultivated for 12 hours and white colonies were picked inside a clean bench for

amplification.

For the amplification reactions, T7 and SP6 promoter regions of the pGEM-T Easy vector were used as starting points for the reaction. The primers applied are as suggested by Promega (T7: 3'CATTATGCTGAGTGATATCCCG5'; SP6: 3'TAAGATATCACAGTGGATTTA5'). The thermal cycle was: one cycle of initiation under 94^{o}C for 3 minutes, followed by 35 cycles of 94^{o}C for 30 sec, 55^{o}C grade for 30 sec, and 72^{o}C for 1 minute, and one cycle of 72^{o}C for 10 minutes. After this, successfully amplified samples were sequenced, using the primers present in table 3.

Sequences alignment and phylogenetic analysis -- forward and reverse sequences were assembled using ATGC v. 4.3.5 (Genetyx Corporation) and aligned using MEGA v. 7.0 (Kumar *et al.* 2016). The most suitable evolutionary model for the datasets was calculated using jModelTest v. 2.1.7 (Darriba *et al*. 2012). As outgroup lineages, sequences of *H. caudiculatum* from Brazil were used.

Bayesian inference analysis was conducted using MrBayes v.3.2.5 (Huelsenbeck & Ronquist 2001, Ronquist & Huelsenbeck 2003). Markov Chain Monte Carlo method (Geyer & Keramidas, 1991) was applied for one million generations,

priors were set for equal and the posterior probability was sampled at each 1000 generations, being the first 25% discarded as *burn-in*. Maximum Likelihood analysis was performed using RAxML (Stamatakis 2006), using the GTRCAT model and over a 1000 bootstrap replicates. Trees were edited using FigTree v. 1.4.2 (Rambaut 2012) and Illustrator v. 18.1.1.

RESULTS AND DISCUSSION

In total, 31 different samples were cloned and at least 10 different clone colonies were sequenced for each sample. Ultimately, 66 different sequences were obtained covering about 554 bp of part of *LEAFY* intron 1 and exon 2. These sequences were applied to phylogenetic analyses, under Bayesian inference and Maximum Likelihood methods. The resulting tree is shown in figure 8, being the BI posterior probabilities shown over each obtained node, and the ML bootstrap rate under each node.

Although the overall topology of the tree suggests a pattern similar to the one found with chloroplast markers, important incongruences are also found here (Fig. 8), demanding explanations. Within the "AN" clade, incongruences appear dispersed

through the tree, while within the “PSA” clade, incongruent sequences appear grouped in distinct clades (marked in red). In both cases, incongruences cannot be explained simply by hybridization, especially because of the geographical origin of the samples. Once specimens from Japan, China and Malaysia appear mixed with Costa Rica lineages, the sympatric coexistence of lineages is discarded and, thus, hybridization is not taken as likely to have occurred.

In the case of the incongruences present at the “PSA” clade, the conflicting sequences appear clustered together in 2 groups (A and B, fig. 8), indicating that they are significantly distinct from other sequences included in the PSA clade. Moreover, branches within these clades are very short, indicating that the sequences within them are similar to each other and that very few variations are present. The phylogeny topology observed is a result of variations that are exclusively shared by the sequences included in groups A and B (appendix 2). These variations are, however, not informative inside of each of these groups, resulting in very short branches and restricting the resolution of these clades.

Despite being a low-copy gene, the occurrence of duplications in the *LEAFY*

region is a possibility here, as reported by Archambault & Bruneau (2004), who have found evidences of occurrence of duplication events and also pseudogenes at the molecular region in Caesalpinioideae (Leguminosae). Groups A and B observed in figure 8 can represent clusters of paralog sequences, in a scenario as represented in Fig. 7C. However, due to the short branches inside of these groups, further considerations are restricted. In order to solve this, new primers might be designed based on sequences other than those included in groups A and B. Using these newly designed primers, one may conduct the cloning experiments as performed here, expecting a more congruent topology for the phylogeny to be acquired.

Further analysis

Taking the premise that the incongruent sequences included in groups A and B correspond to paralog sequences, a phylogenetic analysis was performed with a dataset excluding these sequences (Fig. 9). The parameters applied here were the same as the ones applied to the tree of figure 8. The resulting tree has a topology more congruent to that obtained with plastid markers, although some individual sequences

emerged in non-expected positions.

These sequences correspond to *H. myriocarpum* from Costa Rica and Bolivia that appear clustered with *H. wrightii* from Japan, *H. polyanthos* from Marquesas, Malaysia and China that appear within the Neotropics clade, and *H. polyanthos* from Brazil appearing together with *H. polyanthos* from Bolivia. These occurrences suggest a scenario like shown in fig. 7B, but still the geographical distance between the samples limit the range of explanations that can be applied here. It is possible that some kind of introgressive event has occurred between ancestors of these lineages in the past, but further investigations are necessary to bring concrete reasons for these events.

Nevertheless, no further evidence of gene introgression is observed here, what gives support to the pattern obtained through the phylogenetic analysis using plastid data. If introgressive events are not further observed between these groups, this data might evidence that plastid markers phylogeny is directly correlated with the subgenus *Mecodium* phylogeny. In other words, phylogenetic clusters observed here might be understood as independent lineages that need to be described separately.

CONCLUSIONS

Albeit nuclear markers investigations within ferns are still not widely explored, the development of new primers specific to these plants, as well the validity of these markers for phylogenetic studies, have been reported. In this chapter, nuclear *LEAFY* marker regions were sequenced and applied for a phylogenetic analysis aiming to address possible evolutionary backgrounds for the tree topology observed with plastid markers (chapter 2). As a result, incongruent tree topologies were obtained, indicating that paralog sequences were included in the analysis.

To solve this, further investigations are still necessary. Using the obtained data, primers specific to the congruent sequences can be designed and used for new phylogenetic analysis. In order to explain the expectations of such analysis, this chapter shows another analysis where the possibly paralog sequences are excluded from the dataset. The resulting tree shows a more congruent topology, with only some sequences emerging in not expected positions. Taking as a premise that the excluded sequences, in fact, correspond to paralog sequences, this data does not consistently support the occurrence of gene introgressions within the subgenus *Mecodium*. Together with distributional, morphological and ecological information, these results can support a

revision of the subgenus, as discussed in the following chapters.

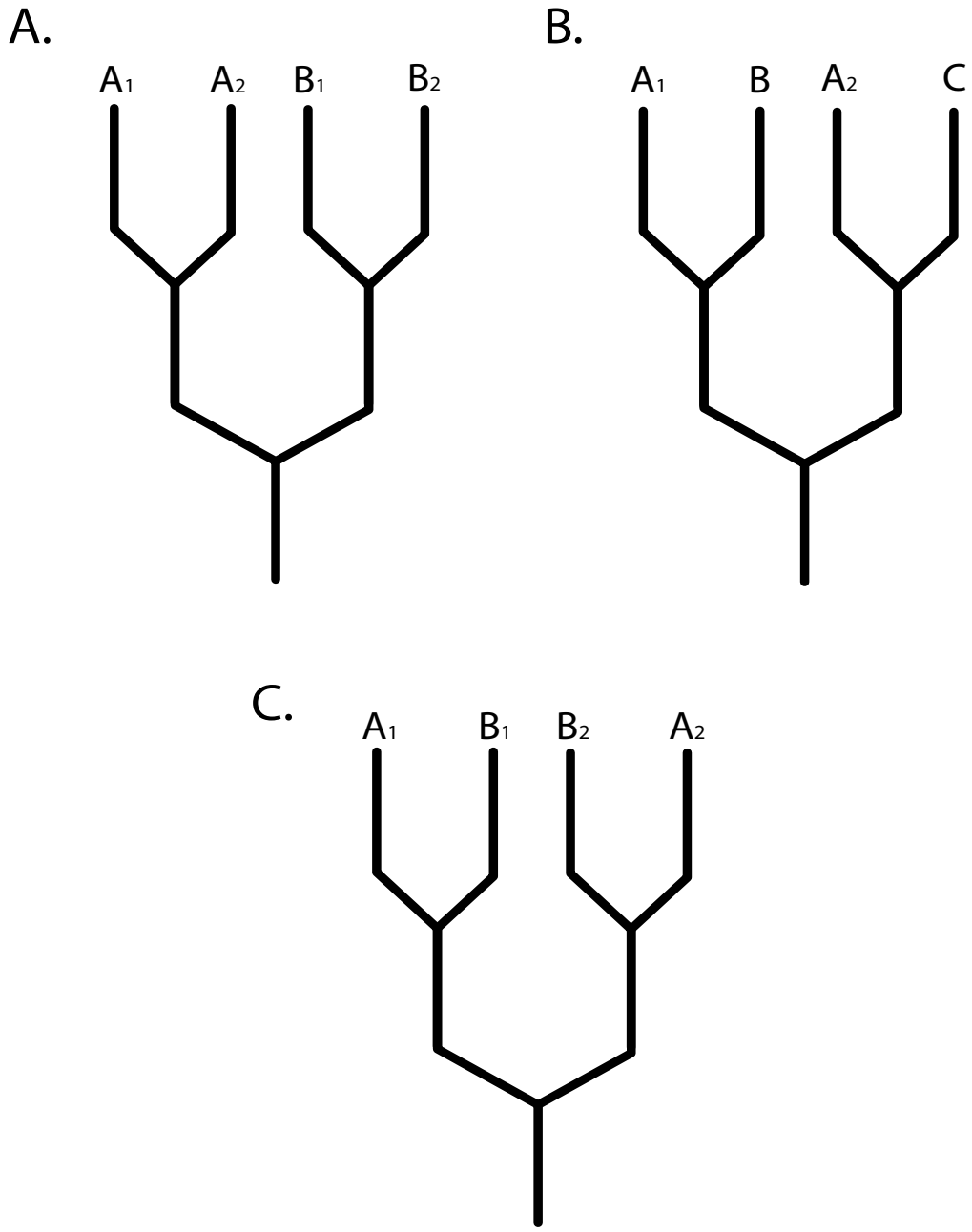

Figure 7: Schematic explanation of the possible tree topologies to be expected. A. Monophyletic topology: species A and B, represented by 2 specimens each, are grouped independently; B. Polyphyletic topology: species A is polyphyletic, and this may be explained by the occurrence of cryptic species, polymorphisms or genetic introgressions; C. Paralogy: expected topology occurs more than one time inside of the tree, indicating the inclusion of paralog sequences in the dataset.

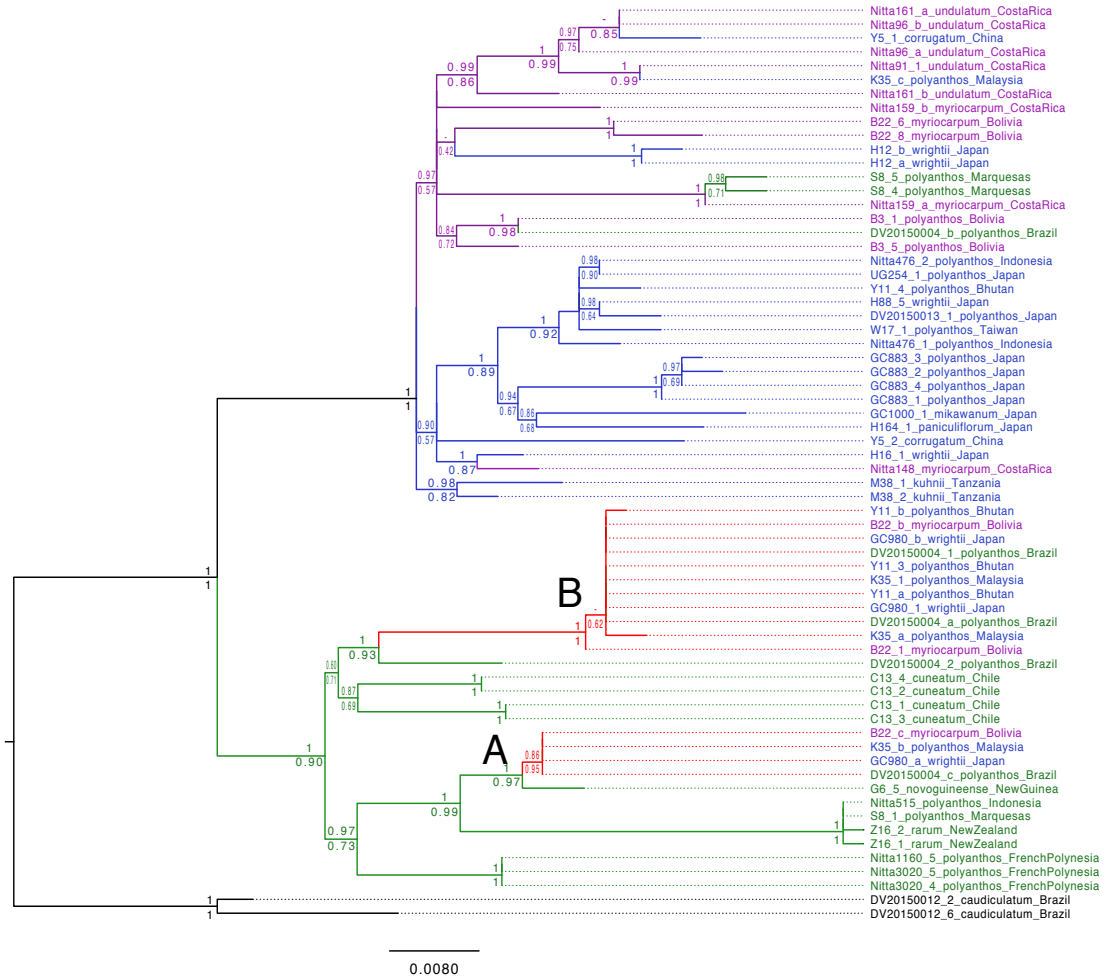

Figure 8: Phylogenetic tree for the subgenus *Mecodium*, focusing on the *Hymenophyllum polyanthos* complex. Sequences of DNA for the nuclear *LEAFY* first intron regions are compared here. Values over the branches indicate the Bayesian inference posterior probability, while values below the branches indicate the ML probability. Clade marked in black represent outgroups, and colored taxa are in accordance to groups obtained in figure 3 analysis. The clades marked in red represent taxa that are discussed in detail, probably indicating paralog sequences.

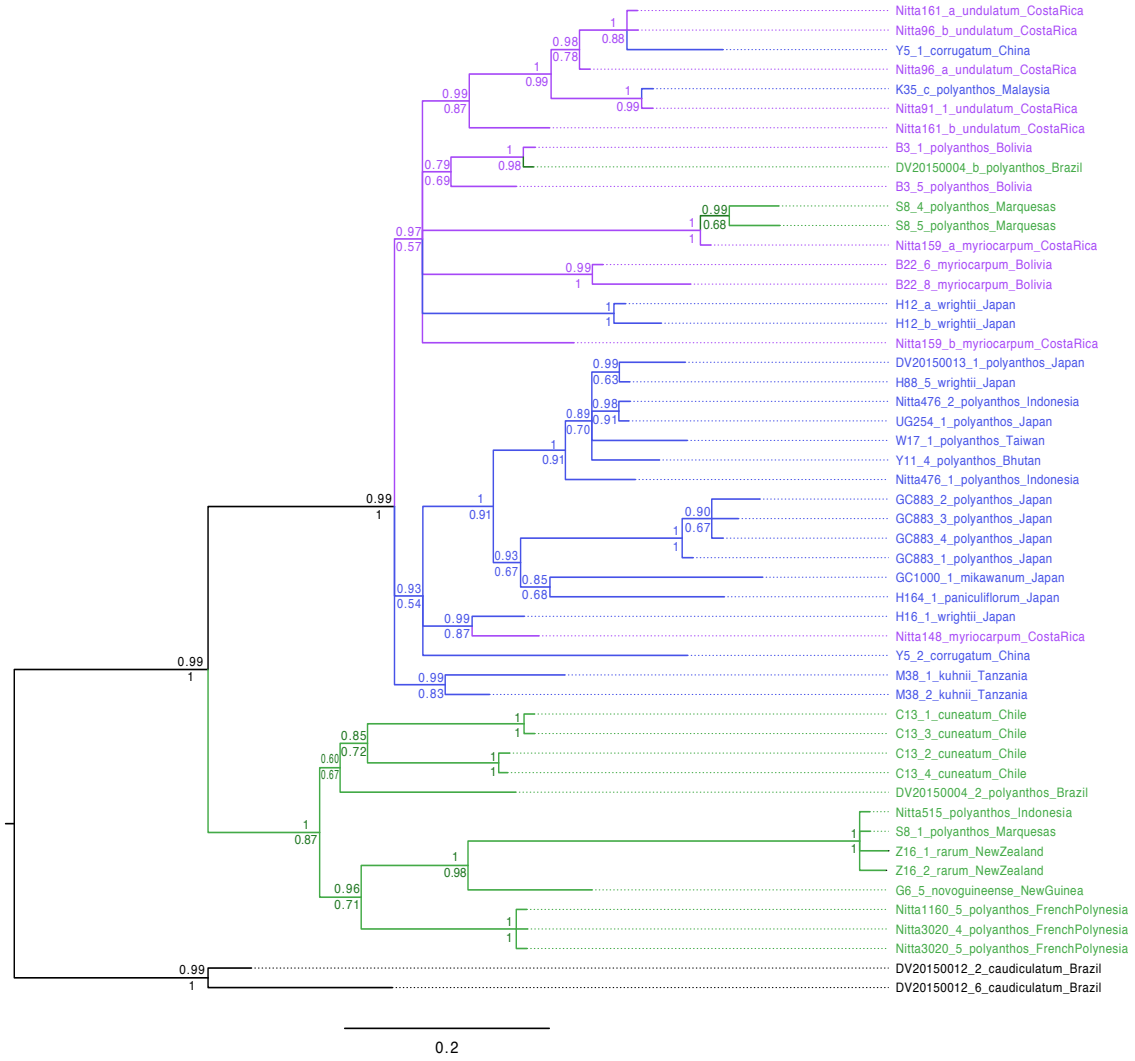

Figure 9: Phylogenetic tree for the subgenus *Mecodium*, focusing on the *Hymenophyllum polyanthos* complex. Sequences of DNA for the nuclear *LEAFY* first intron regions are compared here, but sequences hypothesized as paralog were excluded in this analysis. Values over the branches indicate the Bayesian inference posterior probability, while values below the branches indicate the ML probability. Clade marked in black represent outgroups, and colored taxa are in accordance to groups obtained in figure 3 analysis.

Chapter 4 – Morphological and distributional patterns within the *Hymenophyllum polyanthos* (Sw.) Sw. complex

INTRODUCTION

Overview of the body structure of Hymenophyllaceae

Traditionally the filmy ferns were classified into two genera (*Hymenophyllum* and *Trichomanes*) based mainly on the sori structure of these plants (Morton 1968). Recent investigations have circumscribed 9 different genera within the family (Ebihara *et al*. 2006), elucidating that other morphological traits are also relevant for the taxonomy of the group.

As discussed by Dubuisson *et al*. (2003), all extant basal leptosporangiate ferns (*i.e.* Osmundaceae family and gleicheniaceous ferns) are terrestrial, with exception of the filmy ferns. The epiphytic habit appears within the filmy ferns together with the emergence of long-creeping, wiry rhizomes bearing few to none roots. Nevertheless, within the different lineages of filmy ferns, the terrestrial habit can still be observed, hypothesized either as a plesiomorphic conversion, or an evolutionary reversion (Dubuisson *et al.* 2003).

Epiphytic plants with long-creeping, wiry rhizomes usually represent the

“hymenophylloid ferns”, while the “trichomanoid ferns” can bear creeping, erect or ascending rhizomes, including hemi-epiphytic and terrestrial species. Appendices on the rhizomes are also variable between these 2 morpho-groups: rhizomes glabrous or bearing light-colored hairs can be observed in “hymenophyloid ferns”, while blackish-hairs are present in the “trichomanoid ferns”.

Several authors have described the rhizome stele structure (Boodle 1900; Hennequin 2004; Le Thomas 1961; Ogura 1938), being reported several types according to the position and number of vascular cells. According to these reports, some of the “trichomanoid ferns” present more ‘massive steles’, composed by a ring of metaxylem enclosing parenchyma and protoxylem (Boodle 1900). On the other hand, “hymenophylloid ferns” usually bear simpler steles, including the ‘reduced’, ‘dorsi-ventral’ and ‘subcollateral’ types (Ebihara *et al.* 2007, Hennequin *et al.* 2006), suggesting a reduction of the vascular system.

The leaf structure is divided between the stipe (= petiole) and the lamina. The petiole of filmy ferns is non-articulate to the rhizome and can be winged or not, depending on the group. Indumenta can also be attached both to the petiole or the

lamina, depending on the species. The lamina can be simple-pinnatifid up to 4-pinnate, oblong, flabellate, trapeziform or ovate-lanceolate (Larsen 2014). Each division of the lamina is called a pinna and its dimensions are also variable within groups. The rachis of the pinna can also be winged or not. Regarding the lamina dimensions, Dubuisson *et al.* (2003) proposes that epiphytic/ saxicolous taxa in *Trichomanes* exhibit a reduced body size (dwarfness) as a probable strategy for adaptation to hygrophilous environments.

Sori are terminal to the veins and composed by an indusium covering a receptacle, which bears the sporangia. A bivalved pattern of indusium is more commonly found within the "hymenophylloid ferns", while a tubular type is more common within the "trichomanoid ferns". Traditionally, the indusium shape was used to circumscribe the genus *Hymenophyllum s.l.* and *Trichomanes s.l.* (Morton 1968). The position of the sori is also correlated with the group, being of three kinds: catadromous (growing over a distal vein and blocking further growth of the segment), paratactic (growing over a proximal or distal vein and, thus, not blocking the further growth of the leaf) or pantotactic (growing on the margins of the leaf and occurring only on the genus

Cardiomanes) (Hennequin 2004, Larsen 2014, Prantl 1875).

Morphological traits in Mecodium

Within *Hymenophyllum*, the subgenus *Mecodium* is the less distinguishable morphologically. Even after its circumscription by Ebihara *et al.* (2006) based on molecular evidence, the only synapomorphy pointed for the genus is the chromosome number (x = 28). Under this circumscription, the subgenus is correspondent to the *Hymenophyllum polyanthos* complex and its relative species, which covers a broad geographical distribution, covering both New and Old World regions (Fig. 4).

Hymenophyllum polyanthos s.l. has been consistently evidenced as a non-monophyletic lineage in chapters 2 and 3, being the type clade circumscribed to the lineages distributed in the neotropics and sister to *H. undulatum* (Fig. 6). As a consequence, any another lineage of polyanthos that emerges in the analysis is up to further taxonomical treatment. Moreover, at least two big lineages are present inside the complex (correspondent to the "Pacific/ S. America" clade and the "Asia/ Neotropics" clade, Fig. 6).

Research Goals

Based on the groups obtained through the phylogenetic analysis of DNA markers, one can observe if the reported wide phenotype variation is still maintained when separating individuals according to these phylogenetic taxa. If, after analysis, different lineages of *H. polyanthos s.l.* emerge as morphologically similar, the investigated traits might be defined as homoplastic within the subgenus and, hence, should be avoided for further taxonomical analysis. On the other hand, the topology of the phylogeny showing that several well-circumscribed species appear interspersed to *H. polyanthos s.l.* specimens might also indicate that taxonomically informative traits can be found.

The goal of this chapter is to bring a morpho-anatomical analysis compared to the groups obtained through the phylogenies shown in chapters 2 and 3. Ultimately, the objective of this research is to point out taxonomically informative traits that may aid the treatment of the obtained taxa into novel categories within the subgenus *Mecodium* in a future revision work.

MATERIAL AND METHODS

Sampling – Morphological, anatomical and distributional information were obtained both from DNA sampled specimens, and from voucher species from the TNS, TI and SP herbaria (appendix 3). Based on its locality, additional voucher information from the BM and MO online herbaria were associated to one of the groups found in the phylogenetic analysis from chapter 2. For the morphometric measures, three fertile leaves were selected for each specimen. Specimens with damaged leaves, or bearing only sterile leaves were not considered for analysis. In total, 95 samples were analyzed morphologically (PSA Group: n = 18; N Group: n = 22; A Group: n = 55; appendix 4) and 28 samples were used for the anatomical measurements (PSA Group: n = 4; N Group: n = 6; A Group: n = 20; appendix 5).

Morphometric measures – Voucher samples were scanned and measured using ImageJ v. 1.48 (Abràmoff *et al.* 2004). The leaves of the samples were analyzed under 7 parameters as shown in figure 10: petiole length (PL), lamina length (LL), lamina width (LW), lateral pinnae length (LPL), lateral pinnae width (LPW), pinnae distance (PD) and lateral pinnae insertion angle (A). Similar traits were compared before by

Dubuisson (2003) for the genus *Trichomanes*, proving to be of relevance for the proposition of ecological hypothesis. Only primary pinnae were considered by this analysis and pinnatifid plants (*e.g. H. wrightii*) were compared only in regards to the PL, LL and LW parameters. Each parameter was measured in 3 different leaves per sample, and the average of these values is compared. Available vouchers varied in conservation condition, and many were damaged and not considered in this study.

Anatomical assays – For the anatomical assays, both fresh and voucher samples were used (appendix 5). Fresh samples were fixed in FAA (1 : 1 : 8 Formalin - Acetic Acid – ethanol 50%) for one night. In the case of voucher specimens, rehydration was performed by immersing the samples into 1 : 1 water - glycerol solution at 60^{o}C for one night (adapted from Kobayashi & Suzuki 2014).

In the sequence, samples were immersed in growing concentrations of ethanol for dehydration. From the initial condition, samples were moved to a 50% ethanol solution for two hours, followed by 60%, 70%, 80%, 90%, 95% and 100% ethanol solutions for two hours each. After this, samples were immersed in 100% ethanol once again for 12 hours.

Following the dehydration, samples were progressively immersed in TechnoVit 7100 resin. For this, the immersion time and ratio sequences were of: 2 : 1 ethanol - TechnoVit solution for 3 hours; 1 : 1 ethanol - TechnoVit solution for 3 hours; 1 : 2 ethanol - TechnoVit solution for 12 hours; 100% TechnoVit solution for 24 hours; and again 100% TechnoVit solution for 3 days. After the immersion in resin, samples were hardened and cut using an automated microtome. Rhizome samples were cut transversaly 3 μ m thick and observed under a light microscope. Cortical parenchyma tissue was danified in most of the samples and, for that reason, measurements on the rhizome sizes were based on the diameter of the medule, counting from the sclerenchyma tissue layer. Besides that, the sclerenchyma tissue layer thickness and the number of fully developed metaxylem cells were recorded for analysis, as shown in figure 10.

Statistical analysis & description of groups – The data acquired was compared at first with a Principle Component Analysis (PCA), using Minitab v. 17 (Ryan *et al*. 1994). Parameters were compared as ratios between the lamina length and lamina width (LL:LW), the lamina length and the petiole length (LL:PL), the lamina length and the

lateral pinnae distance (LL:ND) and the lateral pinnae length and lateral pinnae width (LPL:LPW). Insertion angle of lateral pinnae (A) was the only parameter to be compared separately.

Additionaly, data were compared through boxplots and the significance of the observed differences were tested through multistate-ANOVA tests using R language (R Core Team, 2006). Morphology and anatomy data sets were addressed separately and compared to the groups obtained in chapter 2 analyses (*i.e.* A group, N group and PSA group). For some samples, missing data were included, corresponding to pinatifid leaves which the measurement of lateral pinnae was not possible (appendix 4). Four models (Pillai's trace, Wilks' lambda Hotteling's trace and Roy's root) were tested for each data set, resulting in similar results between the different models (table 5). For the pairwise comparison of groups, adjustments of the *p*-values were performed under the Holm method. Based on these results, descriptions for the circumscribed groups are presented at the end of the chapter.

RESULTS AND DISCUSSION

Morphological parameters

In total, 18 samples (= 54 measurements/ parameter) of "PSA" clade, 22 samples (= 66 measurements/ parameter) of "N" clade and 55 samples (= 165 measurments/ parameter) of "A" groups were analysed (appendix 4). Figure 11 shows the score plot for the first two components of the Principal Component Analysis for each analyzed specimen and according to the groups obtained in the phylogeny of chapter 2. The Eigenvalue for each component is represented in figure 12 and the principal coefficients for each component are shown in table 4, according to each relevant variable.

Overall, individuals from the Pacific-South America clade appear distributed in the direction of the upper right part of the graph, while Asia-Neotropics clade represents are more concentrated in the lower left part of the graph (Fig. 11), suggesting a cluster based on the combination of both components, in special principal component 2. Looking at the coefficients for the first two most influential principal components, one can observe that for PC1 the LL/LW and the LL/PD variables were most influential

(coefficient = 0.574344659 and 0.557302741 respectively), while for the PC2 the pinnula angle and LPL/LPW (coefficient = -0.586987493 and 0.785249654 respectively) were the most relevant variables (Table 4).

Figure 13 shows a comparison of the average variance of the five investigated parameter ratios and between the three groups obtained with the phylogenetic analysis. Independently from the parameter ratio to be investigated, “A” groups and “N” clade present overlapping measurements, suggesting that they are morphologically similar to each other. Despites the maximum and minimum values represented by the bars over the boxplots, the quartile limits indicates that the variation from the average measurements is not wide. On the other hand, “PSA” clade plants present more divergent values when compared to “A” groups and “N” clade plants. Also, the quartile limits are broader in “Pacific” plants’ boxplots, indicating a wider range of variation.

The MANOVA results are reported in table 6. Significant differences were observed between A groups and PSA clade, were MANOVA (Pillai’s Trace = 0.60744, $F_{5,62}$ = 19.188, $p < 0.001$), and between N clade and PSA clade (Pillai’s Trace = 0.72673, $F_{5,34}$ = 18.084, $p < 0.001$). On the other hand, no significant difference were

observed between the A groups and the N clade (Pillai's Trace = 0.13384, $F_{5,66}$ = 2.0398, p = 0.08436), suggesting that while the PSA clade is considerably different from the other groups, A groups and N clade are similar in morphology.

Overall, these results suggest that plants from the "PSA" clade have more closely appressed pinna, inserted at an angle up to 50 degrees in comparison to the rachis costa. This renders more slender leaves, reflecting in the LL/LW ratio, which is also significantly different between the "AN" and "PSA" clades (Fig. 13, Table 6). Besides this, LL/PL and LL/PD ratios were significantly different between "A" groups and "PSA" clade plants, indicating that "PSA" plants bear longer and more frequently divided fronds than "A" groups' plants.

Anatomical observations

The measured parameters for anatomical traits can be observed in appendix 5 and are summarized in boxplots in figure 14. All of the observed specimens beared a protostelic 'dorsi-ventral' stele as described by Ebihara *et al*. 2007. This kind of stele is characterized by the presence of protoxylem and metaxylem organized relatively to the dorsi-ventral position of the rhizome.

Several assays presented a damaged cortex, due to the fragility of cortical parenchyma cells. For this reason, the diameter of the rhizome was measure from the sclerenchyma strands and mentioned here as 'inner diameter' (Fig. 10). The 'inner diameter' varied from about 0.2 mm to about 1 mm between samples. The vascular bundle is small, composed by few metaxylem cells (appendix 5, Fig. 14).

Differently from the morphological parameters explored on the previous sections, anatomical parameters' MANOVA results do not point to any signficant correlation ($p > 0.01$) between the observed parameter and the analysed pairwise groups, *i.e.* A *vs* N (Pillai's Trace = 0.26435, $F_{3,19} = 2.2758$, $p = 0.1126$), A *vs* PSA (Pillai's Trace = 0.044776, $F_{3,17} = 265562$, $p = 0.8492$) and N *vs* PSA (Pillai's Trace = 0.57611, $F_{3,6} = 2.7182$, $p = 0.1374$). This suggests that anatomic traits as explored here are not valid for taxonomic purposes when comparing the groups obtained in chapter 2 analyses.

CONCLUSIONS

In this chapter, morphological and anatomical measurements were compared

to the phylogenetic groups obtained in chapter 2 in order to propose a new system for the subgenus *Mecodium* as a whole. The lineages supported by the molecular analysis were compared by different morphological parameters and, as a result, potential diagnosis traits emerged from the analysis. Between the morphological traits, the pinnae insertion angle appeared as the most correlated trait, although some other measurement ratios also showed less promenient correlation. On the other hand, anatomical parameters investigated here did not show correlation to the lineages found in chapter 2, indicating that variations on anatomy evolved similarly between the investigated lineages. The results of this chapter may support a new taxonomic treatment for the subgenus *Mecodium* as a whole. This topic is explored in the next chapter.

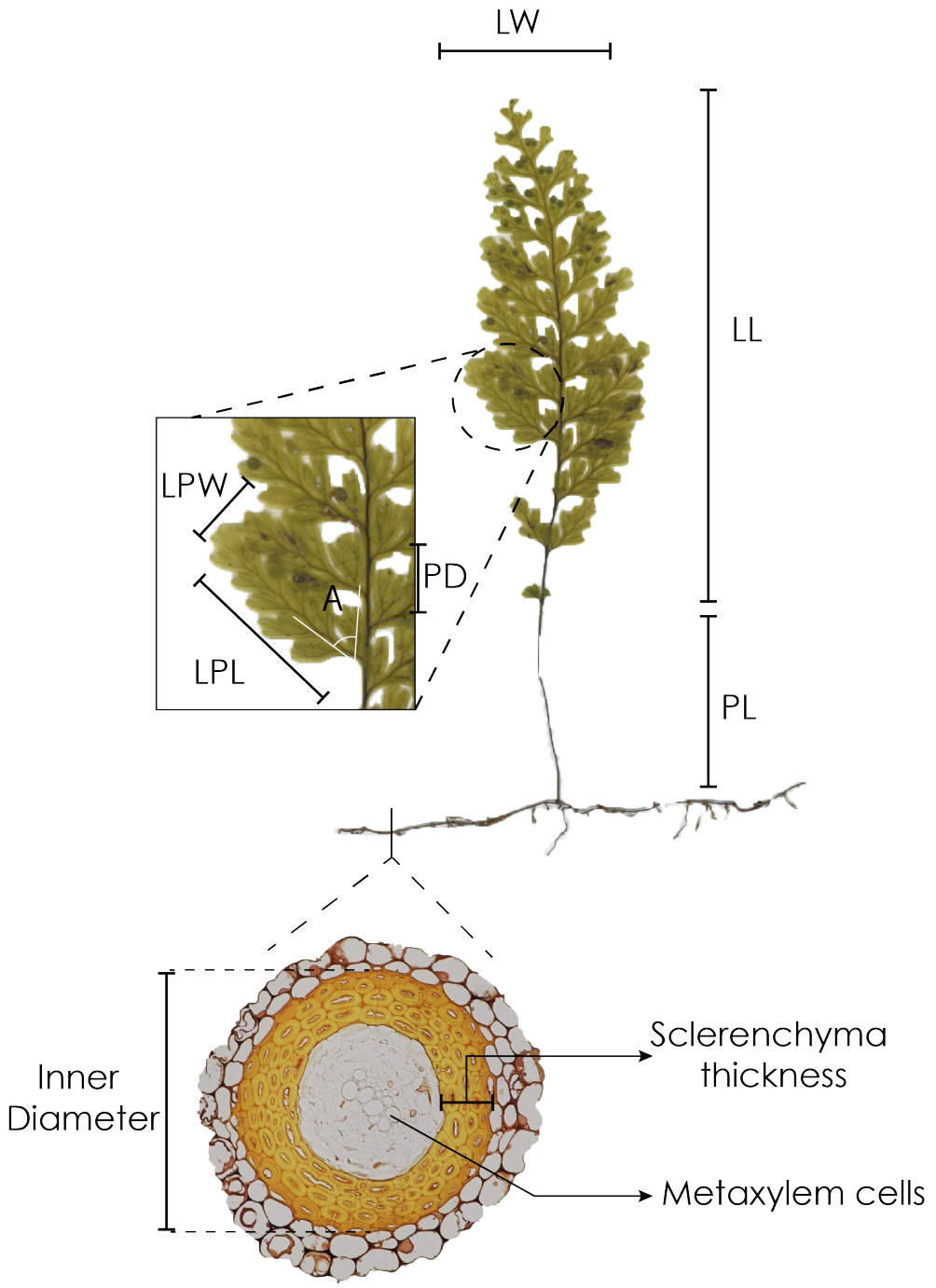

Figure 10: Scheme of the morphological (upper part) and anatomical (lower part) parameters explored. LL: Lamina Length, LW: Lamina Width, PL: Petiole Length, PD: Pinnae Distance, A: Angle of insertion, LPW: Lateral Pinnae Length, LPW: Lateral Pinnae Width.

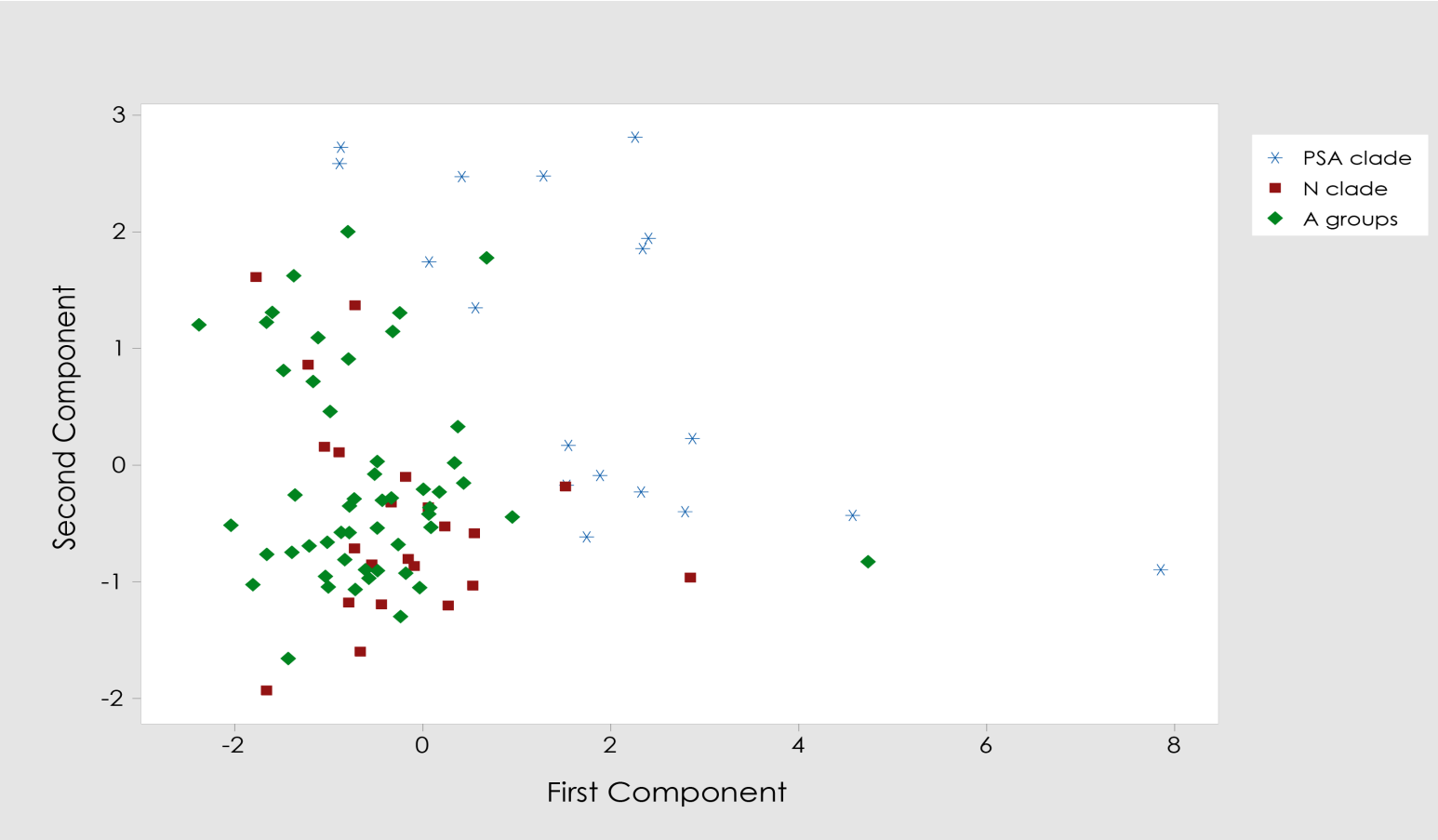

Figure 11: Score plot for the Principal Component Analysis of 5 morphological parameters (*i.e.* LL:LW, LL:PL, LL:PD, LPL:LPW and A, as explained in the methods section). Each specimen is compared according to the 1st and 2nd principal components, and represented according its position in the phylogeny presented in chapter 2 (*i.e.* PSA clade, N clade and A groups).

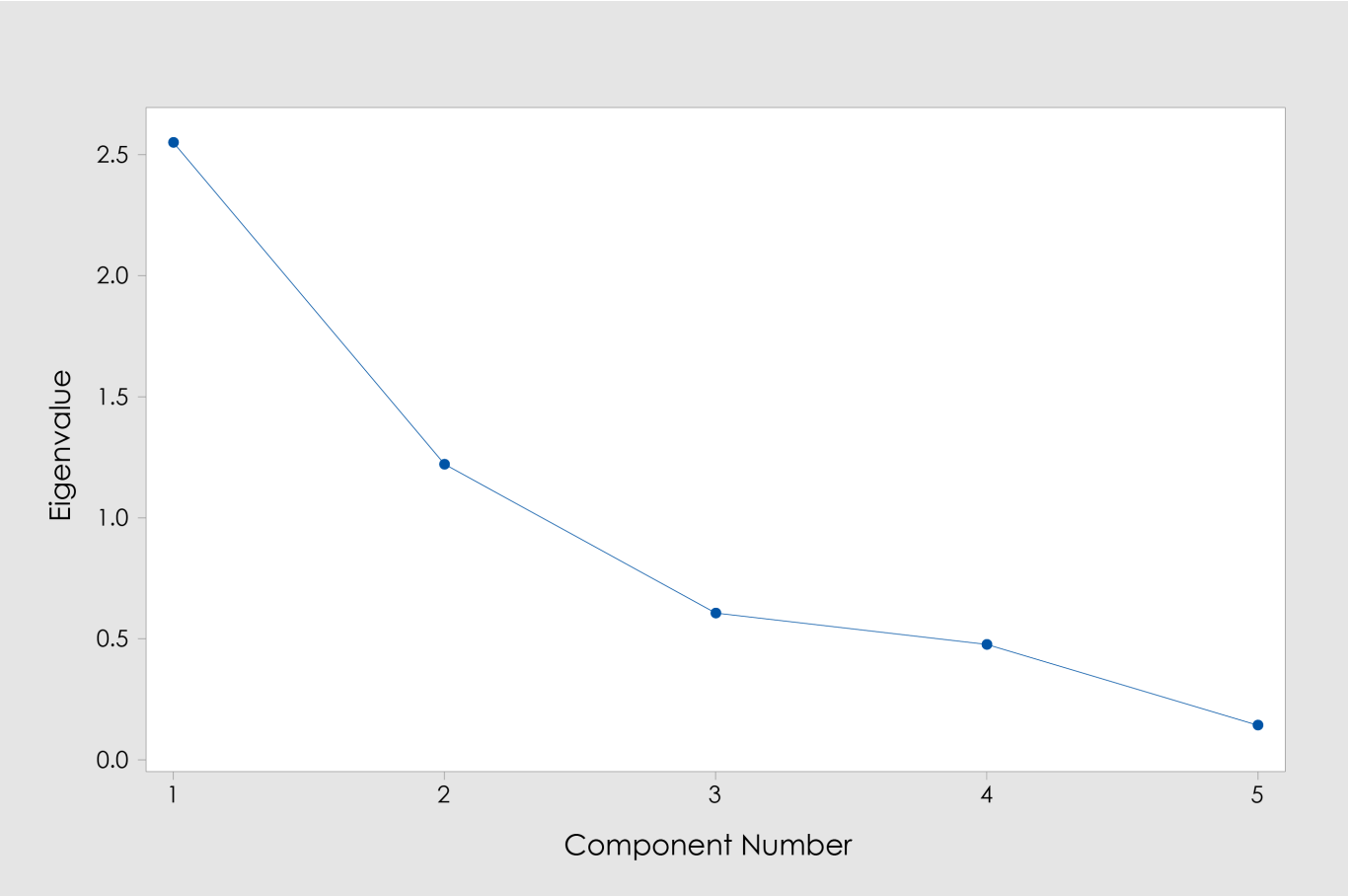

Figure 12: Plot of the Eigenvalue of each principal component from the PCA for the 5 parameters explored here. (*i.e.* LL:LW, LL:PL, LL:PD, LPL:LPW and A, as explained in the methods section). The graph shows that the first two components have the highest values and, thus, are the most influent for the clustering analysis.

Table 4: List of Principal Components and its values for each parameter used in the PCA. LL: Lamina Length; LW: Lamina Width; PL: Petiole Length; PD: Lateral Pinulle Distance; LPL: Lateral Pinnae Length; LPW: Lateral Pinnae Width.

Parameter	PC1	PC2	PC3	PC4	PC5
LL/LW	0.574344659	-0.052208221	0.273075752	0.272669492	-0.720057981
LL/PL	0.501190544	0.043125034	-0.087333055	-0.858987048	0.038242099
LL/PD	0.557302241	-0.185049173	0.287262628	0.317257396	0.687021792
Angle	-0.315503441	-0.586987493	0.686238344	-0.285867009	-0.057098601
LPL/LPW	-0.093851173	0.785249654	0.603622141	-0.073623345	0.069244872

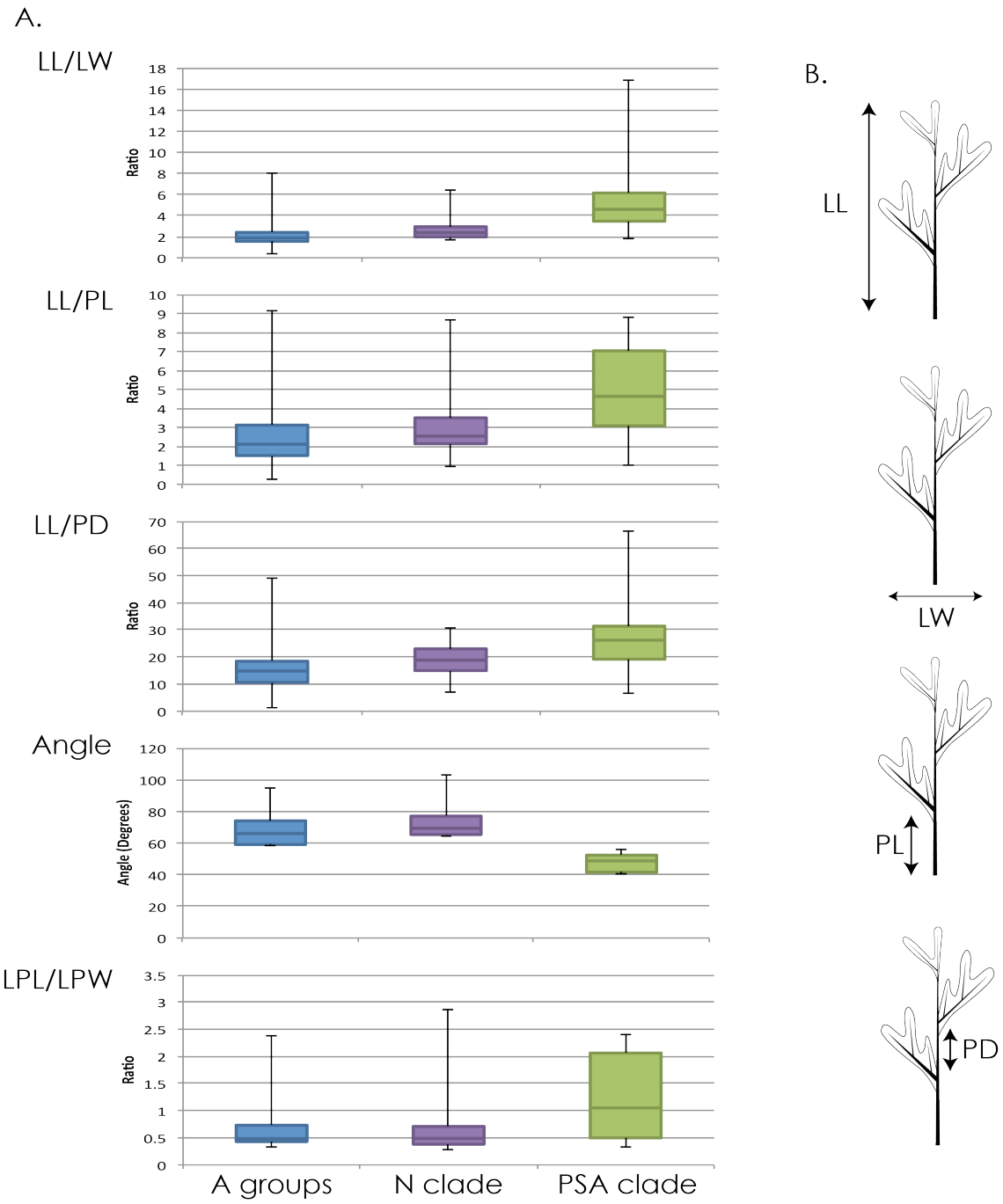

Figure 13: A. Boxplots showing variation of each parameter between the different lineages obtained through the phylogenetic analysis. From left to right, boxplots represent "A" groups (in blue, 55 samples, n = 165 measurements/parameter), "N" clade (in purple, 22 samples, n = 66 measurements/parameter) and "PSA" clade (in green, 18 samples, n = 54 measurements/parameter). From top to bottom, the five parameters used for the PCA are compares (*i.e.* LL:LW, LL:PL, LL:PD, LPL:LPW and A, as explained in the methods section). B. On the right side, a schematic drawing shows the kind of measurement represented by the initials.

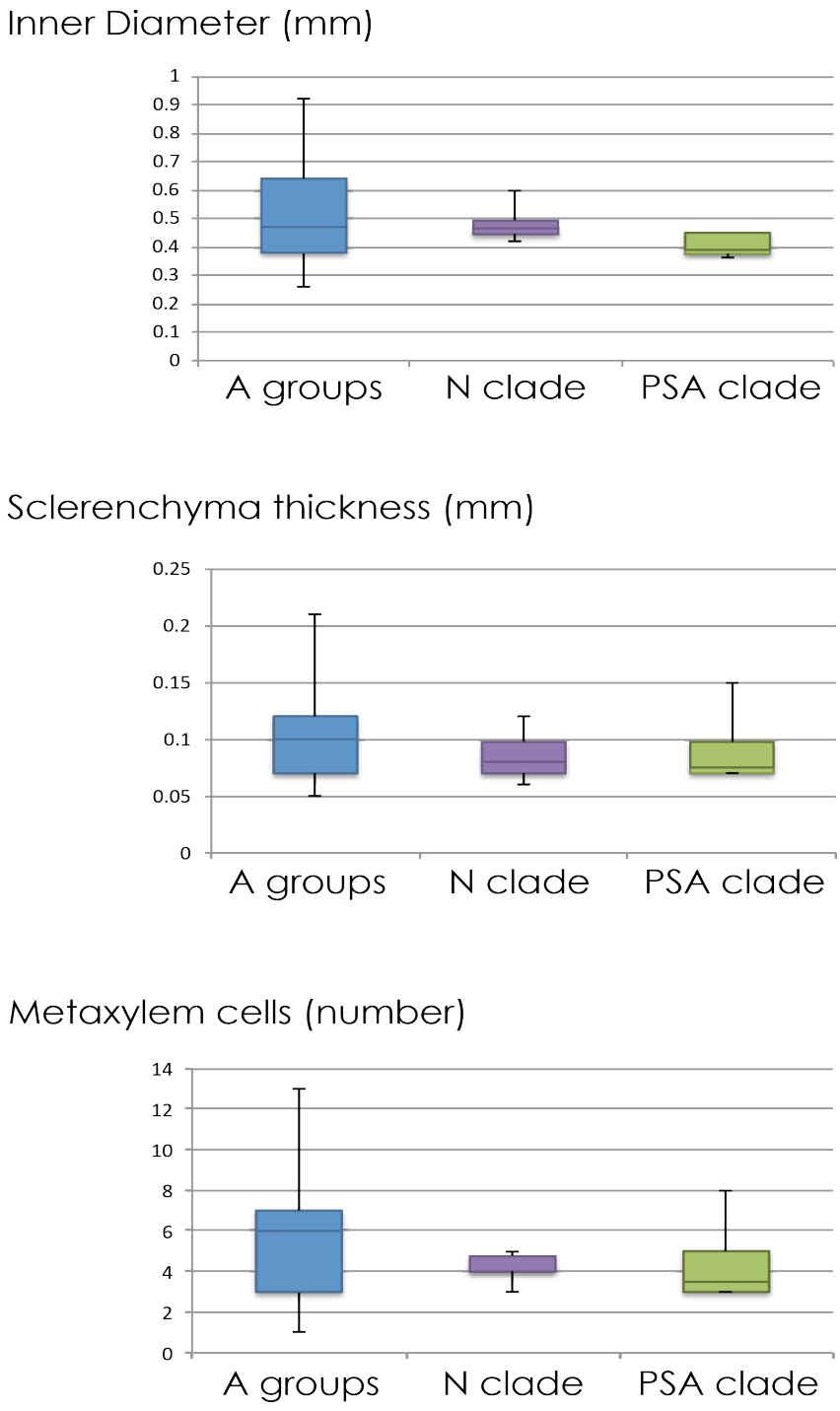

Figure 14: Boxplots showing variation of each anatomical parameter between the different lineages obtained through the phylogenetic analysis. From left to right, boxplots represent "A" groups (in blue, 20 samples), "N" clade (in purple, 6 samples) and "PSA" clade (in green, 4 samples). From top to bottom, the parameters are: inner diameter of the rhizome (mm); thickness of the sclerenchyma band (mm); and number of full developed metaxylem cells.

Table 5: Multivariate test for four different models using R language and using as factors the groups obtained in the phylogeny of chapter 2 (*i.e.* A group, N group and PSA group). The table compares each test result between the two data sets applied here (*i.e.* morphology and anatomy data sets). For the morphology data set, the *p* values obtained were lower than 0.001 for all tests, indicating that the morphological observations are significantly betweent the addressed groups. For the anatomy, all tests show no significant difference between groups.

Test	Df		Value		Approx. *F*		Hypothesis Df		Error Df		*p*-value	
	Morphology	Anatomy	Morphology	Anatomy	Morphology	Anatomy	Morphology	Anatomy	Morphology	Anatomy	Morphology	Anatomy
Pillai's Trace	2	2	0.68904	0.28599	8.8302	1.2792	10	6	168	46	<0.001*	0.2855
Wilks' Lambda	2	2	0.3665	0.72449	10.82	1.2822	10	6	166	44	<0.001*	0.2851
Hotelling's Trace	2	2	1.577	0.36581	12.931	0.36581	10	6	164	6	<0.001*	0.287
Roy's Root	2	2	1.4742	0.32069	24.766	2.4586	5	6	84	23	<0.001*	0.08845

Table 6: MANOVA results for the morphology and anatomy data sets taking as factors the groups obtained in chapter 2 analyses (*i.e.* A group, N group and PSA group). The table shows a comparison of the Pillai's trace test, F-statistics and p-value for each pairwise combination of the groups investigated. Significant differences (p-value < 0.05) are denoted by * marks.

Morphology							
Factor	Df	Pillai's Trace	Approx. *F*	Hypothesis Df	Error Df	*p*-value	Adjusted (Holm)
A vs. N	1	0.13384	2.0398	5	66	0.08436	0.08436
A vs. PSA	1	0.60744	19.188	5	62	1.732E-11*	5.196E-11*
N vs. PSA	1	0.72673	18.084	5	34	9.902E-09*	1.9804E-08*

Chapter 5 – Taxonomic treatment for the subgenus *Mecodium* including the re-validation of *H. sturmii* Bosch. in Brazil

DIVISION OF SUBGENUS *MECODIUM*

By combining the phylogeny results obtained from the molecular analysis of chapters 2 and 3 with the morphometric measurements present in chapter 4, it is evident that the subgenus *Mecodium* is at least divided into 2 lineages: one composed by plants from the Asia-Pacific and South America, and another composed by plants from the Neotropics, Africa and Asia. Hennequin *et al*. (2006) previously estimated these results as possible, but only with the molecular and morphological measurements presented here such hypothesis could be conclusively assessed. Based on this, I suggest the division of the subgenus into 2 sections, as follows.

TAXONOMICAL TREATMENT FOR THE SUBGENUS *MECODIUM*

1) Section *Cuneatae* Vasques *sect. nov.* (Fig. 15-16)

Type: *H. cuneatum* Kunze

Distribution: Chile, Argentina, Brazil (Central to South), La Reunion, Malesia-Papuasia, Australia, Pacific islands.

Diagnosis: Insertion angle of lateral pinnae up to 50°.

Corresponds to the "Pacific - South America" (PSA) clade, including species from Malesia-Papuasia, Australia, Pacific islands and the southern part of South America. Species of this section have more narrow leaves, due the insertion angle of lateral pinnae on the rachis.

Included species: *H. abruptum* Hook.*, *H. copelandii* C.V. Morton*, *H. cuneatum* Kunze, *H. darwinii* Hook.*, *H. fendlerianum* Sturm*, *H. inaequale* (Poir.) Desv., *H. mnioides* Baker*, *H. novoguineense* (Rosenst.) K. Iwats., *H. rarum* R. Br., *H. viridissimum* Feé*.

Asterisks (*) mark species not sampled in the phylogenetic analysis and included here based on distribution and morphological patterns.

2) Section *Mecodium* (Fig. 17-19)

Type: *H. polyanthos* (Sw.) Sw.

Distribution: Venezuela, Peru, Bolivia, Colombia, Paraguay, Brazil (N, NE, Central regions), Mexico, Guatemala, Honduras, El Salvador, Nicaragua, Costa Rica, Panama, Guyana, Surinam, French Guyana, Ecuador, USA, Canada, Alaska, Tanzania,

Madagascar, India, China, Japan, Korea, Malesia countries.

Diagnosis: Insertion angle of lateral pinnae higher than 50°.

Corresponds to the Neotropics (including plants from North and Central America, as well the northern part of South America), African and Asian groups (AN clade). It includes the type species for subgenus *Mecodium*, *Hymenophyllum polyanthos*, which receives a new circumscription here.

Species included: *H. apiculatum* Mett. ex Kuhn, *H. axillare* Sw.*, *H. brevifrons* Kunze*, *H. corrugatum* H. Christ, *H. fumarioides* Bory ex Willd.*, *H. kuhnii* C. Chr., *H. myriocarpum* Hook., *H. ooides* F. Muell. & Baker*, *H. paniculiflorum* C. Presl, *H. polyanthos* (Sw.) Sw., *H. recurvum* Gaudich.*, *H. siliquosum* H. Christ*, *H. undulatum* Sw., *H. wrightii* Bosch.

Asterisks (*) mark species not sampled in the phylogenetic analysis and included here based on distribution and morphological patterns.

HYMENOPHYLLUM POLYANTHOS

Considering the system proposed in the previous section, a further taxonomical treatment specific for *H. polyanthos* samples that have emerged as polyphyletic in this analysis is necessary. As explored before, the type for *H. polyanthos*

is reported from Jamaica and, having that as a premise, the “type clade” is pointed in figure 6, sister to *H. undulatum*. This type clade is described following this section. The remaining *H. polyanthos s.l.* occurrences need taxonomic treatment, as explained in the following.

In the “Pacific - S. America” (PSA) clade, at least three different lineages of *H. polyanthos s.l.* need taxonomic treatment: i) PSA1: the Brazilian specimens, sister to *H. cuneatum* from Chile, and closely related to the La Reunion specimens; ii) PSA2: from Malaysia and Indonesia, closely related to *H. novoguineense* from New Guinea; and iii) PSA3: from French Polynesia, sister to *H. rarum* from New Zealand.

The “Asia” groups remain not completely resolved herein, but the actual topology already points to the existence of at least 5 lineages of *H. polyanthos s.l.* needing treatment: J1: sister to *H. mikawanum* from Japan; IBC, MI and JT: including specimens from Japan, Taiwan, Malaysia, Indonesia, Cambodia and Buthan, the specimens included here are grouped with *H. paniculiflorum* and may still be further divided into more specific lineages based on further molecular evidence; J2 and J3: represented herein by two samples from Japan that emerge in a grade in the acquired

phylogeny; and M: from Malaysia (Fig. 6).

Finally, the "N" clade remais represente by three species: *H. myriocarpum*, *H. undulatum* and *H. polyanthos sensu stricto*, described as follows:

Hymenophyllum polyanthos (Sw.) Sw., J. Bot. (Schrader) 1800 (2): 102. 1802. *Trichomanes polyanthos* Sw. Prodr. 137. 1788. Type: "Jamaica", *O. P. Swartz s.n.* (holotype S06-1597; isotypes B-W 20235, BM000936765, S-R-2978, S-R-6211, S-R- 6212).

Hymenophyllum clavatum Sw., J. Bot. (Schrader) 1800(2): 101. 1801. TYPE: "Jamaica", *O. P. Swartz, s.n.* (holotype SBT10582; isotype B -W 20237).

Habit epiphyte. **Rhizomes** long-creeping, ca. 0.5 mm in diameter, glabrous. **Fronds** monomorphic, 1-pinnate, up to 7 cm long, ca. 2 cm wide; **stipes** approximately 1/2 the length of the frond, glabrous; **pinnae** not articulate, ovate-lanceolate, up to about 7 pairs per pinna, insertion angle of ca. 65^{o}, lamina membranaceous, free-veined, middle pinnae bigger than proximal and distal ones, margins entire; **sori** terminal to the veins, on receptacules involved by a bivalvated annuli, ca. 0.1 cm x 0.15 cm, trapezoid.

Distribution: Jamaica, Costa Rica, Bolivia, North Brazil

HYMENOPHYLLUM STURMII BOSCH. IN BRAZIL

The description of Brazilian Flora has started in the XIX century, with works

of naturalists such as A. Cogniaux, C.D. Martius, A.G. Eichler and I. Urban responsible for the edition of the *Flora Brasiliensis*, the most important taxonomical work for Brazil, including more than 22000 species descriptions, being ca. 6000 of them new entries (Cogniaux *et al*. 1883).

It is estimated that more than 250 thousand species of plants are known Worldwide, and about 14% of these species occur in Brazil (Peixoto & Morim 2003, Shepherd 2003). As exposed by Tryon (1972), the Brazilian region (together with the Mexican and Andean) corresponds to a center of diversity and endemism, concentrating most part of the continental species of America. Nevertheless, works of Flora in Brazil have constantly brought new species, indicating that there is still much of the diversity to be discovered in the region.

Based on the results of the molecular phylogenetic analysis explored in chapter 2, *Hymenophyllum polyanthos* (Sw.) Sw. is restricted to the Neotropics, thus becoming necessary new circumscriptions of any other occurrences around the world. Herein, I explore a treatment for the Brazilian occurrence of *H. polyanthos s.l.* (*i.e.* PSA1 clade in fig. 6), which is described as a revalidated species: *Hymenophyllum*

sturmii Bosch

Hymenophyllum sturmii Bosch Nederlandsch Kruidkundig Archief 5(2): 152. 1861 (Fig. 20) – Syntype: Brasil, Hab. Brasilia (Rio de Janeiro, Sierra dos Orgaos, etc.), *C. Gaudichaud s.n.* (not observed), *A. Vauthier s.n.* (not observed), *H. K. Beyrich s.n.* (not observed).

Plants epiphyte. Rhizomes long-creeping, ca. 0.3mm in diam, glabrous. Fronds monomorphic, 1-pinnate, ca. 10 cm long, laminae ca. 2 cm wide; stipes approximately 1/5 the length of the frond, not winged, glabrous; pinnae ovate-lanceolate, ca. 1cm x 0.4cm, light-green, glabrous, insertion angle of ca. 40^{o}, not articulate, free-veined, middle pinnae bigger than proximal and distal ones, margins entire; sori terminal to the veins, on receptacules involved by a bivalvated annuli, ca. 0.2 cm x 0.2 cm, orbicular.

Diagnosis: *Hymenophyllum sturmii* is firstly reported by Bosch in 1861 as a plant from the "Serra dos Órgãos" montaneous formations in Rio de Janeiro, Brazil, and very similar to *H. polyanthos* (Bosch, 1861). *H. sturmii* is closely related to the Chilean species *H. cuneatum*, being differentiated by the format of the pinna, which is trapeziform to trianguliform in *H. cuneatum* and lanceolate to oblong in *H. sturmii,* and by the format of the annuli, which is cuneate in *H. cuneatum* and round in *H. sturmii*.

Distribution: Argentina, Brazil (South, Southeast and Central regions) and Bolivia.

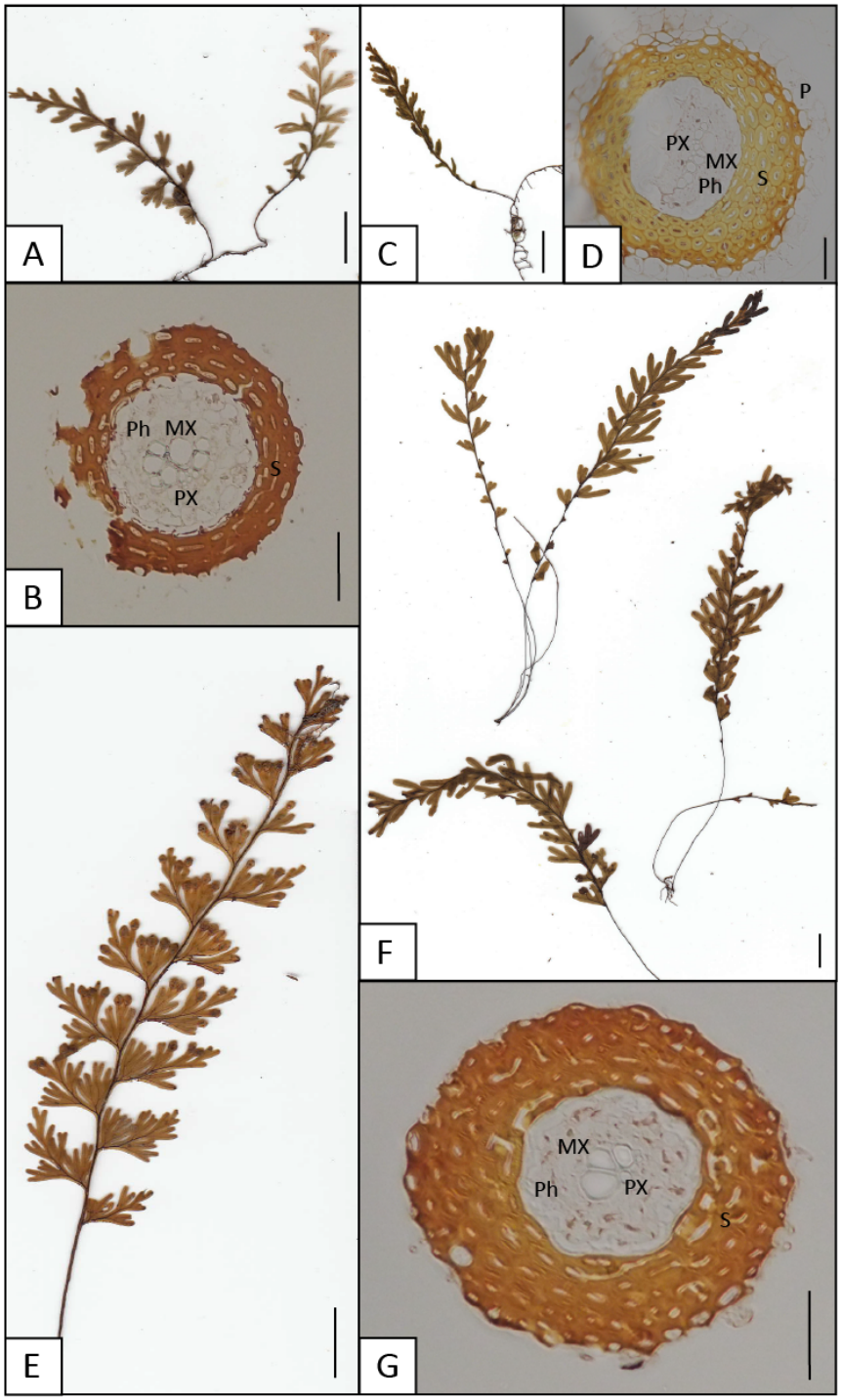

Figure 15: Specimens representative from section *Cuneatae* Vasques *sect. nov.*. A-B: *H. cuneatum* (C13) from Chile, A: lamina detail (scale = 1cm), B: transversal assay of the rhizome (scale = 0,1mm); C-D: *H. polyanthos* (K12) from Malaysia, C: lamina detail (scale = 1cm), D: transversal assay of the rhizome (scale = 0,1mm); E: *H. polyanthos* (K40) from Malaysia, lamina detail (scale = 1cm); F-G: *H. rarum* (Z16) from New Zealand, F: lamina detail (scale = 1cm), G: transversal assay of the rizhome (scale = 0,1mm). On the anatomical assays pictures, the initials stand for: PX: protoxylem, MX: metaxylem, Ph: phloem, S: schlerenchyma, P: parenchyma.

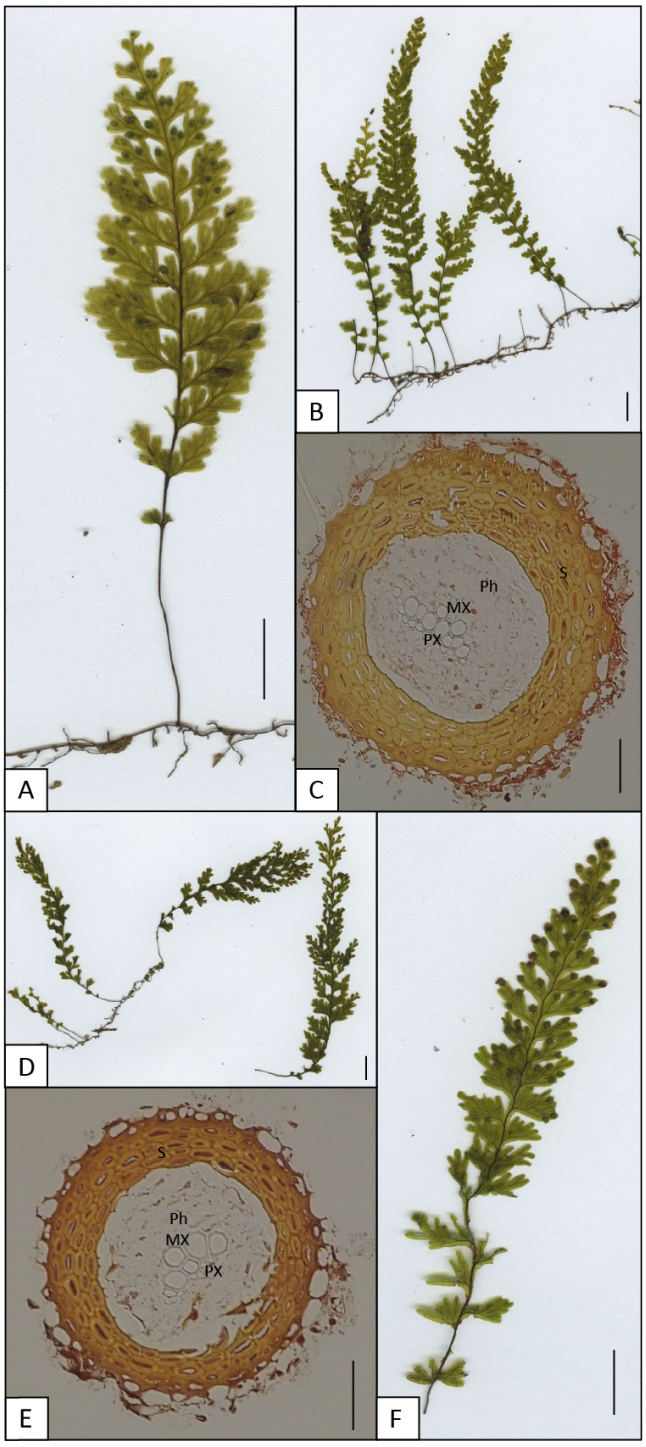

Figure 16: Specimens representative from section *Cuneatae* Vasques *sect. nov.*, in specific Brazil. A: *H. polyanthos* (DV2015001), lamina detail (scale = 1cm); B-C: *H. polyanthos* (DV20150011), B: lamina detail (scale = 1cm), C: transversal assay of the rhizome (scale = 0,1mm); D-E: *H. polyanthos* (RH756), D: lamina detail (scale = 1cm), D: transversal assay of the rhizome (scale = 0,1mm); F: *H. polyanthos* (DV20150004), lamina detail (scale = 1cm). On the anatomical assays pictures, the initials stand for: PX: protoxylem, MX: metaxylem, Ph: phloem, S: schlerenchyma, P: parenchyma.

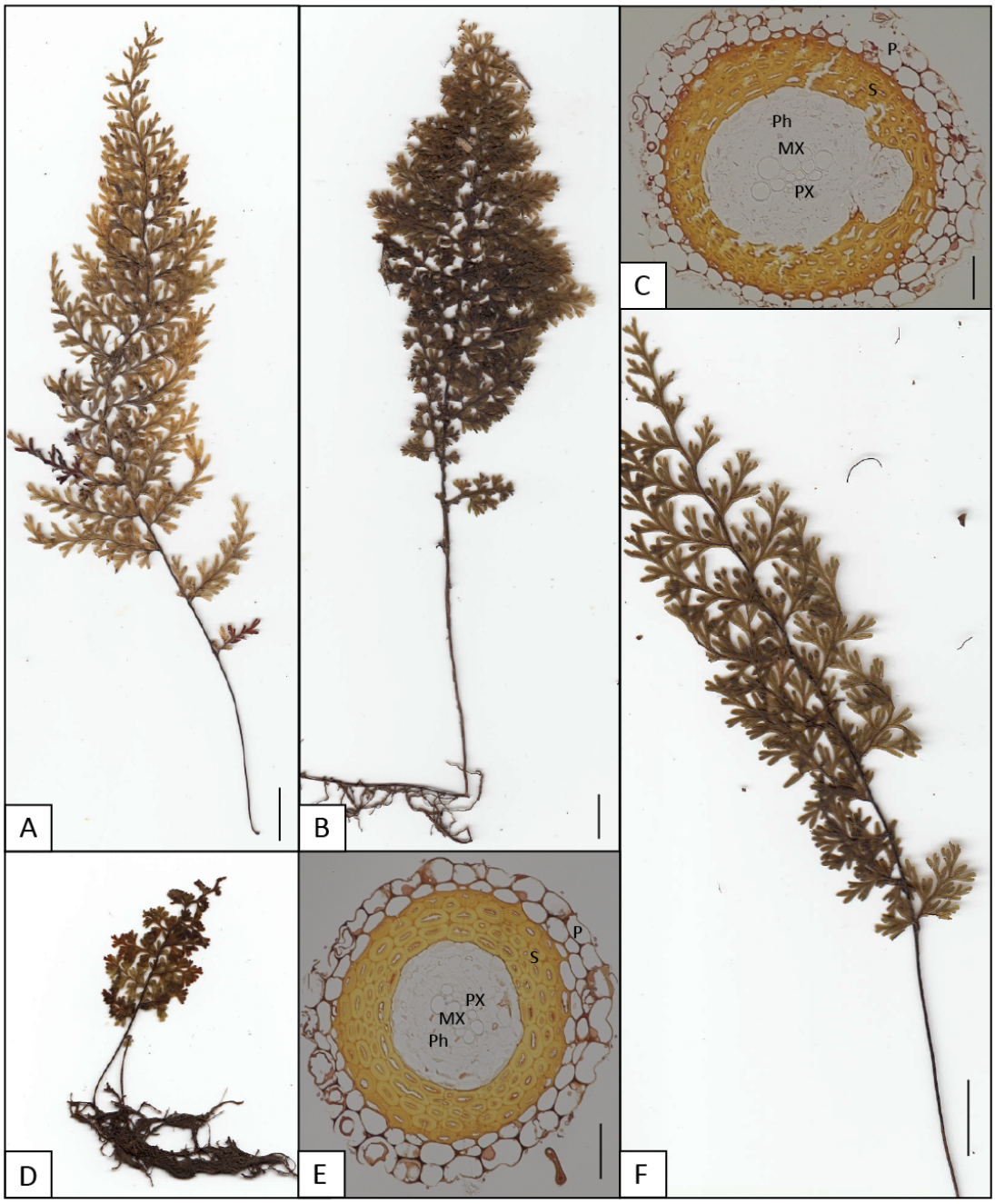

Figure 17: Specimens representative from section *Mecodium* C. Chr., "Neotropics clade". A: *H. myriocarpum* (B25) from Bolivia, lamina detail (scale = 1cm); B-C: *H. myriocarpum* (B22) from Bolivia, B: lamina detail (scale = 1cm), C: transversal assay of the rhizome (scale = 0,1mm); D-E: *H. myriocarpum* (B16) from Bolivia, D: lamina detail (scale = 1cm), D: transversal assay of the rhizome (scale = 0,1mm); F: *H. polyanthos* (B3) from Bolivia, lamina detail (scale = 1cm). On the anatomical assays pictures, the initials stand for: PX:protoxylem, MX: metaxylem, Ph: phloem, S: schlerenchyma, P: parenchyma.

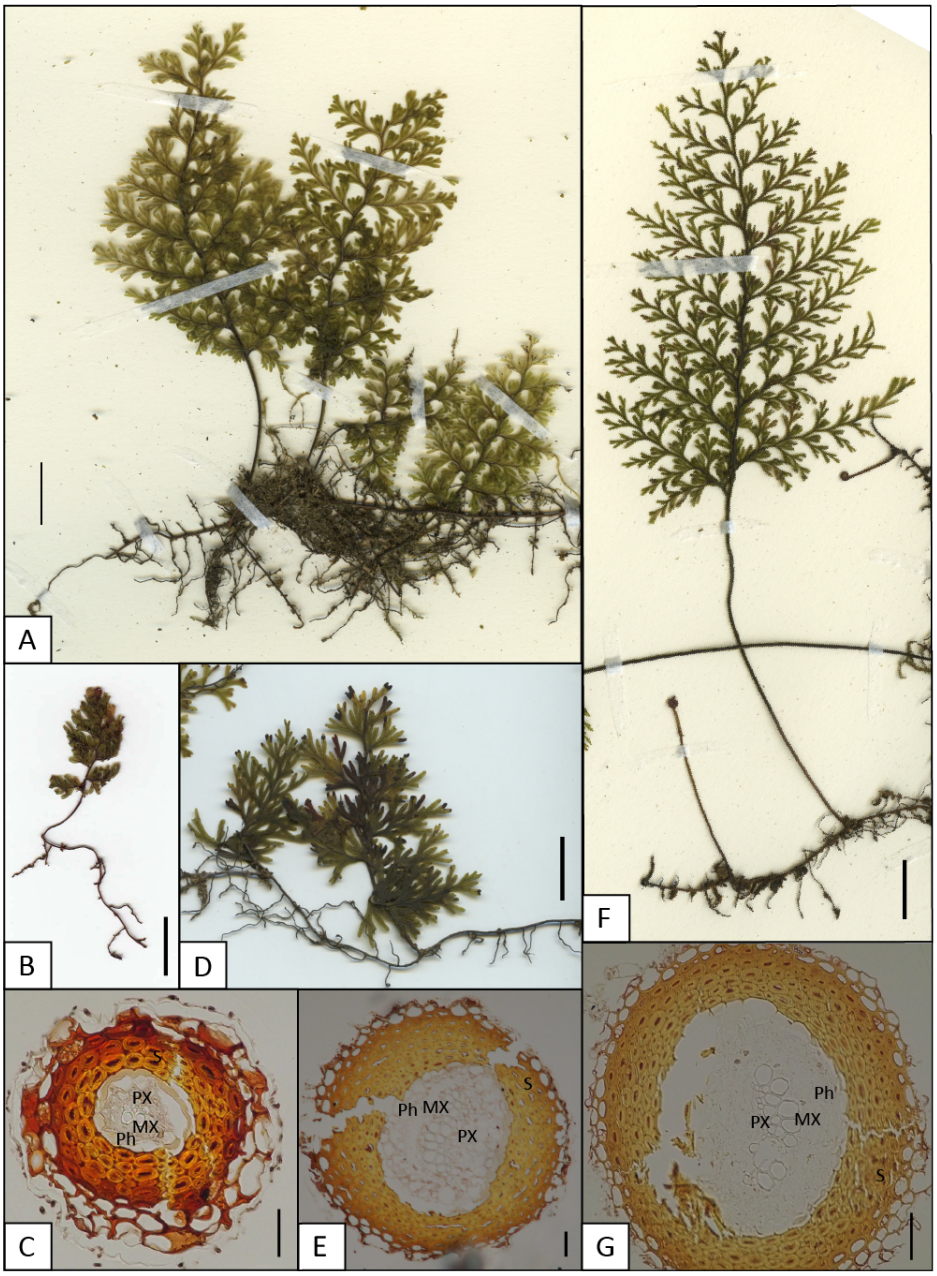

Figure 18: Specimens representative from section *Mecodium* C. Chr., "Asia" groups. A: *H. polyanthos* (W17) from Taiwan, lamina detail (scale = 1cm); B-C: *H. paniculiflorum* (K41) from Malaysia, B: lamina detail (scale = 1cm), C: transversal assay of the rhizome (scale = 0,1mm); D-E: *H. polyanthos* (K36) from Malaysia, D: lamina detail (scale = 1cm), D: transversal assay of the rhizome (scale = 0,1mm); F-G: *H. polyanthos* (W16) from Taiwan, F: lamina detail (scale = 1cm), G: transversal assay of the rhizome (scale = 0,1mm). On the anatomical assays pictures, the initials stand for: PX: protoxylem, MX: metaxylem, Ph: phloem, S: schlerenchyma, P: parenchyma.

Figure 19: Other specimens representative from section *Mecodium* C. Chr., "Asia" groups. A-B: *H. polaynthos* (K35) from Malaysia, lamina detail (scale = 1cm), C: transversal assay of the rhizome (scale = 0,1mm); C-D: *H. paniculiflorum* (K18) from Malaysia, C: lamina detail (scale = 1cm), D: transversal assay of the rhizome (scale = 0,1mm); E-F: *H. mykawanum* (GC1000) from Japan, E: lamina detail (scale = 1cm), F: transversal assay of the rhizome (scale = 0,1mm); G-H: *H. polyanthos* (K34) from Malaysia, G: lamina detail (scale = 1cm), H: transversal assay of the rhizome (scale = 0,1mm). On the anatomical assays pictures, the initials stand for: PX: protoxylem, MX: metaxylem, Ph: phloem, S: schlerenchyma, P: parenchyma.

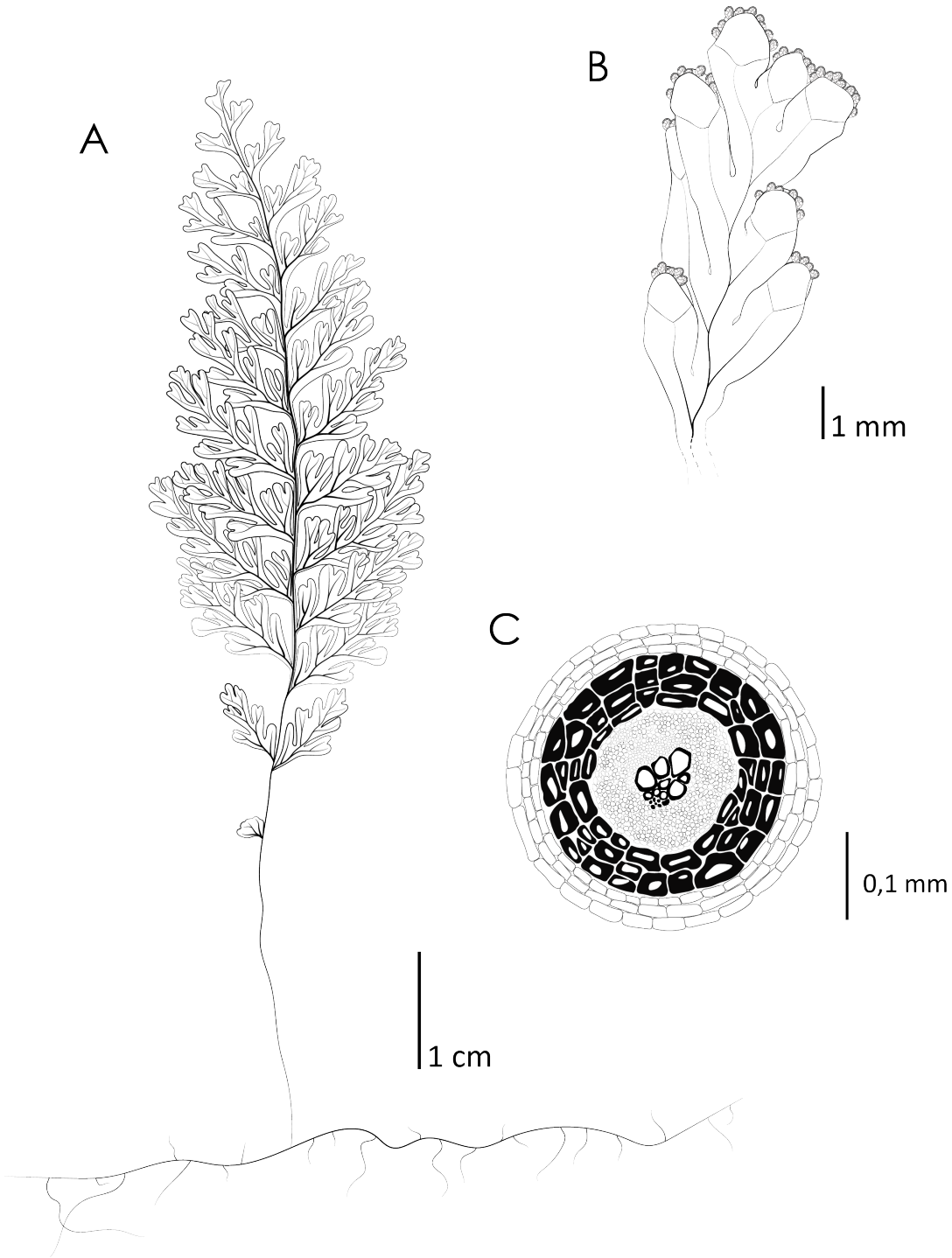

Figure 20: *Hymenophyllum sturmii* (Bosch) Vasques, from Brazil. A: body representation showing one sterile leaf; B: fertile frond detail showing the sori bearing sporangia; C: transversal assay of the rhizome showing from outside to the inner part: cortex parenchyma, cortex sclerenchyma, core parenchyma, vascular bundle with metaxylem turned to the dorsal side.

General Discussion and Conclusions

PHYLOGENETIC RELATIONS WITHIN SUBGENUS MECODIUM

As explained in chapter 1, *Mecodium* corresponds to a name that has been circumscribed several times during the taxonomical history of the family Hymenophyllaceae. The actual circumscription, based on Ebihara *et al*. (2006) and Hennequin *et al*. (2006), places the name as a subgenus within the genus *Hymenophyllum*, and set its boundaries around the subcosmopolitan *Hymenophyllum polyanthos* and its relatives. The monophyly of the subgenus is reinforced by the results of chapters 2 and 3 (Fig. 3 and 8), even when compared to neighbor subgenera (Fig. 5). Additionally, these results indicate that the species within the subgenus have diversified initially into 2 big lineages: one lineage composed of plants from Malesia, Pacific and South America; and another composed of plants from Africa, Asia, Malesia and the Neotropics. This distributional pattern was suggested before by Hennequin *et al*. (2006), but with few pieces of evidence at the time. The broad range of markers and samples applied here brings new evidence towards this hypothesis.

THE POLYPHYLETIC STATE OF H. POLYANTHOS

Still in chapter 2, the phylogenetic analysis based on plastidial markers indicates that, as expected, *Hymenophyllum polyanthos sensu lato* does not correspond to a monophyletic lineage. The subcosmopolitan species emerged interspersed to other represents of subgenus *Mecodium*, thus being described as a "polyphyletic species" (Fig. 3). This discussion is deepened in chapter 3, based on the review presented by Funk & Omland (2003). According to these authors, the phenomenon of "polyphyletic species" can be explained from different points of view, usually requiring further evidence for evaluation. In chapter 3, molecular data from *LEAFY* nuclear marker are compared in order to address the occurrence of gene introgression events within the subgenus *Mecodium* (one of the possible explanations for the polyphyletic condition of *H. polyanthos* samples obtained in chapter 2). In general, the results of chapter 3 do not support the hypothesis of occurrence of gene introgression events to explain the patterns obtained in chapter 2, indicating that *H. polyanthos*' polyphyletic state still might have been a result of poorly distinguishable traits between global specimens, what caused the

inclusion of different lineages under the same name (Fig. 8, 9). Further research is still necessary and additional evidence from other nuclear markers, or of different regions of the *LEAFY* marker may confirm this hypothesis in the future.

PHYLOGENETIC VALUE OF *LEAFY* MARKER

Although phylogenetic studies including nuclear markers in the analysis are common with groups of angiosperms, within ferns' groups such studies are still few. One of the main reasons is that polyploids are quite common within ferns, what brings difficulties to the isolation and sequencing of nuclear markers in these plants. Nevertheless, reports showing that low-copy genes (such as the *LEAFY* marker) can aid phylogenetic studies are becoming more common. In chapter 3, the results of experiments of cloning and sequencing of *LEAFY* sequences for subgenus *Mecodium* samples are reported. Although apparently informative for phylogenetic investigations, possibly paralog sequences were also acquired in the analysis, requiring further investigations to fully address this marker. The development of specific primers for the acquired sequences may solve the problem with the emergence of paralog sequences

and bring, at last, conclusive results to the polyphyly of *H. polyanthos*. Nevertheless, this is the first time *LEAFY* marker sequences are acquired for Hymenophyllaceae, what brings new possibilities for phylogenetic studies within the family.

MORPHOLOGICAL, ANATOMICAL AND DISTRIBUTIONAL ASPECTS

Taking the premise that results from chapters 2 and 3 indicate that *H. polyanthos s.l.* corresponds to a polyphyletic grouping of different phylogenetic lineages, chapter 4 brings a comparison of morpho-anatomical parameters between the lineages obtained with the phylogenetic analysis. Although the simple body of filmy ferns imposes limits to the description of taxonomically informative traits, the principal component analysis applied in chapter 4 brings insights on possible diagnostic traits for the obtained lineages (Fig. 13, 14, Tables 5, 6). The most correlate parameter here was the insertion angle of pinnae on the leaf, showing that plants from PSA clade have more closely appressed pinnae than those from AN clade. The assays on the anatomy of the rhizome of these plants, however, showed no potential correlation of parameters with the acquired groups.

TAXONOMIC REVISION AND FUTURE PROSPECTS

Finally, in chapter 5, results from chapters 2, 3 and 4 are merged in the suggestion of a new classification within the subgenus *Mecodium*, diving it into 2 sections: section *Cuneatae* Vasques *sect. nov.*, including plants from PSA clade (Fig. 15-16); and section *Mecodium* C. Chr. *comb. nov.*, including plants from the AN clade (Fig. 17-19). The type clade for *H. polyanthos* is set inside the Neotropics clade (Fig. 6), based on the location of the original type for the species (*i.e.* from Jamaica). Additionally, the clade PSA1 (Fig. 6) from Brazil is re-circumscribed to *H. sturmii* Bosch, species initially described for the Rio de Janeiro region, but later synonymized to *H. polyanthos* (Fig. 20).

In this study, the combination of molecular, morphological and distributional data analysis with the revision of the taxonomical transformations within the subgenus *Mecodium* have brought a new level of detail to the comprehension of the diversity within the group. Basing the discussion on the results of the phylogenetic analyses applied here, it is evident that *H. polyanthos s.l.* corresponds to a non-monophyletic

grouping and that further treatment of the subgenus *Mecodium* might bring a better solution to the taxonomy of the group. In the near future, the comparison of additional nuclear sequences to this analysis may bring to a conclusion the phylogenetic condition of *H. polyanthos s.l.*, opening ways to novel works towards a taxonomical revision of the complex.

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Bibliography

Abràmoff, M.D., P.J. Magalhães & S.J. Ram (2004). Image processing with ImageJ. Biophotonics international, 11(7), p. 36-42.

Archambault, A. & A. Bruneau (2004). Phylogenetic utility of the *LEAFY/FLORICAULA* gene in the Caesalpinioideae (Leguminosae): gene duplication and a novel insertion. *Systematic Botany*, *29*(3), 609-626.

Adjie, B., S. Masuyama, H. Ishikawa & Y. Watano (2007). Independent origins of tetraploid cryptic species in the fern *Ceratopteris thalictroides*. *Journal of Plant Research* 120: 129–138.

Álvarez, I. & J.F. Wendel (2003). Ribosomal ITS sequences and plant phylogenetic inference. Molecular phylogenetics and evolution, 29(3), 417-434.

Boodle, L. A. 1900. Comparative anatomy of the Hymenophyllaceae, Schizaeaceae and Gleicheniaceae. I. On the anatomy of *Hymenophyllum*, Annals of Botany 14: 455–496.

Bosch, R.B. van den 1861. Hymenophyllaceas novas. In Nederlandsch Kruidkundig

Archief. Verslangen en Mededelingen der Nederlandsche Botanische Vereeniging 5(2): 152.

Chen, C. W., L.Y. Kuo, C.N. Wang & W.L. Chiou (2012). Development of PCR primer sets for intron 1 of the low-copy gene *LEAFY* in Davalliaceae. American Journal of Botany, 99(6), 223-225.

Cogniaux, A., C.D. Martius, A.G. Eichler & I. Urban (1883). Flora brasiliensis. Flora brasiliensis, 14.

Copleand, E.B. (1937). Hymenophyllum. Philipp. J. Sci., 64, 1-188.

Copeland, E.B. (1947). Genera Filicum-the genera of ferns. Genera Filicum-the genera of ferns.

Darriba, D., G.L. Taboada, R. Doallo & D. Posada (2012). ModelTest 2: more models, new heuristics and parallel computing. Nature methods, 9 (8), 772.

Dubuisson, J.Y., S. Hennequin, E.J. Douzery, R.B. Cranfill, A.R. Smith & K.M. Pryer (2003). RbcL phylogeny of the fern genus Trichomanes (Hymenophyllaceae), with special reference to neotropical taxa. International Journal of Plant Sciences, 164(5), 753-761.

Duffy, A.M., M.C. Stensvold & D.R. Farrar (2015). Independent gametophytes of Hymenophyllum wrightii in North America: Not as rare as we thought. American Fern Journal, 105(1), 45-55.

Ebihara, A., K. Iwatsuki, T.A. Ohsawa & M. Ito (2003). *Hymenophyllum paniense* (Hymenophyllaceae), a new species of filmy fern from New Caledonia. Systematic Botany, 28(2), 228-235.

Ebihara, A., S. Hennequin, K. Iwatsuki, P.D. Bostock, S. Matsumoto, R. Jaman, J.Y. Dubuisson & M. Ito (2004). Polyphyletic origin of *Microtrichomanes* (Prantl) Copel. (Hymenophyllaceae), with a revision of the species. Taxon, 53(4), 935-948.

Ebihara, A., H. Ishikawa, S. Matsumoto, S. J. Lin, K. Iwatsuki, M. Takamiya, Y. Watano & M. Ito. (2005). Nuclear DNA, chloroplast DNA, and ploidy analysis clarified biological complexity of the *Vandenboschia radicans* complex (Hymenophyllaceae) in Japan and adjacent areas. American Journal of Botany 92: 1535–1547.

Ebihara, A., J.Y. Dubuisson, K. Iwatsuki, S. Hennequin & M. Ito (2006). A taxonomic

revision of Hymenophyllaceae. Blumea-Biodiversity, Evolution and Biogeography of Plants, 51, 221-280.

Ebihara, A., K. Iwatsuki, M. Ito, S. Hennequin & J.Y. Dubuisson (2007). A global molecular phylogeny of the fern genus *Trichomanes* (Hymenophyllaceae) with special reference to stem anatomy. Botanical Journal of the Linnean Society, 155(1), 1-27.

Ferguson, D. & T. Sang (2001). Speciation through homoploid hybridization between allotetraploids in peonies (*Paeonia*). Proceedings of the National Academy of Sciences, 98(7), 3915-3919.

Frohlich, M. W., & Parker, D. S. (2000). The mostly male theory of flower evolutionary origins: from genes to fossils. Systematic Botany, 25(2), 155-170.

Funk, D. J. & K.E. Omland (2003). Species-level paraphyly and polyphyly: frequency, causes, and consequences, with insights from animal mitochondrial DNA. Annual Review of Ecology, Evolution, and Systematics, 397-423.

Geyer, C.J. & E.M. Keramidas (Ed.)(1991). Markov chain Monte Carlo maximum likelihood. Computing Science and Statistics: Proceedings from 23rd

Symposium on the Interface, p. 156-163. Fairfax Station, Interface Foundation.

Hasebe, M., M. Ito, R. Kofuji, K. Ueda & K. Iwatsuki (1993). Phylogenetic relationships of ferns deduced from rbcL gene sequence. Journal of molecular Evolution, 37(5), 476-482.

Hasebe, M., T. Omori, M. Nakazawa, T. Sano, M. Kato & K. Iwatsuki (1994). RbcL gene sequences provide evidence for the evolutionary lineages of leptosporangiate ferns. Proceedings of the National Academy of Sciences, 91(12), 5730-5734.

Hasebe, M., P.G. Wolf, K.M. Pryer, K. Ueda, M. Ito, R. Sano, G.J. Gastony, J. Yokoyama, J.R. Manhart, N. Murakami, E.H. Crane, C.H. Haufler & W.D. Hauk (1995). Fern phylogeny based on rbcL nucleotide sequences. American Fern Journal, 134-181.

Hennequin, S., A. Ebihara, M. Ito, K. Iwatsuki & J.Y. Dubuisson (2003). Molecular systematics of the fern genus *Hymenophyllum sl* (Hymenophyllaceae) based on chloroplastic coding and noncoding regions. Molecular Phylogenetics and Evolution, 27(2), 283-301.

Hennequin, S. 2004. Le genre *Hymenophyllum* Sm. (Hymenophyllaceae, Filicopsida): systématique phylogénétique, évolution morphologique et histoire biogéographique. Doctoral thesis, l'Université Pierre et Marie Curie. 266 pp.

Hennequin, S., A. Ebihara, M. Ito, K. Iwatsuki & J.Y. Dubuisson (2006). New insights into the phylogeny of the genus Hymenophyllum sl (Hymenophyllaceae): revealing the polyphyly of Mecodium. Systematic Botany, 31, 271-284.

Hennequin, S., A. Ebihara, J.Y. Dubuisson & H. Schneider (2010). Chromosome number evolution in Hymenophyllum (Hymenophyllaceae) with special reference to the subgenus Hymenophyllum. Molecular Phylogenetics and Evolution, 55 (1), 47-59.

Hofer, J. M. & T.N. Ellis (1998). The genetic control of patterning in pea leaves. Trends in plant science, 3(11), 439-444.

Hoot, S.B. & W.C. Taylor (2001). The utility of nuclear ITS, a *LEAFY* homolog intron, and chloroplast atpB-rbcL spacer region data in phylogenetic analyses and species delimitation in *Isoetes*. American Fern Journal, 91(3), 166-177.

Huelsenbeck, J.P. & F. Ronquist (2001). MRBAYES: Bayesian inference of phylogeny.

Bioinformatics, 17, 754-755.

Ishikawa, H., Y. Watano, K. Kano, M. Ito & S. Kurita (2002). Development of primer sets for PCR amplification of the PgiC gene in ferns. Journal of plant research, 115(1), 65-70.

Iwatsuki, K. (1961). The occurrence of Mecodium wrightii in Canada. American Fern Journal, 51(3), 141-144.

Iwatsuki K. (1984). Studies on the Systematics of Filmy Ferns VII. A scheme of classification based chiefly on the Asiatic Species. Acta Phytotax. Geobot 35(4), 165-179.

Iwatsuki, K. (1990). Hymenophyllaceae. In: Kramer, K.U. & P.S. Green. The Families and Genera of Vascular Plants - Pteridophytes and Gymnosperms (p. 157-163). Berlin, Heidelberg: Springer.

Kobayashi, K. & M. Suzuki (2014). Identification Methods of Plant Materials of Excavated Weavings. Bulletin of the National Museum of Japanese History, 187, 457-467.

Kumar, S., G. Stecher & K. Tamura (2016). MEGA 7: Molecular Evolutionary Genetic Analysis version 7.0 for bigger datasets. 1-5.

Kuo, L. Y., F.W. Li, W.L. Chiou & C.N. Wang (2011). First insights into fern *matK* phylogeny. Molecular Phylogenetics and Evolution, 59(3), 556-566.

Larsen, C. (2014). Estudios sistemáticos y biogeográficos en Hymenophyllum (Hymenophyllaceae) en Sudamérica Subtropical y Templada (Doctoral dissertation, Facultad de Ciencias Naturales y Museo).

Lehtonen S. (2011). Towards resolving the complete fern tree of life. PLoS One 6: e24851.

Le Thomas, A. (1961). Etude anatomique du rhizome et du pe´tiole des Hymenophyllaceae d'Afrique Occidentale et de la re´gion malgache. Bulletin de la Socie´te´ Scientifique de Bretagne 36: 217– 264.

Morton, C.V. (1968). The genera, subgenera, and sections of the Hymenophyllaceae. Contributions from the United States National Herbarium, 38 (5), 153-214.

Nadot, S., R. Bajon & B. Lejeune (1994). The chloroplast gene *rps4* as a tool for the study of Poaceae phylogeny. Plant Systematics and Evolution, 191(1-2), 27-38.

Ogura, Y. (1938). Anatomie der Vegetationsorgane der Pteridophyten. Berlin: Gebru¨der Borntra¨ger.

Peixoto, A. L. & M.P. Morim (2003). Coleções botânicas: documentação da biodiversidade brasileira. Ciência e Cultura, 55(3), 21-24.

Prantl, K.B. 1875. Untersuchungen zur Morphologie der Gefässcryptogamen, I. Die Hymenophyllaceaen, die niedrigste Entwicklungsreihe der Farne. Engelmann. Leipzig, Germany.

Pryer, K. M., H. Schneider, A.R. Smith, R. Cranfill, P.G. Wolf, J.S. Hunt & S.D. Sipes (2001). Horsetails and ferns are a monophyletic group and the closest living relatives to seed plants. *Nature*, *409*(6820), 618-622.

Pryer, K. M., E. Schuettpelz, P.G. Wolf, H. Schneider, A.R. Smith & R. Cranfill (2004). Phylogeny and evolution of ferns (monilophytes) with a focus on the early leptosporangiate divergences. American Journal of Botany, 91(10), 1582-1598.

Rambaut, A. (2012). Tree Figure Drawing Tool Version 1.4.0. UK: Institute of Evolutionary Biology, University of Edinburgh.

Raymond, O., F. Piola & C. Sanlaville-Boisson (2002). Inference of reticulation in outcrossing allopolyploid taxa: caveats, likelihood and perspectives. Trends in Ecology & Evolution, 17(1), 3-6.

R Core Team (2006). R: A language and environment for statistical computing. R foundation for statistical computing, Vienna, Austria. URL https://www.R-project.org/.

Ronquist, F. & J.P. Huelsenbeck (2003). MRBAYES 3: Bayesian phylogenetic inference under mixed models. Bioinformatics, 19, 1572-1574.

Rothfels C.J., A. Larsson, L.-Y. Kuo, P. Korall, W.-L. Chiou & K.M. Pryer (2012). Overcoming deep roots, fast rates, and short internodes to resolve the ancient rapid radiation of eupolypod II ferns. Systematic Biology 16: 490 – 509.

Ryan, T.A., B.L. Joiner & B.F. Ryan (1994). *Minitab™*. John Wiley & Sons, Inc.

Sano, R., M. Takamiya, M. Ito, S. Kurita & M. Hasebe (2000). Phylogeny of the lady fern group, tribe Physematieae (Dryopteridaceae), based on chloroplast rbcL gene sequences. Mol. Phylogenet. Evol. 15, 403–413.

Schneider H., A.R. Smith, R. Cranfill, T.J. Hildebrand, C.H. Haufler & T.A. Ranker

(2004a). Unraveling the phylogeny of polygrammoid ferns (Polypodiaceae and Grammitidaceae): exploring aspects of the diversification of epiphytic plants. Molecular phylogenetics and evolution, 31(3): 1041-1063.

Schneider H, E. Schuettpelz, K.M. Pryer, R. Cranfill, S. Megallon & R. Lupia (2004b). Ferns diversified in the shadow of angiosperms. Nature 428: 553 – 557.

Schneider H, L. He, S. Hennequin & X.-C. Zhang (2013). Towards a natural classification of Pteridaceae: inferring the relationships of enigmatic pteridoid fern species occurring in the Sino-Himalaya and Afro-Madagascar. Phytotaxa 77: 49–60.

Schuettpelz, E., P. Korall & K.M. Pryer (2006). Plastid atpA data provide improved support for deep relationships among ferns. Taxon 55, 897–906.

Schuettpelz, E. & K.M. Pryer (2007). Fern phylogeny inferred from 400 leptosporangiate species and three plastid genes. *Taxon*, *56* (4), 1037-1037.

Serizawa, S. (1983). A new species of *Mecodium* (Hymenphyllaceae) from central Honshu of Japan. Journ. Jap. Bot. 58 (2): p. 62-65.

Shepherd, G. J. (2003). Conhecimento de diversidade de plantas terrestres do Brasil. Ministério do Meio Ambiente (MMA), Brasília.

Shreve, F. (1911). Studies on Jamaican Hymenophyllaceae. Botanical Gazette, 184-209.

Smith, A.R., K.M. Pryer, E. Schuettpelz, P. Korall, H. Schneider & P.G. Wolf (2006). A classification for extant ferns. Taxon 55, 705–731.

Smith, A.R., K.M. Pryer, E. Schuettpelz, P. Korall, H. Schneider & P.G. Wolf (2008). Fern classication. In: Ranker, T.A., Haufler, C.H. (Eds.), Biology and Evolution of Ferns and Lycophytes. Cambridge University Press, New York, pp. 159–174.

Souza-Chies, T. T., G. Bittar, S. Nadot, L. Carter, E. Besin & B. Lejeune (1997). Phylogenetic analysis of Iridaceae with parsimony and distance methods using the plastid gene *rps4*. Plant Systematics and Evolution, 204(1-2), 109-123.

Stamatakis, A. (2006). RAxML-VI-HPC: maximum likelihood-based phylogenetic analysis with thousands of taxa and mixed models. Bioinformatics, 22 (21), 2688-2690.

Steil, W.N. (1939). Apogamy, apospory, and parthenogenesis in the pteridophytes. The Botanical Review, 5, 433-453.

Stolze, R.G. & R.M. Tryon (1989). Pteridophyta of Peru. Part I. 1. Ophioglossaceae – 12. Cyatheaceae. Feldiana Botany 20, p. 49-78.

Swartz, O.P. (1788). Nova genera & species plantarum seu prodromus descriptionum vegetalium, maximam partem incognitorum quæ sub itinere in Indiam occidentalem annis 1783-1787 digessit Olof Swartz. MD. in bibliopolis Acad. M. Swederi.

Swarts, O. (1801). Journal für die Botanik. Germany: Göttingen.

Taylor, T.M.C. (1967). Mecodium wrightii in British Columbia and Alaska. American Fern Journal, 57(1), 1-6.

Tryon, R. (1972). Endemic areas and geographic speciation in tropical American ferns. Biotropica, 121-131.

Van den Heede, C.J., R.L.L. Viane, & M.W. Chase (2003). Phylogenetic analysis of *Asplenium* subgenus *Ceterach* (Pteridophyta: Aspleniaceae) based on plastid

and nuclear ribosomal ITS dna sequences. American Journal of Botany 90(3): 481–495.

Wolf, P. (1997). Evaluation of *atpB* nucleotide sequences for phylogenetic studies of ferns and other pteridophytes. American Journal of Botany, 84(10), 1429-1440.

Appendices

Appendix 1: List of samples used for the molecular analysis (Chapter 2). Samples are organized by species name, deposited herbarium name, Collector name, location and identification number.

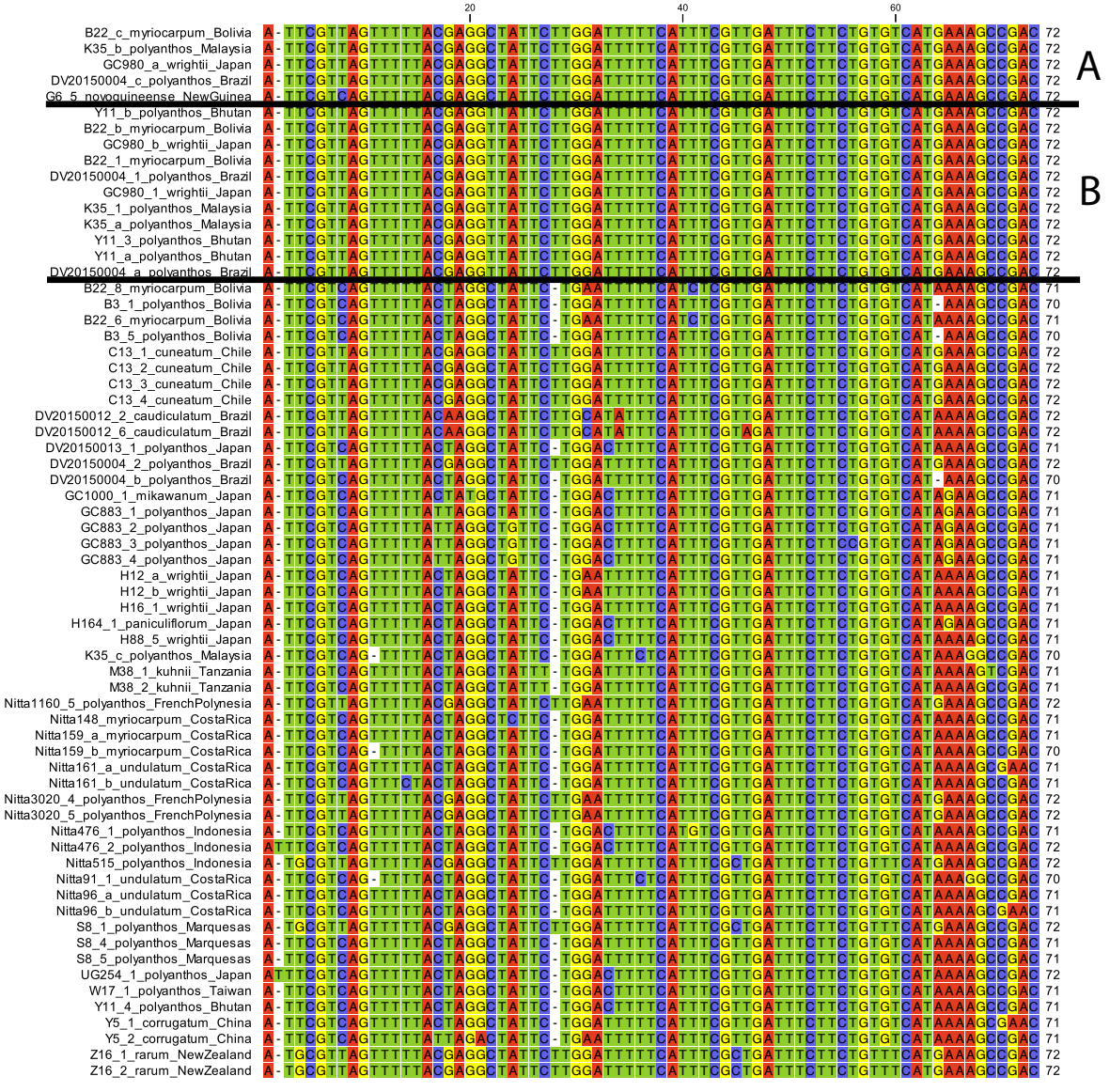
Abrodictyum rigidum: Duke, J.Y. Dubuisson HV 1997-3, no date, Venezuela, AY775447, AY095137. ***Hymenophyllum hygrometricum:*** P, F, J.Y. Dubuisson HR-1999-13, no date, La Reunion, AY775451, AY095118. ***Hymenophyllum apiculatum:*** J.Y. Dubuisson HV 1997-03, no date, Venezuela, AY775438, AY775411. ***Hymenophyllum armstrongii***: TI, CHR, A. Ebihara 01122-03, no date, New Zealand, AB162691; UC, A.R. Smith 2610, no date, AY095128. ***Hymenophyllum asplenioides***: DV20150008, D.T. Vasques 2015-0008, 22.II.2015, Serra da Bocaina, Sao Paulo, near to the entrance gate to the park 1562m. ***Hymenophyllum atrovirens***: A. Ebihara 040119-01, no date, New Zealand, AB496575, AB496595. ***Hymenophyllum caudiculatum***: DV20150012, D.T. Vasques 2015-0012, 23.II.2015, Serra da Bocaina, Sao Paulo, near to the cottage (Campos da Bocaina) trail 1590m. ***Hymenophyllum corrugatum***: Y5, G.Miehe & U.Wuendisch 94-220-11, no date, China, Xizang, SE Tibet, AB191443. ***Hymenophyllum cruentum***: TI, T.A. Ohsawa 2015, no date, Chile, AB191455; P, F, M. Wedin H38, no date, AY095133. ***Hymenophyllum cuneatum***: C13, A.Ebihara 021223-07, no date, Chile. ***Hymenophyllum demissum***: RBG, B.G. Glasgow, 830, no date, New Zealand, AY775441, AY775416. ***Hymenophyllum devolii***: ED19587, UG221, s.n., no date, Taiwan, Taitung. ***Hymenophyllum dilatatum***: TI, A. Ebihara 011219-06, no date, New Zealand, AB191444; UC, W.C. Taylor 90584, no date, AY095138. ***Hymenophyllum flabellatum***: A. Ebihara 0111216-02, no date, New Zealand, AB083279; s.n., no date, French Polynesia, AY775417. ***Hymenophyllum heimii***: F. Rakotondrainibe 6008, no date, Madagascar, AY775443, AY775419. ***Hymenophyllum hirsutum***: J.Y. Dubuisson HR-1999-6, no date, La Reunion, AY775450, AY775432. ***Hymenophyllum imbricatum***: S. Matsumoto 01-758, no date, Vanuatu, AB496566, AB496587. ***Hymenophyllum inaequale***: P, F, J.Y. Dubuisson HR-1999-9 no date La Reunion, AB217848, AY095122. ***Hymenophyllum kuhnii:*** M38, G.Rouhan 517, no date, Tanzania, AB496577, AB496597. ***Hymenophyllum lyallii***: TI, CHR, A. Ebihara, 011221-06, no date, New Zealand, AB162684; TI, CHR, A. Ebihara 011221-06, no date, New Zealand, AB496589. ***Hymenophyllum mikawanum***: TNS VS 738136, GC1000, A. Ebihara, S. Serizawa & H. Miyazaki AC2009-2172, 13.VI.2009, Japan, Aichi prefecture. ***Hymenophyllum minimum:*** A. Ebihara 011222-09, no date, New Zealand, AB496572, AB496592. ***Hymenophyllum myriocarpum:*** Sundue and J. Nitta 1483, 20.I.2008, Costa Rica, Heredia, Rio Cuarto, La Selva Biological Station 60m; Sundue and J. Nitta 1475, 17.I.2008,

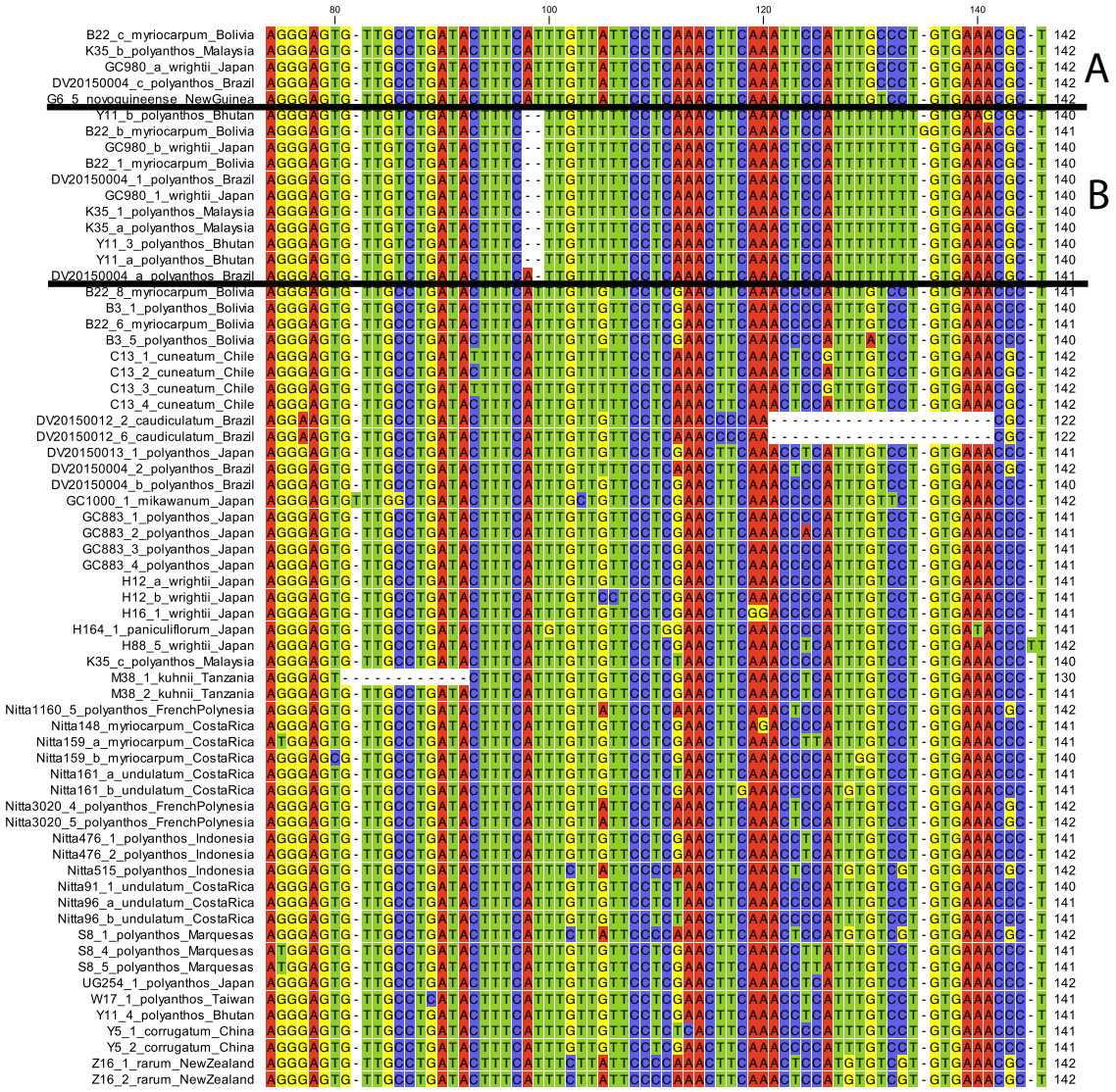
Costa Rica, San Jose, Villa Mills, Cerro de la Muerte 3354m; J. Nitta, J. Condack, F. Matos, C. Rothfels, M. Sundue, A. Vasco 94, 27.I.2008, Costa Rica, San Jose, Cerro de la Muerte 3093m; J. Nitta, J. Condack, F. Matos, C. Rothfels, M. Sundue, A. Vasco 90, 25.I.2008, Costa Rica, Alajeula, road to Vulcan Poas 2244m; J. Nitta 2325, no date, Costa Rica, Alajeula, San Ramon, Nectandra Biological Preserve; J. Nitta 159, 8.III.2008, Costa Rica, Puntarenas Reserva Biologico Durika 2102m; J. Nitta 110, 1.II.2008, Costa Rica, San Jose, Chirripo National Park 3083m; B3, Asakawa 174-4, 2001, Bolivia; B25, Asakawa 208-3, 2001, Bolivia; B22, Asakawa 196-2, 2001, Bolivia; B20, Asakawa 184-2, 2001, Bolivia; B19, Asakawa 184-1 2001, Bolivia; B16, Asakawa 180-3, 2001, Bolivia; J. Nitta 148, 24.II.2008, Costa Rica, Alajeula, San Ramon 1203m. ***Hymenophyllum novoguineense***: G6, R.J.Johns 9637, no date, New Guinea. ***Hymenophyllum paniculiflorum***: TNS VS 738125, UG253, A. Ebihara, S. Serizawa & H. Miyazaki AC2009-2183, 13.VI.2009, Japan, Aichi Prefecture, Toyota city; TNS VS 776536, UG252, A. Ebihara, C. Tsutsumi, G. Kokubugata & C.-I. Huang TW2008-1879, 20.VI.2008, Taiwan, Yilan; TAIF VS, UG225, s.n., no date Taiwan, Yilan; TNS VS 766206, K41, A.Sugawara, A.Ebihara, T.Nakamura & S.Matsumoto, SB2007-61, 1.II.2007, Malaysia, SabahMt., KinabaluMasilau, Nepenthes Trail; K18, Arikawa, 316A, no date, Malaysia, Mt. Kinabalu; H164, A.Ebihara 040404-03, no date, Japan, Saitama prefecture, Chichibu. ***Hymenophyllum pectinatum***: TI, T.A. Ohsawa 2017, no date, Chile, AB191450; P, F, M. Wedin H41, no date, AY095134. ***Hymenophyllum polyanthos***: TNS VS, Y11, Bhutan 59, no date, Bhutan; Y10, Rocher, no date, Bhutan; TNS VS 762064, W17, A.Ebihara, M.Yokota, G.Kokubugata, S.Kobayashi & K.Yasuda TW2006-187, 6.XII.2007, Taiwan, Pingtung Chuenr Hsiang, Jinshui-ying; TNS VS 762067, W16, A.Ebihara, M.Yokota, G.Kokubugata, S.Kobayashi & K.Yasuda TW2006-184, 6.XII.2007, Taiwan, Pingtung Chuenr Hsiang, Jinshui-ying; TNS VS 763148, UG258, s.n., 30.IX.2006, Japan, Kagoshima, Kumage; TNS VS 776487, UG257, A. Ebihara, T. Oka & T. Oka NN2008-1967, 16.IX.2008, Japan, Nagano prefecture; TNS VS 768184, UG256, Atsushi Ebihara, Mihoko Uzawa, Naoko Mizukami & Kanako Tokutome 1355, 6.X.2007, Japan, Saitama prefecture, Chichibu; TNS VS 766417, UG255 , A. Ebihara, S. Fujimoto & K. Ohora KI2007-1261, 25.VIII.2007, Japan, Wakayama; TNS VS 764218, UG254, A.Ebihara, C.Tsutsumi, M.Kato, G.Kokubugata, T.Komatsu & H.Yamashita AM2007-395, 20.V.2007, Japan, Kagoshima, Ooshima-gun; TAIF VS , UG226, s.n., no date, Taiwan, Hsinchu; S8, Wood 10456, no date, Marquesas; RH756, R.Y. Hirai, J. Prado & R. da Silva Cruz 756, 12.XII.2014, Brazil, São Paulo, Santo André 850m; J. Nitta 631, 22.VIII.2010, French Polynesia, Society Islands, Moorea 372m; J. Nitta 170, 11.III.2008, Costa Rica, San Jose; J. Nitta 135, 19.II.2008, Costa Rica, Alajeula; J. Nitta 9, 8.X.2006, French Polynesia, Society Islands, Moorea, Mt. Rotui; J. Nitta 78, 27.XI.2007, French Polynesia, Society Islands, Borabora; J. Nitta 631, 22.VIII.2010, French Polynesia, Society Islands, Moorea, face of Mt. Tohiea above town of Maatea 372m; J. Nitta 6,

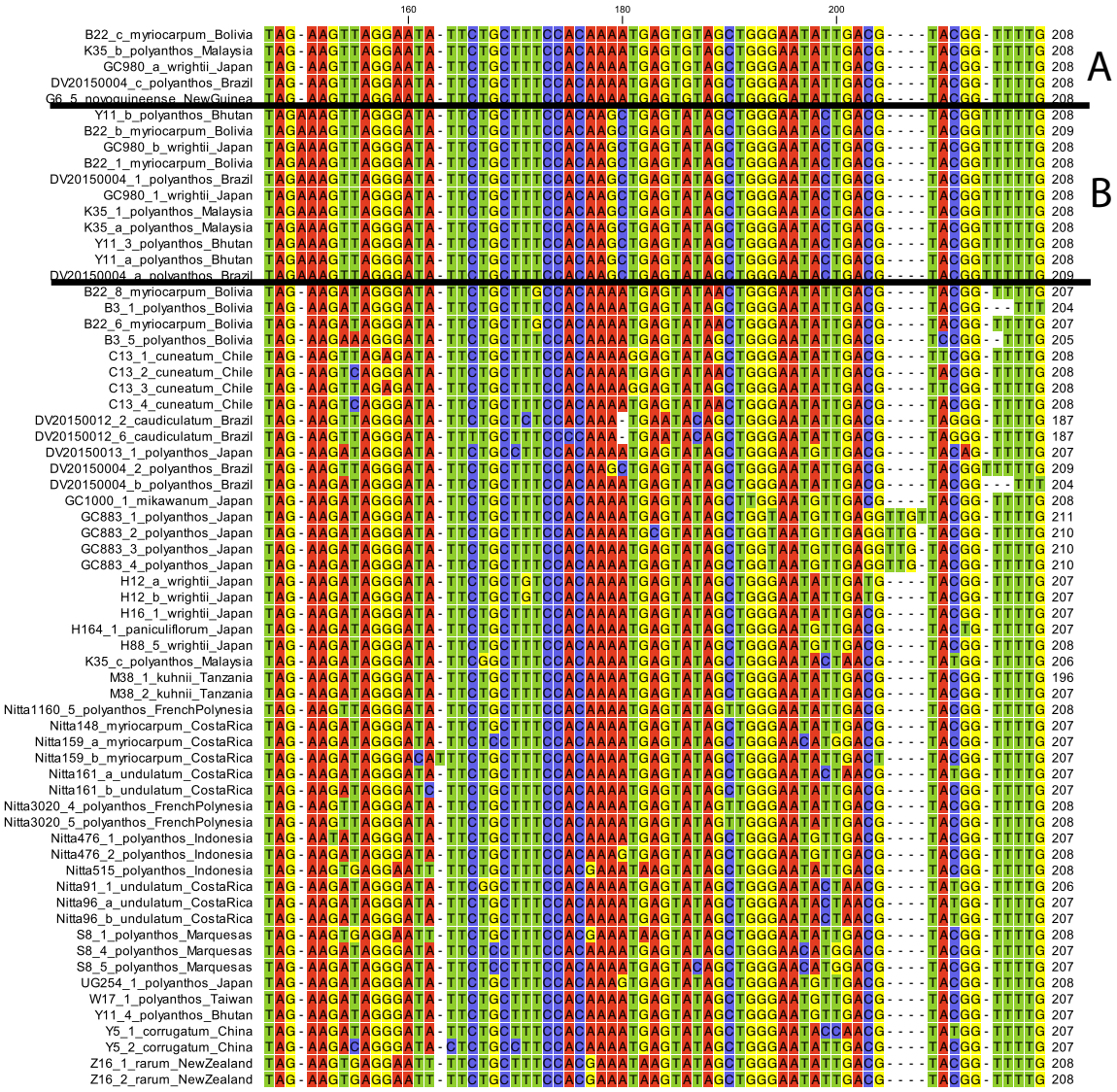
8.X.2006, French Polynesia, Society Islands, Moorea, Mt. Rotui 711m; J. Nitta, U. Hapid 537, 27.IV.2009, Indonesia, Java, Gunung Halimun, trail to Gunung Kendeng 1648m; J. Nitta, U. Hapid 528, 27.IV.2009, Indonesia, Java, Gunung Halimun, trail to Gunung Kendeng 1350m; J. Nitta, U. Hapid 515, 26.IV.2009, Indonesia, Java, Gunung Halimun, trail to Gunung Kendeng 1113m; J. Nitta, U. Hapid 476, 23.IV.2009, Indonesia, Java, Gunung Gede, trail to waterfalls 1500m; J. Nitta, U. Hapid 475, 23.IV.2009, Indonesia, Java, Gunung Gede, trail to waterfalls 1500m; J. Nitta, Li-Yaung Kuo 426, 26.XI.2008, Taiwan, Pingtung County, Li Long Shan Trail 842m; J. Nitta, Li-Yaung Kuo 424, 26.XI.2008, Taiwan Pingtung County, Li Long Shan Trail 813m; J. Nitta 42, 26.X.2006, French Polynesia, Society Islands, Moorea, Mt. Mouaputa 578m; J. Nitta 3020, 10.VII.2013, French Polynesia, Society Islands, Moorea, Mt. Mouaputa 794m; J. Nitta 2824, 1.VII.2013, French Polynesia, Society Islands, Moorea, Mt. Mouaputa 411m; J. Nitta 2770, 24.VI.2013, French Polynesia, Society Islands, Moorea, Mt. Mouaputa 646m; J. Nitta 2645, 17.VI.2013, French Polynesia, Society Islands, Moorea, Mt. Rotui 830m; J. Nitta 2311, no date, Costa Rica, Alajeula, San Ramon, Nectandra Biological Preserve; J. Nitta, Suzanne Vinette 1806, 20.VIII.2012, French Polynesia, Society Islands, Moorea, Mt. Rotui; J. Nitta 170, 11.III.2008, Costa Rica, San Jose, San Gerardo de Dota 2364m; J. Nitta, Suzanne Vinette, Ravahere Taputuarai 1537, 9.VIII.2012, French Polynesia, Society Islands, Moorea, Mt. Tohiea 1170m; J. Nitta, Suzanne Vinette 1438, 3.VIII.2012, French Polynesia, Society Islands, Moorea, Mt. Tohiea 1000m; J. Nitta, Suzanne Vinette 1395, 30.VII.2012, French Polynesia, Society Islands, Moorea, Mt. Tohiea 800m; J. Nitta, Suzanne Vinette 1254, 21.VII.2012, French Polynesia, Society Islands, Moorea, Mt. Tohiea 1109m; J. Nitta, Suzanne Vinette 1160, 18.VII.2012, French Polynesia, Society Islands, Moorea, Mt. Tohiea 800m; TNS VS 762710, KS19, A.Ebihara, Y.Hirayama, G.Kokubugata, Y.Saito & M.Uzawa KS2007-287, 24.II.2007, Japan, Miyazaki prefecture, Kitago-cho, Inohae Ravine; TNS VS 762571, KS1, A.Ebihara, Y.Hirayama, G.Kokubugata, Y.Saito & M.Uzawa, KS2007-116, 18.II.2007, Japan, Kagoshima prefecture, Satsuma-cho, Mt. Shibi; TNS VS 766210, K45, A.Sugawara, A.Ebihara, T.Nakamura & S.Matsumoto SB2007-94, 3.II.2007, Malaysia, Sabah, Mt. Alabu; TNS VS 766209, K44, A.Sugawara, A.Ebihara, T.Nakamura & S.Matsumoto SB2007-92, 3.II.2007, Malaysia, Sabah, Mt. Alabu; TNS VS 766208, K43, A.Sugawara, A.Ebihara, T.Nakamura & S.Matsumoto SB2007-76, 2.II.2007, Malaysia, Sabah, Mt. Kinabalu, Silau Silau trail; TNS VS 766205, K40, A.Sugawara, A.Ebihara, T.Nakamura & S.Matsumoto SB2007-60, 1.II.2007, Malaysia, Sabah, Mt. Kinabalu, Masilau, Nepenthes trail; TNS VS 766204, K39, A.Sugawara, A.Ebihara, T.Nakamura & S.Matsumoto, SB2007-52, 1.II.2007, Malaysia, Sabah, Mt. Kinabalu, Masilau, Nepenthes trail; TNS VS 766200, K36, A.Sugawara, A.Ebihara, T.Nakamura & S.Matsumoto SB2007-16, 31.I.2007, Malaysia, Sabah, Mt. Kinabalu, between Timophon gate and Kandis shelter; TNS VS 766199, K35, A.Sugawara, A.Ebihara, T.Nakamura & S.Matsumoto SB2007-13,

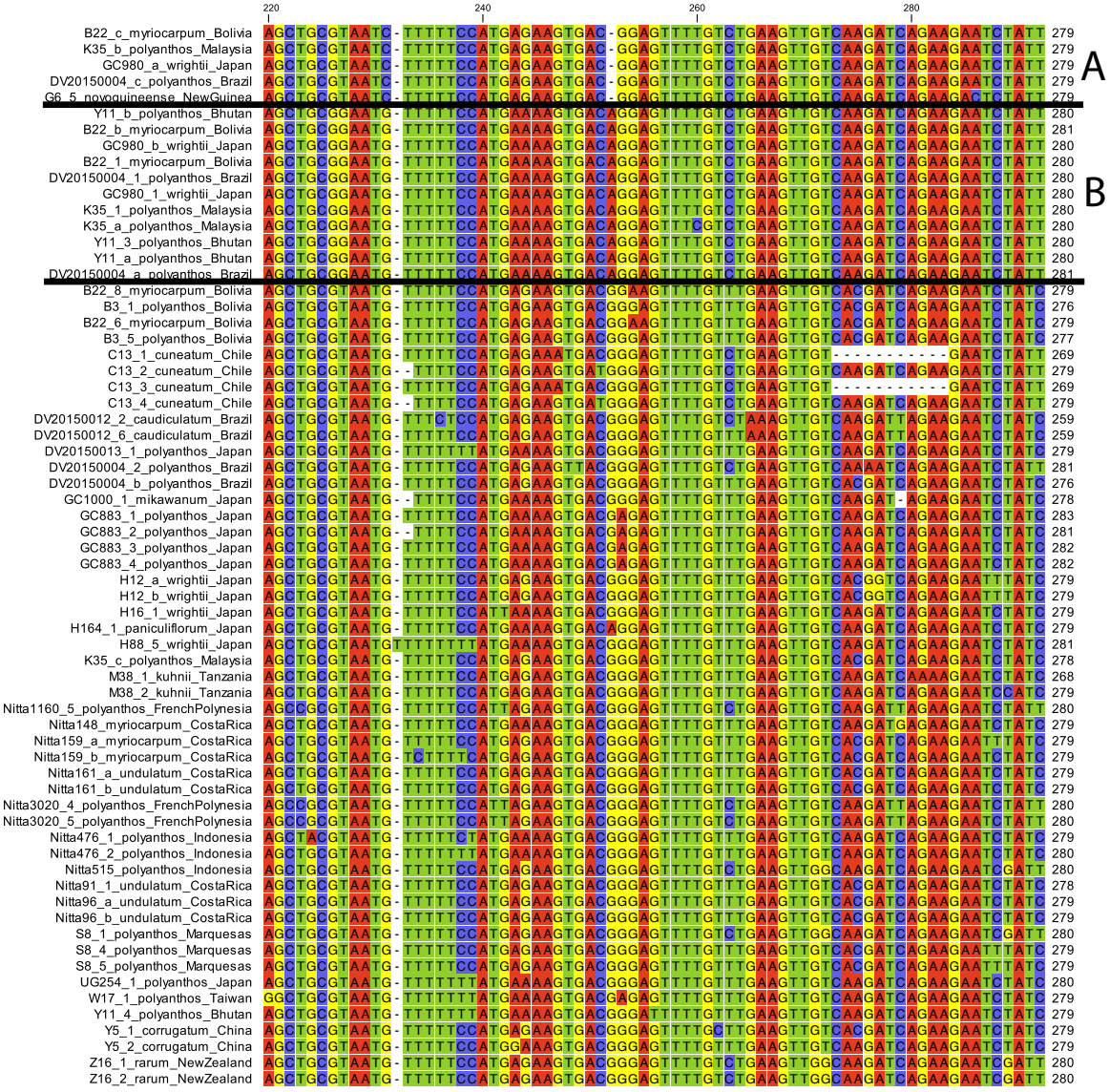
31.I.2007, Malaysia, Sabah, Mt. Kinabalu, between Timophon gate and Kandis shelter; K12, Yamada 011129-01, no date, Malaysia; I5, Asakawa 21, no date, Indonesia; TNS VS 1161468, HY59, s.n. 1755, no date, Cambodia; TNS VS 1161451, HY16, s.n. 1497, no date, Cambodia; H40, Fujimoto, s.n., no date, Japan, Aomori prefecture, AB574717; H17, Morino 000616, 16.VI.2000, Japan, Kanagawa prefecture, Tanzawa; TNS VS 773469, GC883, Atsushi Ebihara, Chie Tsutsumi & Katsuyuki Kawahara KS2008-1726, 16.V.2008, Japan, Kagoshima prefecture, Kumame-gun, Yakushima; DV20150013, D.T. Vasques & A. Ebihara 2015-0013, 22.V.2015, Japan, Tokyo, Okutama 360m; DV20150011, D.T. Vasques, J. Prado, R.Y. Hirai & D.G. Gissi 2015-0011, 23.II.2015, Brazil, São Paulo, São José do Barreiro 1590m; DV20150004, D.T. Vasques, J. Prado, R.Y. Hirai & D.G. Gissi 2015-0004, 22.II.2015, Brazil, São Paulo, São José do Barreiro 1562m; DV20150001, D.T. Vasques, J. Prado, R.Y. Hirai & D.G. Gissi 2015-0001, 21.II.2015, Brazil, São Paulo, São José do Barreiro 1564m; J.Y. Dubuisson s.n., no date, La Reunion, AY775445, AY775424; s.n. 40, no date, Tahiti, AB217846, AY775425. ***Hymenophyllum rarum***: Z16, A. Ebihara, 011217-09, no date, New Zealand, AB496571, GU200689. ***Hymenophyllum revolutum***: D. Callen, no date, New Zealand, GU200675, GU200690. ***Hymenophyllum rufescens***: A. Ebihara 011221-08, no date, New Zealand, AB496570, AB496591. ***Hymenophyllum scrabum:*** A. Ebihara 011223-05, no date, New Zealand, AB083278, AY775428. ***Hymenophyllum serrulatum***: A. Ebihara 000223-0009, no date, Malaysia, AB496565, AB496586; ***Hymenophyllum tenellum***: P, Duke, J.Y. Dubuisson HR-1999-27, no date, La Reunion, AB191453, AY095126. ***Hymenophyllum tunbrigense***: S. Hennequin 2004-2, no date, Portugal, GU200679, GU200694. ***Hymenophyllum undulatum***: J. Nitta, J. Condack, F. Matos, C. Rothfels, M. Sundue, A. Vasco 96, 27.I.2008, Costa Rica, San Jose, Cerro de la Muerte 3093m; J. Nitta, J. Condack, F. Matos, C. Rothfels, M. Sundue, A. Vasco 91, 25.I.2008, Costa Rica, Alajeula, road to Volcan Poas 2244m; J. Nitta & David Barrington 2019, 12.I.2013, Costa Rica, Alajeula, San Ramon, Nectandra Biological Preserve 2143m; J. Nitta 161, 9.III.2008, Costa Rica, Puntarenas, Reserva Biologico Durika 2500m. ***Hymenophyllum valvatum***: DV20150014, D.T. Vasques & A. Ebihara 2015-0014, 22.V.2015, Okutama, Japan, Tokyo 360m. ***Hymenophyllum villosum***: TI, A. Ebihara 011223-01, no date, New Zealand, AB191454; D. Callen, no date, New Zealand, AY775429. ***Hymenophyllum wilsonii***: F. Katzer 1, no date, United Kingdom, GU200678, GU200693. ***Hymenophyllum wrightii:*** H16, A. Ebihara 000618-1, 18.VI.2000, Japan, Gunma prefecture; H12, A. Ebihara, 000901-1, 1.IX.2000, Japan, Nagano prefecture, AB083277, AY775430; GC980, A. Ebihara, A. Yamaoka & Y. Fukazawa 081220-12, no date, Japan, Tokyo; TNS VS 765790, GC435, A.Ebihara, Y.Tsujita & Y.Horii TH2007-911, 7.VIII.2007, Japan, Akita prefecture, Yurihonjyou city; H88, A. Ebihara 000901-02, no date, Japan, Nagano prefectur

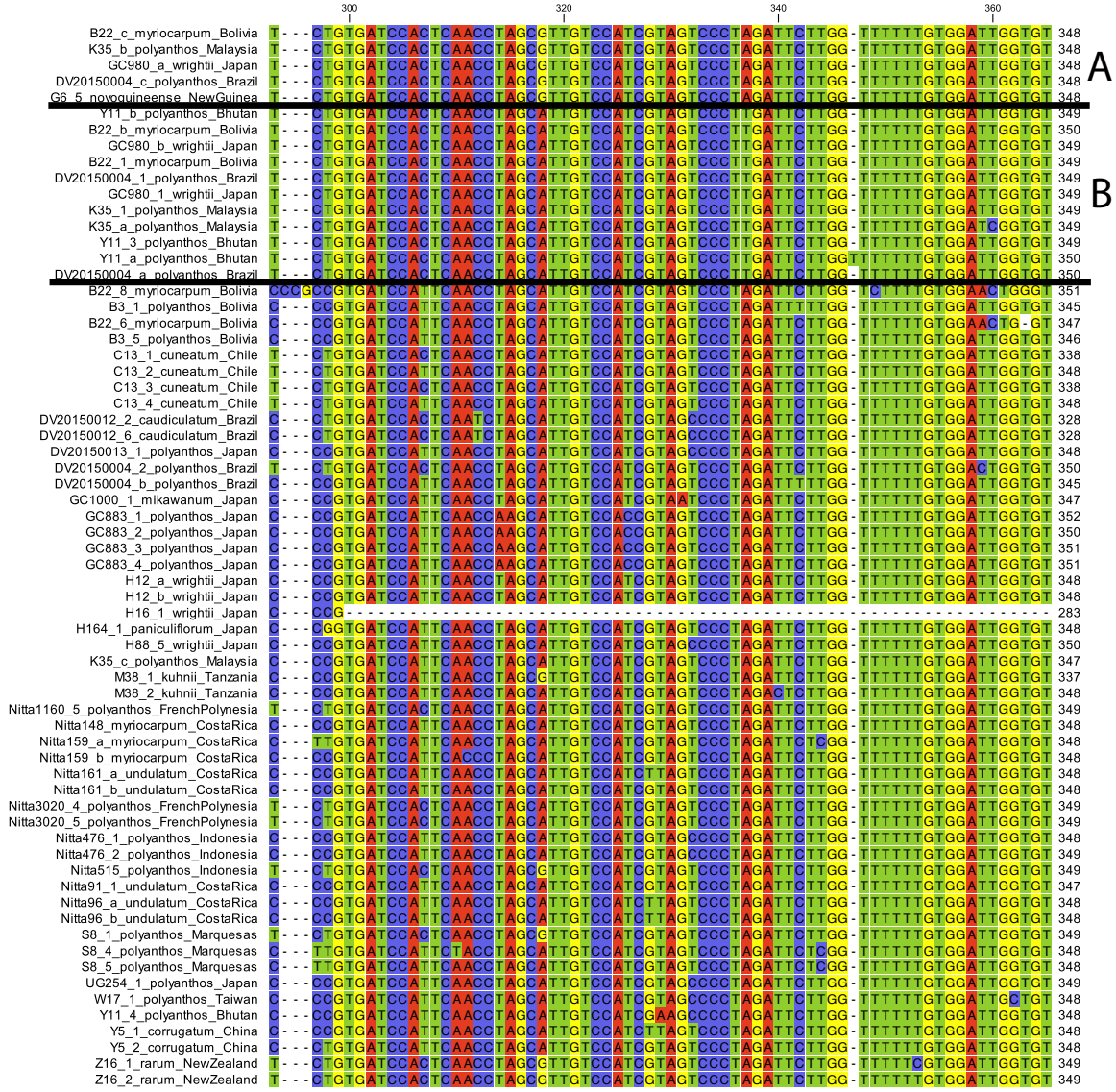
Appendix 2: Nucleotide sequences alignment used for the phylogenetic investigation based on *LEAFY* nuclear marker (Chapter 3). The groups A and B denote paralog sequences found in the analysis, as shown in figure 9.




80
100
120
140
A
B


160
180
200
A
B


220
240
260
280
A
B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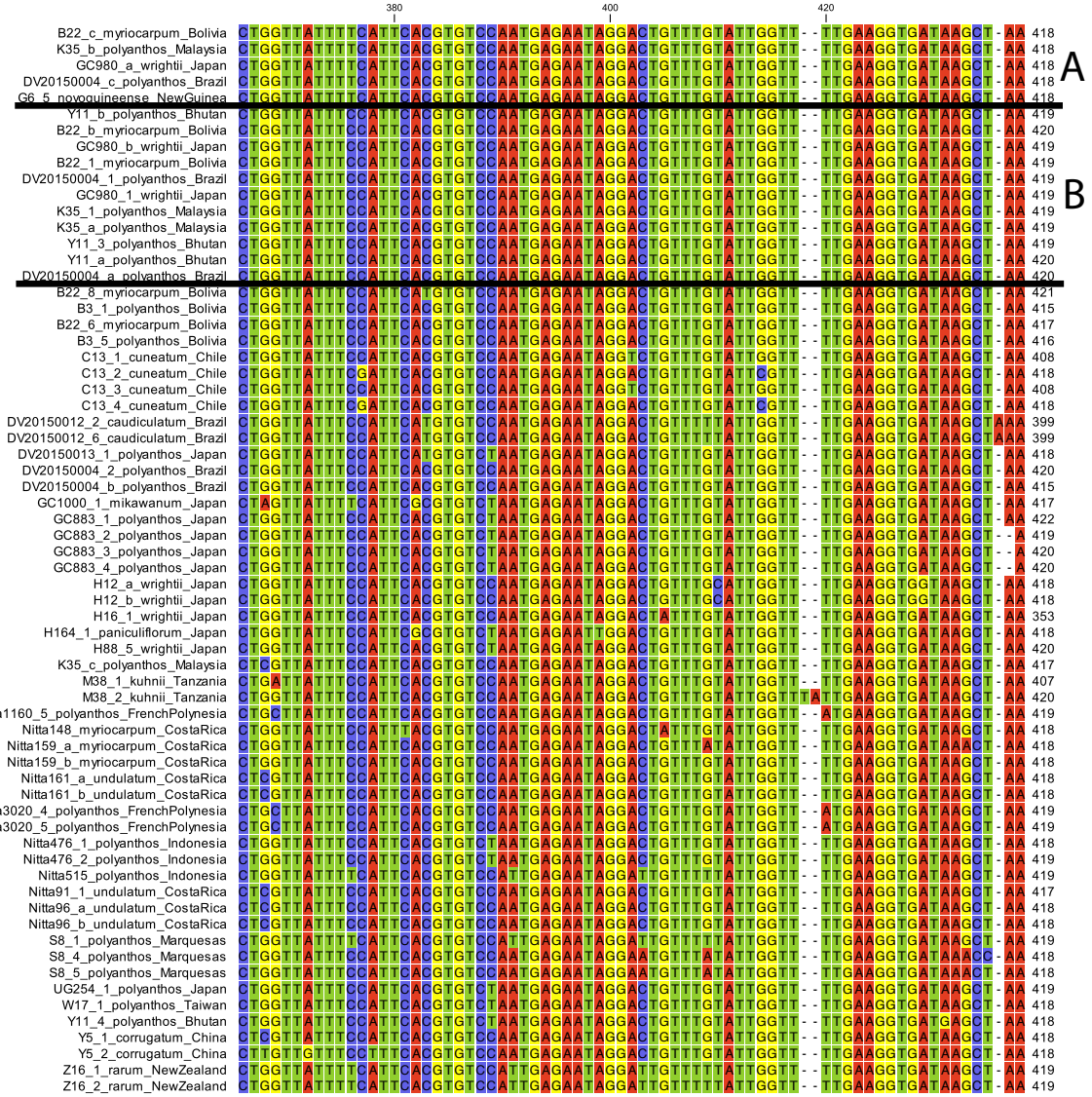
A
B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400
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Y11_b_polyanthos_Bhutan CTGGTTATTTTCCATTCACGTGTCCAATGAGAATAGGACTGTTTGTATTGGTT--TTGAAGGTGATAAGCT-AA 419
B22_b_myriocarpum_Bolivia CTGGTTATTTCCATTCACGTGTCCAATGAGAATAGGACTGTTTGTATTGGTT--TTGAAGGTGATAAGCT-AA 420
GC980_b_wrightii_Japan CTGGTTATTTCCATTCACGTGTCCAATGAGAATAGGACTGTTTGTATTGGTT--TTGAAGGTGATAAGCT-AA 419
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K35_a_polyanthos_Malaysia CTGGTTATTTCCATTCACGTGTCCAATGAGAATAGGACTGTTTGTATTGGTT--TTGAAGGTGATAAGCT-AA 419
Y11_3_polyanthos_Bhutan CTGGTTATTTCCATTCACGTGTCCAATGAGAATAGGACTGTTTGTATTGGTT--TTGAAGGTGATAAGCT-AA 419
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C13_2_cuneatum_Chile CTGGTTATTTCGATTCACGTGTCCAATGAGAATAGGACTGTTTGTATTCGTT--TTGAAGGTGATAAGCT-AA 418
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C13_4_cuneatum_Chile CTGGTTATTTCGATTCACGTGTCCAATGAGAATAGGACTGTTTGTATTCGTT--TTGAAGGTGATAAGCT-AA 418
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DV20150004_b_polyanthos_Brazil CTGGTTATTTCCATTCACGTGTCCAATGAGAATAGGACTGTTTGTATTGGTT--TTGAAGGTGATAAGCT-AA 415
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GC883_2_polyanthos_Japan CTGGTTATTTCCATTCACGTGTCTAATGAGAATAGGACTGTTTGTATTGGTT--TTGAAGGTGATAAGCT--A 419
GC883_3_polyanthos_Japan CTGGTTATTTCCATTCACGTGTCTAATGAGAATAGGACTGTTTGTATTGGTT--TTGAAGGTGATAAGCT--A 420
GC883_4_polyanthos_Japan CTGGTTATTTCCATTCACGTGTCTAATGAGAATAGGACTGTTTGTATTGGTT--TTGAAGGTGATAAGCT--A 420
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H16_1_wrightii_Japan CTGGTTATTTCCATTCACGTGTCCAATGAGAATAGGACTATTTGTATTGGTT--TTGAAGGTGATAAGCT-AA 353
H164_1_paniculiflorum_Japan CTGGTTATTTCCATTCGCGTGTCTAATGAGAATTGGACTGTTTGTATTGGTT--TTGAAGGTGATAAGCT-AA 418
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Nitta148_myriocarpum_CostaRica CTGGTTATTTCCATTTACGTGTCCAATGAGAATAGGACTATTTGTATTGGTT--TTGAAGGTGATAAGCT-AA 418
Nitta159_a_myriocarpum_CostaRica CTGGTTATTTCCATTCACGTGTCCAATGAGAATAGGACTGTTTATATTGGTT--TTGAAGGTGATAAACT-AA 418
Nitta159_b_myriocarpum_CostaRica CTGGTTATTTCCATTCACGTGTCCAATGAGAATAGGACTGTTTGTATTGGTT--TTGAAGGTGATAAGCT-AA 418
Nitta161_a_undulatum_CostaRica CTCGTTATTTCCATTCACGTGTCCAATGAGAATAGGACTGTTTGTATTGGTT--TTGAAGGTGATAAGCT-AA 418
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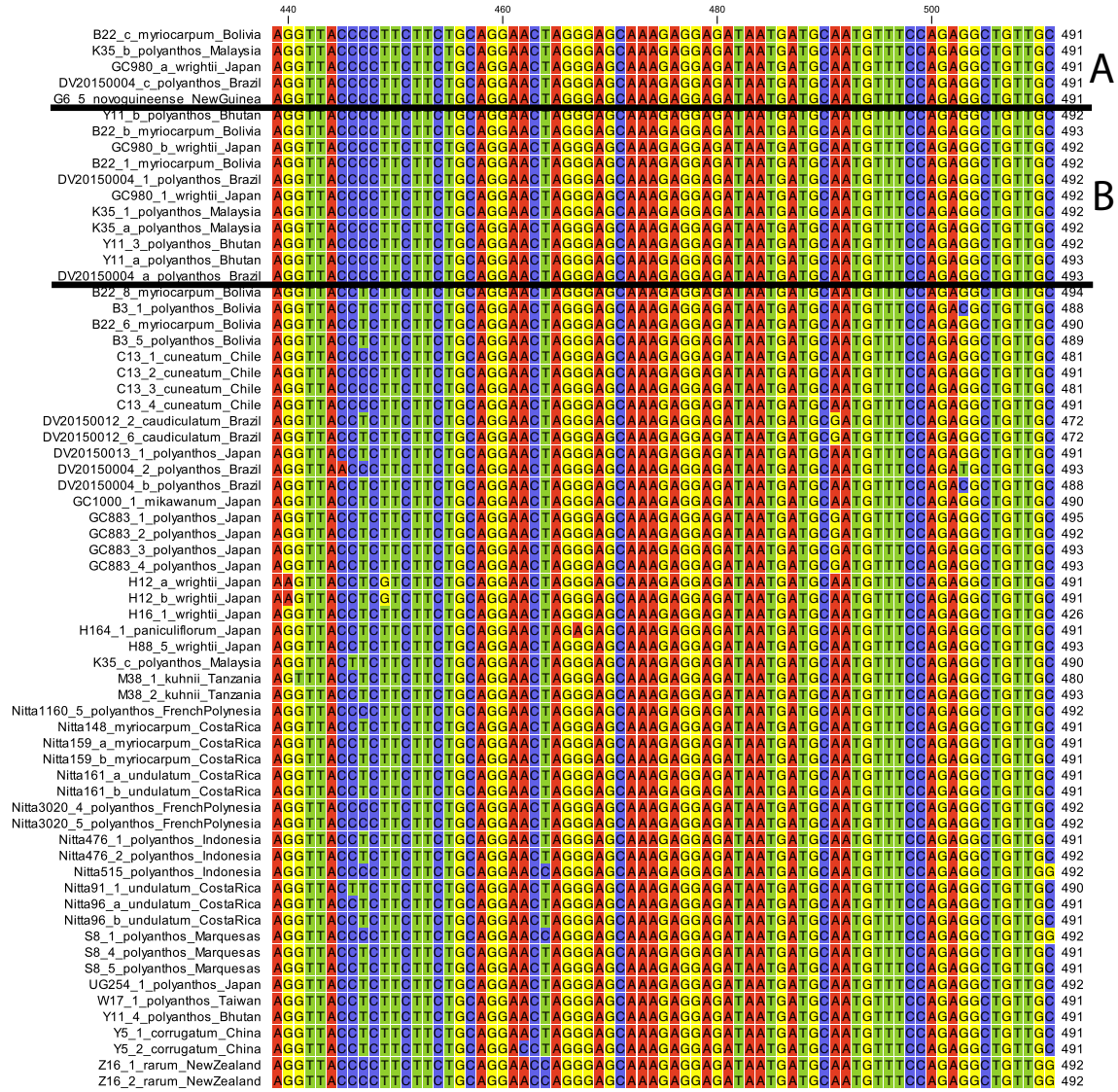
A
B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460
480
500
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K35_b_polyanthos_Malaysia 491
GC980_a_wrightii_Japan 491
DV20150004_c_polyanthos_Brazil 491
G6_5_novoguineense_NewGuinea 491
A
Y11_b_polyanthos_Bhutan 492
B22_b_myriocarpum_Bolivia 493
GC980_b_wrightii_Japan 492
B22_1_myriocarpum_Bolivia 492
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GC980_1_wrightii_Japan 492
K35_1_polyanthos_Malaysia 492
K35_a_polyanthos_Malaysia 492
Y11_3_polyanthos_Bhutan 492
Y11_a_polyanthos_Bhutan 493
DV20150004_a_polyanthos_Brazil 493
B
B22_8_myriocarpum_Bolivia 494
B3_1_polyanthos_Bolivia 488
B22_6_myriocarpum_Bolivia 490
B3_5_polyanthos_Bolivia 489
C13_1_cuneatum_Chile 481
C13_2_cuneatum_Chile 491
C13_3_cuneatum_Chile 481
C13_4_cuneatum_Chile 491
DV20150012_2_caudiculatum_Brazil 472
DV20150012_6_caudiculatum_Brazil 472
DV20150013_1_polyanthos_Japan 491
DV20150004_2_polyanthos_Brazil 493
DV20150004_b_polyanthos_Brazil 488
GC1000_1_mikawanum_Japan 490
GC883_1_polyanthos_Japan 495
GC883_2_polyanthos_Japan 492
GC883_3_polyanthos_Japan 493
GC883_4_polyanthos_Japan 493
H12_a_wrightii_Japan 491
H12_b_wrightii_Japan 491
H16_1_wrightii_Japan 426
H164_1_paniculiflorum_Japan 491
H88_5_wrightii_Japan 493
K35_c_polyanthos_Malaysia 490
M38_1_kuhnii_Tanzania 480
M38_2_kuhnii_Tanzania 493
Nitta1160_5_polyanthos_FrenchPolynesia 492
Nitta148_myriocarpum_CostaRica 491
Nitta159_a_myriocarpum_CostaRica 491
Nitta159_b_myriocarpum_CostaRica 491
Nitta161_a_undulatum_CostaRica 491
Nitta161_b_undulatum_CostaRica 491
Nitta3020_4_polyanthos_FrenchPolynesia 492
Nitta3020_5_polyanthos_FrenchPolynesia 492
Nitta476_1_polyanthos_Indonesia 491
Nitta476_2_polyanthos_Indonesia 492
Nitta515_polyanthos_Indonesia 492
Nitta91_1_undulatum_CostaRica 490
Nitta96_a_undulatum_CostaRica 491
Nitta96_b_undulatum_CostaRica 491
S8_1_polyanthos_Marquesas 492
S8_4_polyanthos_Marquesas 491
S8_5_polyanthos_Marquesas 491
UG254_1_polyanthos_Japan 492
W17_1_polyanthos_Taiwan 491
Y11_4_polyanthos_Bhutan 491
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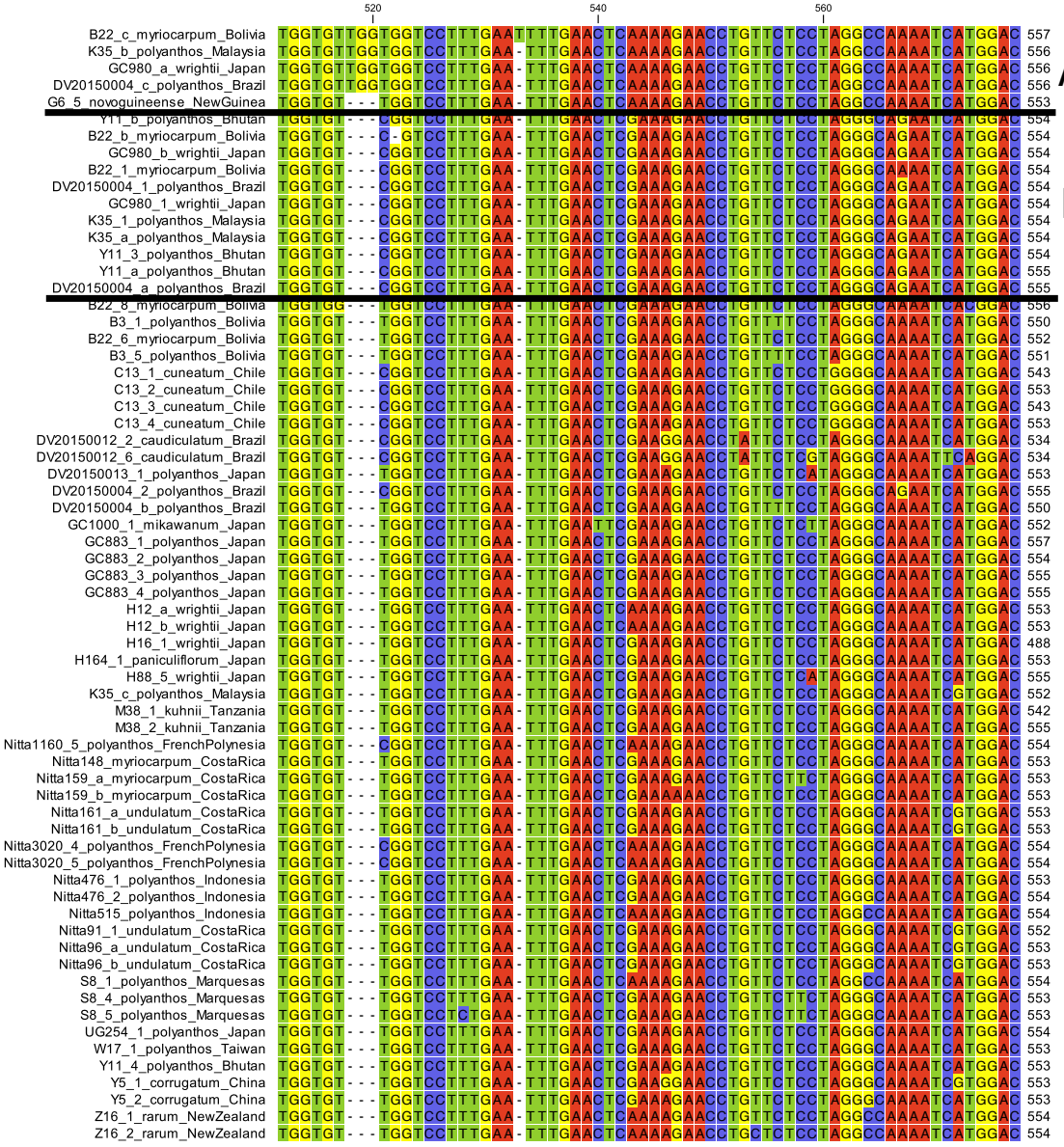
Y5_2_corrugatum_China 491
Z16_1_rarum_NewZealand 492
Z16_2_rarum_NewZealand 492


520
540
560
A
B

Appendix 3: List of samples used for the morphometric analysis (Chapter 4). Samples are organized by species name, deposited herbarium name, Collector name, location and identification number.

***Hymenophyllum cuneatum*:** C13, A. Ebihara 021223-07, no date, Chile. ***Hymenophyllum mikawanum*:** TNS VS 738136, GC1000, A. Ebihara, S. Serizawa & H. Miyazaki AC2009-2172, 13.VI.2009, Japan, Aichi prefecture. ***Hymenophyllum myriocarpum*:** B3, Asakawa 174-4, 2001, Bolivia; B25, Asakawa 208-3, 2001, Bolivia; B16, Asakawa 180-3, 2001, Bolivia; B22 Asakawa 196-2, 2001. Bolivia. ***Hymenophyllum paniculiflorum*:** TNS VS 766206, K41, A.Sugawara, A.Ebihara, T.Nakamura & S.Matsumoto SB2007-61, 1.II.2007, Malaysia, Sabah Mt. Kinabalu Masilau, Nepenthes Trail. ***Hymenophyllum polyanthos*:** DV20150013, D.T. Vasques & A. Ebihara 2015-0013, 22.V.2015, Japan, Tokyo, Okutama 360m; TNS VS 776487, UG257, A. Ebihara, T. Oka & T. Oka NN2008-1967, 16.IX.2008, Japan, Nagano prefecture; TNS VS 1161451, HY16, 1497, no date, Cambodia; TNS VS 766204, K39, A. Sugawara, A. Ebihara, T. Nakamura & S.Matsumoto SB2007-52 1.II.2007 Malaysia Sabah Mt. Kinabalu Masilau, Nepenthes Trail; I5, Asakawa 21, Indonesia; TNS VS 762067, W16, A. Ebihara, M. Yokota, G. Kokubugata, S. Kobayashi & K. Yasuda TW2006-184, 6.XII.2007, Taiwan, Pingtung Chuenr Hsiang Jinshui-ying; TNS VS 762064, W17, A. Ebihara, M. Yokota, G. Kokubugata, S. Kobayashi & K. Yasuda TW2006-187, 6.XII.2007, Taiwan, Pingtung Chuenr Hsiang Jinshui-ying; TNS VS 766417, UG255, A. Ebihara, S. Fujimoto & K. Ohora KI2007-1261, 25.VIII.2007, Japan, Wakayama; TNS VS 766199, K35, A. Sugawara, A. Ebihara, T. Nakamura & S. Matsumoto SB2007-13, 31.I.2007, Malaysia, Sabah Mt. Kinabalu between Timophon Gate and Kandis Shelter; TNS VS 766200, K36, A. Sugawara, A. Ebihara, T. Nakamura & S. Matsumoto SB2007-16, 31.I.2007, Malaysia, Sabah Mt. Kinabalu between Timophon Gate and Kandis Shelter; TNS VS 766209, K44, A.Sugawara, A. Ebihara, T. Nakamura & S. Matsumoto SB2007-92, 3.II.2007, Malaysia, Sabah Mt. Alabu; DV20150001, D.T. Vasques, J. Prado, R.Y. Hirai & D.G. Gissi 2015-0001, 21.II.2015, Brazil, São Paulo, São José do Barreiro, 1564m; DV20150004, D.T. Vasques, J. Prado, R.Y. Hirai & D.G. Gissi 2015-0004, 22.II.2015, Brazil, São Paulo, São José do Barreiro, 1562m; DV20150011, D.T. Vasques, J. Prado, R.Y. Hirai & D.G. Gissi 2015-0011, 23.II.2015, Brazil, São Paulo, São José do Barreiro, 1590m; RH756, R.Y. Hirai, J. Prado & R. da Silva Cruz 756, 12.XII.2014, Brazil, São Paulo, Santo André, 850m; TNS VS766205, K40, A. Sugawara, A. Ebihara, T. Nakamura & S. Matsumoto SB2007-60, 1.II.2007, Malaysia, Sabah Mt. Kinabalu Masilau, Nepenthes Trail; K12, Yamada, 011129-01, no date, Malaysia; Nitta631, J. Nitta 631, 22.VIII.2010, French Polynesia, Society Islands, Moorea, 372m; K. Iwatsuki V-97038, 29.XII.1997, Vietnam, Lam Dong, 1400m; K. Iwatsuki V-98082, 1.I.1998, Vietnam, Lam Dong, 1400-1500m; TI00001290, T. Shimizu, K. Iwatsuki, N. Fukuoka & M. Hutoh M13034, 14.X.1967, Malaysia, Penang,

500m; TI00001293, B. Hayata 1917, Indo-China; N.E.G. Courtwell 187, 29.V.1958, Papua-NewGuinea, Kanosuru; TI00001297, W.T. Tsang 20496, 17.V.1932, Indonesia, Sam Kok Shan; R.J. Johns 6644 10.III.1991, Brunei, Temburong, 1125m; R.J. Johns 6635, 10.III.1991, Brunei, Temburong, 1125m; TI00001298, M. Tagawa, K. Iwatsuki & N. Fukuoka T1293, 4.XII.1965, Thailand, Udawn, 110-1500m; TI00001289, K. Yoda 496, 17.II.1962, Thailand, Mt. Khao Luang, 1000-1300m; TI00001288, E.B. Copeland, no number, I.1909, Philippines Luzon, Mt. Banahaw; TI00001295, T. Nakai, II.1919, Java, Mt. Gede; TI00001284, G. Murata, N. Fukuoka & Sukasdi J-819, 18.VIII.1973, Java, Baturaden-Gunung Slamet; TI00047073, H. Hara, H. Kanai, G. Murata, M. Togashi & T. Tuyama, 17.V.1960, Sikkim, Yoksam, 1700m; TI00047067, H. Hara, H. Kanai, G. Murata, M. Togashi & T. Tuyama, 18.V.1960, Sikkim, Yoksam, Bakkim, 1700-2200m; TI00001457, H. Ohba, M. Wakabayashi, M. Suzuki & S. Akiyama 8351506, 14.IX.1983, Nepal, Khae Khola, Phedi Kharka - Koplang – Khanigaon, 1700-2100m, TI00001466, H. Kanai, H. Ohashi, K. Iwatsuki, H. Ohba, Z. Iwatsuki & P.R. Shakya 872271, 5.VI.1972, Nepal, Hile-Mure-Sinduwa-Bhalikhop-Chitre, 1900-2400m, TI00001471, H. Kanai, H. Ohashi, K. Iwatsuki, H. Ohba, Z. Iwatsuki & P.R. Shakya 873274, 20.VI.1972, Nepal, Topke Gola - Jalang Chhyongo, 3600-4300m; TI00001476, H. Kanai, H. Hara & H. Ohba 852275, 31.VIII.1972, Nepal, Bhorlang- Sundarijar, 1400-2400m; TI00001491, H. Kanai, G. Murata & M. Togashi, 16.XI.1963, Nepal, Baroya Khimty-Thakma Khofkla; TI00001450, H. Ohba, M. Wakabayashi, M. Suzuki, N. Kurisaki, K.R. Rajbhandari & S.K. Wu 8581003, 19.VIII.1985, Nepal, Sagarmatha - Kata Bisana - Goyem - Lamjura – Taktor, 2550-3400m; TI00001458, H. Ohba, H. Kanai, M. Wakabayashi, M. Suzuki & S. Akiyama 8350300, 12.VII.1983, Nepal, Dhaulagiri - Gorepani Deorali, 2650-3170m; TI00001488, H. Hara, S. Kurosawa & T. Tuyam, 14.XI.1963, Nepal , Thakma Khola-Diorali Bhanjang; TI00001486, H. Hara, S. Kurosawa & T. Tuyama, 12.XI.1963, Nepal, Baroya Khimty-Thakma Khofkla; TI00001489, H. Kanai, G. Murata & M. Togashi, 16.XI.1963, Nepal, Baroya Khimty-Thakma Khofkla; TI00001453, H. Ohba, M. Wakabayashi, M. Suzuki, N. Kurisaki, K.R. Rajbhandari & S.K. Wu 8581292, 2.IX.1985, Nepal, Sagarmatha - Sarkari Pati, 3350-3970m; TI00001452, H. Ohba, M. Wakabayashi, M. Suzuki, N. Kurisaki, K.R. Rajbhandari & S.K. Wu 8581172, 25.VIII.1985, Nepal, Sagarmatha, 3970m; M.Kato, Y. Shimizu, N. Murakai, S. Akiyama & X. Cheng 1058, 21.VII.1988, China, Yunnan, Yanbi County, 2350-2600m; TI00047018, W.T. Tsang 24872, 1-16.III.1935, China, Ts'ung-hwa District, Sam Kok Shan; D.E. Boufford & B. Bartholomew 24725, 9.IX.1988, China, Guan Xian, Qinglongzui, 1620m; M.Kato, Y. Shimizu, N. Murakai, S. Akiyama & X. Cheng 1109, 22.VII.1988, China, Yunnan, Yanbi County, 2300-3150m; Gaoligong Shan Biodiversity Survey 16696, 28.IX.2002, China, Yunnan, Gongshan, 2950m; TI00001496, J. Murata 9382, 15.II.1980, Japan, Tokunoshima, 500m; TI00001499, S. Hatusima 18379, 25.VI.1955, Japan, Okinawa, 450m; TI00001500, R. Nozu, 9.XI.1957, Japan, Okinawa;

TI00047002, T. Uchiyama, 16.XII.1900, Japan, Kagoshima, Amami-Oshima, TI00047003, H. Ito, 7.V.1936, Japan, Kagoshima, Amami-Oshima; TI00047005, H. Ito, 7.V.1936, Japan, Kagoshima, Amami-Oshima; TI00047007, H. Ito, 7.V.1936, Japan, Kagoshima, Amami-Oshima; BM000936770, W.A.W. de Beuzeville 6296, III.1897, Trindad Tobago; BM000785349, E.A.C.L.E. Schelpe 5540, 6.VII.1955, Mozambique, Sofala; BM000776979, R. Spruce 4699, VIII.1856, Peru; SP22249, F.C. Hoehne, V.1914, Brazil, Rio de Janeiro, Tijuca; SP22251, M. Wacket 153a, no date, Brazil, São Paulo, Serra do Mar; SP5083, F.C. Hoehne, 22.I.1921, Brazil, Minas Gerais, Santa Barbara; SP448301, J. Prado, H. Tuomisto, K. Ruokolainen & J.N. de Souza 1948, 12.II.2008, Brazil, Amazonas, Presidente Figueiredo; SP443379, E. Schuettpelz, J. Prado, P.B. Schwarsburd & G. Yatskievych 1412, 14.I.2010, Brazil, Minas Gerais, Santo Antonio do Itambé, 865m; SP430123, R.Y. Hirai, J. Prado, J. Vasconcellos Neto & P.R. Polli 642, 16.X.2009, Brazil, São Paulo, Jundiaí, 1233m; SP440645, N.M. Lepsch da Cunha & E. Costa Pereira 402, 28.IX.1989, Brazil, Amazonas, Distrito Agropecuário, 50-150m; SP266246, O. Yano, M.P. Marcelli & T. Ahti 21482, 25.XI.1993, Brazil, Minas Gerais, Itamonte, 2250m; SP175321, M. Kirizawa, E.A. Lopes & A. Custodio Filho 655, 8.XII.1981, Brazil, São Paulo, Casa Grande; SP386691, F.B. Matos, A.M. Amorim, J. Paixão, S. Sant'ana et al. 926, 8.II.2006, Brazil, Minas Gerais, Santa Maria do Salto; SP313553, J. Prado & M.P. Marcelli 786, 21.III.1996, Brazil, São Paulo, Campos do Jordão, 1430m; SP391924, P.H. Labiak, A. Amorim, M. Lopes, A.B. Rodrigues & S. Sant'ana 3712, 11.VIII.2006, Brazil, Bahia, Camacã, 850m; SP337057, P. Labiak 1097, 24.V.1999, Brazil, São Paulo, Salesópolis, 900m; SP382404, F.B. Matos, A. Amorim, J. Jardim, J. Paixão et al. 358, 8.II.2005, Brazil, Bahia, Barro Preto; SP8694, F.C. Hoehne, 13.IX.1923, Brazil, São Paulo, Campos do Jordão; MBG2139818, D.B. Lellinger & J.J. White 990, 7.VII.1970, Costa Rica, Heredia; Nitta135, J. Nitta 135, 19.II.2008, Costa Rica, Alajeula; Nitta170, J. Nitta 170, 11.III.2008, Costa Rica, San Jose; TI00001277, H. Ito, 9.IV.1941, South Pacific Mandate, Kusai, Mt. Matante; TI00001273, S. Sekibe, 18.VIII.1940, South Pacific Mandate, Palao, Aimiriki; TI00001269, T. Tuyama, 25.VIII.1937, South Pacific Mandate, Palao; JP2289, J. Prado & R.Y. Hirai 2289, 16.XI.2012, Brazil, São Paulo, Campos do Jordão, 1888m; RH534, R.Y. Hirai 534, 30.V.2008, Brazil, Minas Gerais, 1300m. ***Hymenophyllum rarum*:** AB496571, Z16, A. Ebihara 011217-09, no date, New Zealand. ***Hymenophyllum wrightii*:** AB083277, H12, A. Ebihara 000901-1, 1.IX.2000, Japan, Nagano prefecture; H16, A. Ebihara 000618-1, 18.VI.2000, Japan, Gunma prefecture; GC980, A. Ebihara, A. Yamaoka & Y. Fukazawa 081220-12, Japan, Tokyo; H88, A. Ebihara 000901-02, no date, Japan, Nagano prefecture

Appendix 4: List of samples observed for the morphometric analysis. Samples are organized according to the topology of the acquired phylogeny. Morphometric data ratio obtained from the each sample is also presented: LL/LW: lamina length : width ratio; LL/PL: lamina length : petiole length ratio; LL/PD: lamina length : pinnula distance ratio; PA: pinnae insertion point angle (in degrees); LPL/LPW: lateral pinnula length : width ratio. N/A stands for "not applicable".

Group	***spp***	**Herbarium ID**	**Other ID**	**Country**	**LL/LW**	**LL/PL**	**LL/PD**	**PA**	**LPL/LPW**
A groups	*mikawanum*	TNS VS 738136	GC1000	Japan	0.9	1.1	5.4	N/A	N/A
A groups	*paniculiflorum*	TNS VS 766206	K41	Malaysia	0.4	0.5	3.8	67.9	1.9
A groups	*polyanthos*		DV20150013	Japan	1.1	1.1	7.1	59.3	1.6
A groups	*polyanthos*	TNS VS 776487	UG257	Japan	1.9	1.9	11.8	68.2	0.3
A groups	*polyanthos*	TNS VS 1161451	HY16	Cambodia	1.9	1.6	12.0	62.1	1.7
A groups	*polyanthos*	TNS VS 766204	K39	Malaysia	2.0	3.7	13.4	53.0	1.5
A groups	*polyanthos*		I5	Indonesia	1.5	1.6	8.8	58.9	2.0
A groups	*polyanthos*	TNS VS 762067	W16	Taiwan	1.7	1.4	16.3	52.3	2.2
A groups	*polyanthos*	TNS VS 762064	W17	Taiwan	1.5	2.7	13.4	74.4	1.8
A groups	*polyanthos*	TNS VS 766417	UG255	Japan	2.8	2.6	18.4	63.5	1.9
A groups	*polyanthos*	TNS VS 766199	K35	Malaysia	1.5	2.8	15.7	65.1	1.7
A groups	*polyanthos*	TNS VS 766200	K36	Malaysia	1.4	1.9	10.4	70.6	1.7
A groups	*polyanthos*	TNS VS 766209	K44	Malaysia	1.5	1.9	9.6	72.2	2.2
A groups	*polyanthos*			Vietnam	2.1	2.3	31.1	57.9	0.7
A groups	*polyanthos*			Vietnam	2.9	2.1	22.3	55.7	0.4

A groups	*polyanthos*	TI00001290	Malaysia	8.0	9.2	49.3	57.7	0.4
A groups	*polyanthos*	TI00001293	Indo-China	3.1	3.2	18.8	53.6	0.5
A groups	*polyanthos*	TI00001297	Indonesia	2.3	3.0	21.6	61.0	0.5
A groups	*polyanthos*		Brunei	2.4	3.1	9.9	57.3	0.5
A groups	*polyanthos*		Brunei	1.8	0.6	9.9	46.7	0.5
A groups	*polyanthos*	TI00001298	Thailand	2.0	3.5	18.5	69.2	0.4
A groups	*polyanthos*	TI00001289	Thailand	2.1	2.9	15.0	62.7	0.5
A groups	*polyanthos*	TI00001288	Philippines	4.0	4.1	23.8	59.7	0.4
A groups	*polyanthos*	TI00001295	Java	1.9	3.9	21.5	64.5	0.4
A groups	*polyanthos*	TI00001284	Java	1.8	1.7	15.9	53.0	0.3
A groups	*polyanthos*	TI00047073	Sikkim	2.2	1.8	15.1	94.8	0.5
A groups	*polyanthos*	TI00047067	Sikkim	2.2	2.1	18.9	77.7	0.5
A groups	*polyanthos*	TI00001457	Nepal	1.5	1.5	12.9	76.4	0.6
A groups	*polyanthos*	TI00001466	Nepal	3.7	2.5	17.7	73.4	0.4
A groups	*polyanthos*	TI00001471	Nepal	4.7	3.5	22.7	59.0	2.4
A groups	*polyanthos*	TI00001476	Nepal	2.7	2.0	13.4	80.4	0.5
A groups	*polyanthos*	TI00001491	Nepal	1.6	1.5	17.8	75.8	0.5
A groups	*polyanthos*	TI00001450	Nepal	2.0	1.5	16.0	67.9	0.5
A groups	*polyanthos*	TI00001458	Nepal	0.9	0.5	6.0	71.1	0.5
A groups	*polyanthos*	TI00001488	Nepal	1.9	1.4	17.7	65.9	0.4
A groups	*polyanthos*	TI00001486	Nepal	1.6	1.0	14.8	70.3	0.4

A groups	*polyanthos*	TI00001489		Nepal	1.6	0.9	26.2	69.7	0.4
A groups	*polyanthos*	TI00001453		Nepal	1.7	1.7	8.8	69.3	0.7
A groups	*polyanthos*	TI00001452		Nepal	1.5	1.7	9.7	87.9	0.7
A groups	*polyanthos*			China	1.5	1.0	10.1	76.7	0.5
A groups	*polyanthos*	TI00047018		China	3.7	2.4	18.8	63.3	0.5
A groups	*polyanthos*			China	3.1	2.2	17.9	76.2	0.6
A groups	*polyanthos*			China	1.8	1.7	30.6	76.2	0.4
A groups	*polyanthos*			China	1.9	2.4	13.5	63.5	0.5
A groups	*polyanthos*	TI00001496		Japan	2.2	2.5	14.2	74.4	0.5
A groups	*polyanthos*	TI00001499		Japan	1.7	3.9	15.6	77.3	0.5
A groups	*polyanthos*	TI00001500		Japan	2.0	4.2	13.0	66.3	0.6
A groups	*polyanthos*	TI00047002		Japan	1.9	4.5	15.0	58.5	0.4
A groups	*polyanthos*	TI00047003		Japan	2.6	1.1	13.7	61.5	0.4
A groups	*polyanthos*	TI00047005		Japan	2.5	4.5	13.2	45.8	0.3
A groups	*polyanthos*	TI00047007		Japan	3.3	2.4	23.8	74.8	0.5
A groups	*wrightii*	AB083277	H12	Japan	0.4	0.3	1.1	N/A	N/A
A groups	*wrightii*		H16	Japan	2.0	4.8	10.4	N/A	N/A
A groups	*wrightii*		GC980	Japan	3.0	7.2	10.9	N/A	N/A
A groups	*wrightii (cf.)*		H88	Japan	0.8	6.6	11.0	N/A	N/A
N clade	*myriocarpum*		B3	Bolivia	1.7	0.9	7.0	65.2	2.2
N clade	*myriocarpum*		B25	Bolivia	2.5	2.4	23.3	81.3	2.9

N clade	*myriocarpum*		B16	Bolivia	1.8	2.2	9.3	65.6	1.5
N clade	*myriocarpum*		B22	Bolivia	2.3	3.0	21.9	98.2	2.4
N clade	*polyanthos*	BM000936770		Trindad Tobago	2.4	2.5	17.6	58.4	0.5
N clade	*polyanthos*	BM000785349		Mozambique	2.8	3.5	12.2	72.1	0.6
N clade	*polyanthos*	SP22251		Brazil2	2.4	4.4	23.4	61.0	0.3
N clade	*polyanthos*	SP5083		Brazil2	2.1	3.6	14.0	83.0	0.5
N clade	*polyanthos*	SP448301		Brazil2	1.9	2.1	14.8	103.4	0.6
N clade	*polyanthos*	SP443379		Brazil2	2.4	3.0	15.1	78.4	0.7
N clade	*polyanthos*	SP430123		Brazil2	2.5	3.5	18.0	91.0	0.5
N clade	*polyanthos*	SP440645		Brazil2	3.1	2.8	26.8	73.0	0.3
N clade	*polyanthos*	SP266246		Brazil2	2.0	2.5	22.6	66.0	0.3
N clade	*polyanthos*	SP175321		Brazil2	1.9	2.4	20.4	73.0	0.5
N clade	*polyanthos*	SP386691		Brazil2	2.0	5.9	16.7	68.9	0.5
N clade	*polyanthos*	SP313553		Brazil 1	3.0	8.5	18.9	58.9	0.4
N clade	*polyanthos*	SP391924		Brazil2	1.8	2.1	24.9	75.8	0.4
N clade	*polyanthos*	SP382404		Brazil2	3.2	2.1	21.9	69.4	0.4
N clade	*polyanthos*	MO2139818		Costa Rica	3.8	1.9	30.3	68.6	0.4
N clade	*polyanthos*		Nitta135	Costa Rica	3.1	2.1	17.6	66.4	0.7
N clade	*polyanthos*		Nitta170	Costa Rica	2.3	2.2	22.7	55.8	0.3
N clade	*polyanthos*		RH534	Brazil2	1.7	2.9	9.4	62.5	0.7
PSA clade	*cuneatum*		C13	Chile	1.8	2.1	6.5	39.5	2.1

PSA clade	*polyanthos*		DV20150001	Brazil1	2.2	2.9	22.5	37.0	2.1
PSA clade	*polyanthos*		DV20150004	Brazil1	4.2	8.4	24.1	41.0	1.7
PSA clade	*polyanthos*		DV20150011	Brazil1	4.5	6.6	31.8	40.5	1.9
PSA clade	*polyanthos*		RH756	Brazil1	4.7	5.4	30.1	29.8	2.2
PSA clade	*polyanthos*	TNS VS 766205	K40	Malaysia	3.8	4.1	15.8	48.7	1.4
PSA clade	*polyanthos*		K12	Malaysia	5.4	5.2	17.6	46.1	2.4
PSA clade	*polyanthos*		Nitta631	French Polynesia	2.9	3.1	18.8	52.6	2.0
PSA clade	*polyanthos*			Papua-NewGuinea	16.8	7.7	66.4	48.7	0.5
PSA clade	*polyanthos*	BM000776979		Peru	6.0	3.9	28.5	50.1	0.5
PSA clade	*polyanthos*	SP22249		Brazil 1	4.9	8.8	28.2	47.0	0.5
PSA clade	*polyanthos*	SP337057		Brazil1	9.3	8.3	44.4	56.2	0.7
PSA clade	*polyanthos*	SP8694		Brazil1	3.6	7.2	20.1	52.0	0.5
PSA clade	*polyanthos*	TI00001277		South Pacific Mandate	6.2	1.7	36.5	56.3	0.4
PSA clade	*polyanthos*	TI00001273		South Pacific Mandate	7.5	3.1	40.8	51.1	0.5
PSA clade	*polyanthos*	TI00001269		South Pacific Mandate	8.1	3.4	30.1	53.2	0.5
PSA clade	*polyanthos*		JP2289	Brazil1	3.4	6.5	23.4	53.2	0.3
PSA clade	*rarum*	AB496571	Z16	New Zealand	2.3	1.0	10.5	43.4	2.3

Appendix 5: List of samples observed for the anatomical analysis. Samples are organized according to the topology of the acquired phylogeny. Anatomical data obtained from the each sample is presented as: inner diameter (mm); sclerenchyma thickness (mm); and metaxylem cells (number).

Region	**Species**	**Herbarium ID**	**Other ID**	**Country**	**Inner diameter (mm)**	**Sclerenchyma thickness (mm)**	**Metaxylem cells**
PSA clade	*cuneatum*		C13	Chile	0.38	0.08	3
PSA clade	*polyanthos*		DV20150011	Brazil	0.6	0.15	8
PSA clade	*polyanthos*		RH756	Brazil	0.4	0.07	4
PSA clade	*rarum*	AB496571	Z16	New Zealand	0.36	0.07	3
N clade	*myriocarpum*		B1	Bolivia	0.42	0.07	3
N clade	*myriocarpum*		B16	Bolivia	0.44	0.09	5
N clade	*myriocarpum*		B19	Bolivia	0.47	0.06	4
N clade	*myriocarpum*		B20	Bolivia	0.5	0.1	4
N clade	*myriocarpum*		B22	Bolivia	0.6	0.12	5
N clade	*myriocarpum*		B3	Bolivia	0.46	0.07	4
A groups	*mikawanum*	TNS VS 738136	GC1000	Japan	0.29	0.05	3
A groups	*paniculiflorum*		H164	Japan	0.34	0.07	3
A groups	*polyanthos*		H173	Japan	0.59	0.1	5
A groups	*wrightii*		H88	Japan	0.26	0.05	1
A groups	*polyanthos*		I5	Indonesia	0.51	0.12	7
A groups	*paniculiflorum*		K18	Malaysia	0.46	0.12	5
A groups	*polyanthos*		K33	Malaysia	0.28	0.06	3

A groups	*polyanthos*		K34	Malaysia	0.38	0.06	7
A groups	*polyanthos*	TNS VS 766199	K35	Malaysia	0.46	0.1	5
A groups	*polyanthos*	TNS VS 766200	K36	Malaysia	0.92	0.21	13
A groups	*polyanthos*	TNS VS 766204	K39	Malaysia	0.65	0.1	8
A groups	*polyanthos*	TNS VS 766205	K40	Malaysia	0.59	0.15	7
A groups	*paniculiflorum*	TNS VS 766206	K41	Malaysia	0.38	0.1	2
A groups	*polyanthos*		K43	Malaysia	0.47	0.09	6
A groups	*polyanthos*	TNS VS 766209	K44	Malaysia	0.67	0.13	8
A groups	*polyanthos*		K45	Malaysia	0.64	0.1	7
A groups	*polyanthos*	TNS VS 762067	W16	Taiwan	0.7	0.13	8