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Ferns and Lycophytes as new challenges

A global review of chromosome number and genome size for the filmy ferns family (Hymenophyllaceae, Polypodiopsida)

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Abstract

Hymenophyllaceae is a fern family comprising around 450 species distributed among nine genera. Genome size and chromosome number have been recurring research target for Hymenophyllaceae in taxonomic and evolutionary studies. However, there is currently a lacks a thorough compilation for this information. The objective of this study was to compile data on chromosome number and genome size for Hymenophyllaceae. A panorama was constructed in order to highlight the observed patterns for the genera and subgenera. The discussed topics also included the geographic areas sampled and the methodological challenges surrounding data acquisition. This study included data on chromosome number and genome size for 158 and 15 species. The family displayed great variation for these characteristics, ranging from $2n = 22$ to 356 for chromosome number and from 2C = 21.47 pg to 73.2 pg for genome size. The genera C*allistopteris*, *Polyphlebium*, *Vandenboschia*, *Crepidomanes* and *Hymenophyllum* have $2n = 72$, or multiples of this value, as the most frequent numbers, *Trichomanes* and *Cephalomanes* mainly have $2n = 64$ (or multiples), and *Didymoglossum* has mostly $2n = 68$ (or multiples). We hope that this review will assist in the development of future research, seeking a better understanding of evolution and taxonomy for the Hymenophyllaceae.

Key words: chromosome count, cytogenetics, Hymenophyllales, Polypodiopsida, total DNA content.

Resumo

Hymenophyllaceae compreende um grupo de samambaias com cerca de 450 espécies organizadas em nove gêneros. Nessa família, o tamanho de genoma e número cromossômico têm sido explorados em estudos taxonômicos e evolutivos. Contudo, não existe atualmente uma compilação desses dados. Assim, o objetivo deste trabalho foi compilar dados sobre o número cromossômico e tamanho do genoma para Hymenophyllaceae. Foi construído um panorama destacando os padrões encontrados nos gêneros e subgêneros. Também discutimos as áreas geográficas amostradas e questões metodológicas que permeiam a aquisição de dados. A pesquisa incluiu o número cromossômico e tamanho de genoma para 158 e 15 espécies, respectivamente. Essas características apresentaram uma grande variação, o número cromossômico variou de $2n = 22$ a 356 e o tamanho do genoma variou de 2C = 21,47 pg a 73,2 pg. Nos gêneros C*allistopteris*, *Polyphlebium*, *Vandenboschia*, *Crepidomanes* e *Hymenophyllum* o valor mais frequente foi $2n = 72$ e múltiplos deste, já em *Trichomanes* e *Cephalomanes* as espécies apresentaram principalmente $2n = 64$ (ou múltiplos), e em *Didymoglossum* a maioria tem $2n = 68$ (ou múltiplos). Por fim, esperamos que esta revisão possa auxiliar no desenvolvimento de pesquisas futuras, proporcionando o melhor entendimento da evolução e taxonomia da família.

Palavras-chave: contagem de cromossomos, citogenética, Hymenophyllales, Polypodiopsida, quantidade total de DNA.

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Introduction

Hymenophyllaceae, the only family in the order Hymenophyllales, is organized into nine genera with approximately 450 species (Ebihara *et al*. 2006; PPG I 2016) and presents the highest abundance and diversity of species found in tropical montane forests (Tryon & Tryon 1982; Iwatsuki 1985; Kornás 1993). Traditionally, Hymenophyllaceae is divided into two subfamilies: Trichomanoideae and Hymenophylloideae (PPG I 2016). Over the years, several postulations were raised regarding the infra-familial classification of Hymenophyllaceae (Copeland 1947; Morton 1968; Picchi-Sermolli 1977), culminating in nine currently recognized monophyletic genera delineated by Ebihara *et al*. (2006). Hymenophylloideae contains only the genus *Hymenophyllum*, whereas Trichomanoideae branches into the eight remaining genera of the family: *Abrodictyum*, *Callistopteris*, *Cephalomanes*, *Crepidomanes*, *Didymoglossum*, *Vandenboschia*, *Polyphlebium* and *Trichomanes* (Ebihara *et al*. 2006).

Cytogenetic research surrounding Hymenophyllaceae began after the 1950s gaining traction through a publication entitled "*Problems of Cytology and Evolution in the Pteridophyta*", a work by Manton (1950). This title marks the initial step towards the widespread utilization of chromosomes in studies of fern evolution (Britton 1974). In Hymenophyllaceae, cytology-based information has significantly contributed to establishing the genera and subgenera within the family (Tindale & Roy 2002), as well as it has greatly stimulated debates regarding taxonomy (Walker 1985). Besides serving as additional criteria for taxon identification, cytogenetics also provides insights that may assist in elucidating the underlying relationships between species (Brathwaite 1975).

The main cytogenetic characteristics employed in this context for Hymenophyllaceae are the chromosome number and genome size. An example of a study using chromosome numbers to instigate a debate on Hymenophyllaceae systematics is the work conducted by Brathwaite (1975). It consists of a comparison of the classifications proposed by Copeland (1938, 1947) and Morton (1968) concerning available cytogenetic information. While Copeland argues for a classification with 34 distinct genera, Morton defends a perspective for two large genera, *Trichomanes* and *Hymenophyllum*. The introduction of chromosome number data to provide further context and substantiate the debate revealed problems in both propositions (Brathwaite 1975). Besides the use in systematics, chromosome number data has been a relevant trait in evolutionary studies, as demonstrated by Hennequin *et al*. (2010). In their research, the authors instigated the evolution of chromosome numbers for the genus *Hymenophyllum* by combining previously published chromosome numbers, newly reported counts and a comparative phylogenetics approach (Hennequin *et al*. 2010).

Together with chromosome number data, genome size has been used, for example, to estimate the ploidy level of species from the complex *Vandenboschia radicans* (Sw.) Copel.. In this case, cytogenetic and phylogenetic data allowed a better understanding of the biological status of taxa from this complex, which originated through reticulate evolution (Ebihara *et al*. 2005). Furthermore, broader fern studies that include Hymenophyllaceae species in their sampling suggest that genome size may be related to a plethora of factors. Clark *et al*. (2016), for example, detected a correlation between genome size and chromosome number, implying an apparent tendency of DNA quantity conservation per chromosome (Clark *et al*. 2016). Moreover, Fujiwara *et al*. (2023) identified a strong phylogenetic signal along the phylogeny of ferns for cytogenetic parameters of holoploid genome size, monoploid genome size and average DNA amount per chromosome. The authors also found that the rate of evolution for the three evaluated parameters was significantly correlated with total species number and diversification (Fujiwara *et al*. 2023).

Even though several studies have explored cytogenetic aspects of Hymenophyllaceae, a review that encompasses both chromosome numbers and genome sizes for the species in this family is non-existent at present. Moreover, there is no up-to-date compilation of taxonomic groups and geographic regions for Hymenophyllaceae species sampled in cytogenetic studies. In this context, the objective of this paper was to develop a comprehensive review of chromosome numbers and genome sizes for species of the Hymenophyllaceae. Building a database of the available data creates a foundation upon which unknown cytogenetic patterns may be unveiled for the group. Additionally, a review on this subject can highlight possible gaps in the current knowledge and direct future studies. This review sought to answer the following questions:

(*i*) Which species from the Hymenophyllaceae have previously reported data on chromosome number and genome size? (*ii*) How are cytogenetic data distributed within taxonomic groups (genera and subgenera)? (*iii*) Which geographic regions have the least studied species diversity? Are there any areas with a notable prevalence of polyploid species? (*iv*) What are the methodological challenges surrounding cytological data acquisition for Hymenophyllaceae?

Material and Methods

Acquisition of cytogenetic data

Cytogenetic data collection for Hymenophyllaceae took place until September 2023 and was carried out via a survey of specific repositories for cytogenetic data, as well as through searches on Web of Science (Institute of Scientific Information, Thomson Scientific) (<https://apps.webofknowledge.com/>). Access to the original publications was determined as an inclusion criterion. Both sporophytic and gametophytic numbers were considered for chromosome counts. The taxonomic classification proposed by Ebihara *et al*. (2006) was adopted in this study. The International Plant Names Index (IPNI 2023) and The Tropicos database (Missouri Botanical Garden 2023) were also consulted for nomenclature verification.

Chromosome number counts were retrieved from the repositories Index to Plant Chromosome Number (Goldblatt & Johnson 1979) and The Chromosome Counts Database (Rice *et al*. 2015). Genome size data was extracted from the Plant DNA C-values Database (Leitch *et al*. 2019). The terms used for queries were the family and genera names. In total, the search in the databases allowed the retrieval of 49 articles.

In order to search for publications on the Web of Science (Institute of Scientific Information, Thomson Scientific) (<https://apps.webofknowledge.com/>), we employed the following combination of terms: ("fern" OR "ferns" OR "Hymenophyllales" OR "Hymenophyllaceae" OR "*Abrodictyum*" OR "*Callistopteris*" OR "*Cephalomanes*" OR "*Crepidomanes*" OR "*Didymoglossum*" OR "*Hymenophyllum*" OR "*Polyphlebium*" OR "*Trichomanes*" OR "*Vandenboschia*") AND ("Cytogenetics" OR "Kariological" OR "Chromosome Number" OR "Chromosome Numbers" OR "Genome Size" OR "Nuclear DNA" OR "DNA content"). Papers were filtered to only include articles containing newly reported chromosome number counts or genome size estimates for Hymenophyllaceae species. Therefore, out of a total of 497 retrieved articles after the initial search, only 13 were selected for this review.

Data analysis

The following information was gathered and analysed from the selected articles: (*i*) description of the studies (year of publication, first author institution and country, general subject of the paper); (*ii*) sampled species (currently accepted names and taxa nomenclature used in the original publications); (*iii*) geographic data (country of the studied populations - only for chromosome number data); (*iv*) chromosome number data (gametophytic and sporophytic chromosome counts, number of cytotypes and ploidy level); (*v*) genome size data (genome size estimate, buffer solution, calibration standard and estimation method). The data here described were utilized to convey the current panorama of Hymenophyllaceae research regarding chromosome number and genome size. The full dataset produced from this review is available in Supplementary Material 1 (available at <https://doi.org/10.6084/m9.figshare.22277602>).

The general subjects we used to classify and group the papers are as follows: (*i*) Strict cytogenetics - publications exclusively concerned with cytogenetics, including reports on chromosome number and genome size; (*ii*) Systematics - publications that provide insights on classification and organization of species alongside cytogenetic data; (*iii*) Taxonomy - publications centred on nomenclature and description of species alongside cytogenetic data; and (*iv*) Evolution - publications that investigate evolutionary relationships between species throughout time alongside cytogenetic data.

In the interest of assess the taxonomic coverage for available chromosome number and genome size data, species diversity estimates for Hymenophyllaceae genera were obtained from Ebihara *et al*. (2006). The ploidy level for each species was estimated according to the base chromosome numbers reported by Ebihara *et al*. (2006). Additionally, this information was complemented with data from Hennequin (2004) for species of *Hymenophyllum* subg. *Hymenophyllum*.

We chose to indicate the sporophytic chromosome number ($2n$), representative of the total chromosome quantity for a species. Due to the existence of univalents, bivalents, trivalents

and quadrivalents, the use of the gametophytic number would not be adequate for comparisons. Some species had chromosome numbers reported by multiple studies. Identical counts for the same species were considered only once.

To verify whether Hymenophyllaceae chromosome numbers follow an equal frequency across the different genera we made use of the chi-squared test with software Jasp *v*. 0.17.2.1 (JASP Team 2023), considering *p*-values < 0.01 as statistically significant.

The chromosome number and genome size data were analysed alongside the phylogenetic structure of the group. The phylogenetic analysis included DNA sequences from 48 species of Hymenophyllaceae and two outgroup species (*Osmundastrum cinnamomeum* (L.) C.Presl and *Osmunda japonica* Thunb.), and its inclusion in analysis aimed to better organize the cytogenetic data and facilitate visualization of the relationships between groups in the family. The markers used correspond to the coding region of the *rbc*L gene and the intergenic spacers *rbc*L-*acc*D, *rps*4-*trn*S and *trn*G-*trn*R. All sequences were sourced from GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>), and their accession numbers can be found in Supplementary Material 2 (available at <https://doi.org/10.6084/m9.figshare.24291094>). Alignment of the DNA sequences was performed with software MAFFT version 7 (Katoh *et al*. 2018). Nucleotide substitution models were independently selected for each marker using PartitionFinder2 (Lanfear *et al*. 2017). The best model for all markers, determined based on the Akaike Information Criterion, was GTR+G. A Maximum Likelihood phylogenetic reconstruction analysis was conducted through RAxMLGUI *v*.2.0 with a concatenated matrix and 100 bootstrap replicates (Edler *et al*. 2021). The topology obtained is in agreement with Del Rio *et al*. (2017) and Hennequin *et al*. (2008). The resulting phylogenetic tree can be viewed in Supplementary Material 3 (available at <https://doi.org/10.6084/m9.figshare.24291127>).

Results and Discussion

Cytogenetic studies of the Hymenophyllaceae

Including searches in Web of Science and cytogenetic databases, a total of 52 research papers containing chromosome number counts published between 1950 and 2018 were included in this review, together with six papers covering genome size estimation published between 2002 and 2021 (Fig. 1). Throughout the years, it is possible to observe a diminishing number of scientific works reporting chromosome numbers, reaching a publication peak between 1960 and 1980. This reduction of studies after the 80s may be attributed to notable advancements in molecular technologies, which lowered the appeal and interest in classic cytogenetic research. As highlighted by Hennequin *et al.* (2010): "...cytological studies have been largely abandoned in the 1980s in favour of molecular studies". Other plant groups retain the same trend. Chromosome number studies for the fern family Gleicheniaceae, for example, also show a clear peak in publications between 1960 and 1980, dwindling afterwards (Lima *et al*. 2021). In the same manner, the order Sapindales, a group of angiosperms, shows an increase in papers reporting chromosome number data during the 70s and a decrease in subsequent decades (Guimarães & Fornl-Martins 2022).

In contrast to chromosome number research, studies on genome size are much more recent. The works of Obermayer *et al*. (2002) mark the first Hymenophyllaceae study containing data of this nature (Fig. 1). In their paper, the authors estimate the total DNA content for *Vandenboschia speciosa* (Willd.) G. Kunkel using the technique of Feulgen microdensitometry. Other genome size estimates for Hymenophyllaceae have been obtained through flow cytometry (Nitta *et al*. 2011; Clark *et al*. 2016; Kim & Kim 2020; Fujiwara *et al*. 2023). Furthermore, genome size studies on other fern families have in some instances begun even more recently, such as with Gleicheniaceae studies starting in 2016 (Lima *et al*. 2021; Clark *et al*. 2016).

One of the reasons for this increase in genome size studies in the last decades is the improvement of analytical techniques. Feulgen microdensitometry, the previous standard method, was substituted by flow cytometry around 1990 (Doležel *et al*. 2007). While the prior method demanded a laborious preparation of samples and frequently resulted in an imprecise output, flow cytometry renders quick and precise genome size estimates (Doležel *et al*. 2007; Temsch *et al*. 2022). Therefore, flow cytometry has been extensively employed in recent efforts to expand genome size data coverage for fern species, as evidenced in Clark *et al*. (2016), Wang *et al.* (2022) and Fujiwara *et al.* (2023).

Studies were also categorized according to the general subject of their approach. Out of the 55 articles included in this review, 33 adhered strictly to cytogenetics, nine were classified as containing cytogenetics and taxonomy aspects, six as cytogenetics and systematics, and five as cytogenetics and evolution. Additionally, two studies fit into more than three categories. From this analysis, it becomes evident that, despite relevance and benefits of an integrative approach, most publications on Hymenophyllaceae cytogenetics focus mainly on reporting chromosome number data and describing cytogenetic characteristics.

Previously published chromosome numbers were found for 158 species of Hymenophyllaceae. In total, approximately 37% of species within the family have a chromosome number count. In contrast, only 15 species have had their genome size estimated (< 4% of taxa). The complete set of data for chromosome numbers and genome sizes compiled in this study can be found in Supplementary Material 1 (available at <https://doi.org/10.6084/m9.figshare.22277602>). The most represented genera in terms of chromosome number data are *Hymenophyllum*, *Crepidomanes* and *Trichomanes*. However, the heterogeneity in species diversity is an influential factor that should be taken into account. For example, even though *Hymenophyllum* has the highest number of chromosome counts, it is also the most diverse group within the family and thus only 29% of its species have this data

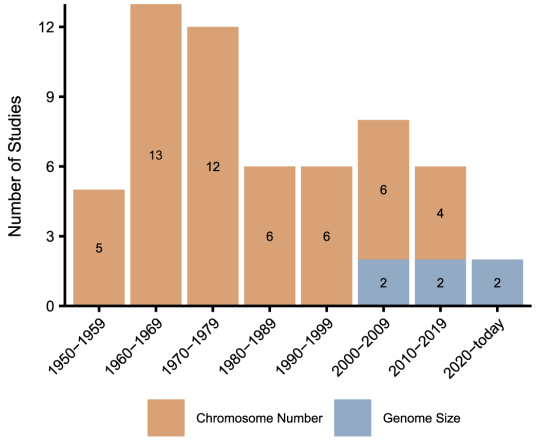

Figure 1 – Distribution of research papers throughout time that were included in the review. Studies are distinguished by type of information available for Hymenophyllaceae: chromosome number and genome size data.

available. Therefore, relative to species diversity, *Hymenophyllum*, *Trichomanes* and *Callistopteris* comprehend the least sampled genera in terms of chromosome number. Along the same line of thought, 24 out of 30 species from *Crepidomanes* underwent chromosome count procedures, resulting in the best-covered genus with 80% of its diversity studied, followed by *Cephalomanes* with 75% and *Vandenboschia* with 73% (Fig. 2).

Regarding genome size, there are no estimates available for the genera *Abrodictyum*, *Callistopteris*, *Didymoglossum* and *Trichomanes*. The highest coverage for genome size comes from *Vandenboschia* (40 %) and *Hymenophyllum* (12.5 %), having data for six and five species, respectively. The number of species with and without cytogenetic data available for chromosome number and genome size is presented in Figure 2 comparing the genera of Hymenophyllaceae.

Hymenophyllaceae displays great variation concerning chromosome numbers with 26 different counts previously reported ($2n$ = 22, 24, 26, 28, 36, 42, 44, 52, 54, 56, 58, 62, 64, 66, 68, 72, 84, 102, 108, 112, 116, 128, 132, 136, 144, 256 and 384). This variation is not uniformly distributed among the genera (χ^2 = 490.162, p-value < 0.01). The *Hymenophyllum* genus exhibits the highest diversity of chromosome numbers within the family (20 different numbers reported), and this diversity is especially apparent for subgenus *Hymenophyllum*. In contrast, the remaining genera within the family exhibit lower diversity in terms of chromosome numbers. The diagram in Figure 3 illustrates the overall synthesis of the data for Hymenophyllaceae chromosome numbers and genome sizes compiled in this review.

The lowest chromosome number found in the family is $2n = 2x = 22$, for *Hymenophyllum peltatum* (Poir.) Desv., while the highest is $2n = 12x = 384$ for a hybrid between the species *Trichomanes crispum* L. and *Trichomanes robustum* E. Fourn. (Brownlie 1958; Walker 1966, 1973, 1985; Manton & Vida 1968; Tindale & Roy 2002). The most frequent number for the family is $2n$ = 72 (40% of species), followed by $2n$ = 42 (8% of species), and $2n$ = 128 and 144 (both holding 6% of species). On the opposite side of the spectrum, the least common chromosome numbers are $2n$ = 22, 28, 56, 84, 116 and 384, all of which were found for single species.

When directly comparing the two subfamilies of Hymenophyllaceae, namely Hymenophylloideae and Trichomanoideae, we can observe an expressively higher variation of chromosome

numbers for Hymenophylloideae. It is important to highlight that while Hymenophylloideae only contains one genus (*Hymenophyllum*), Trichomanoideae is divided into eight genera. Still, out of the trichomanoids, the genera *Abrodictyum* and *Trichomanes* retain the higher diversity of chromosome numbers.

Analysing the previously reported chromosome numbers it can be noted that the sporophytic number of 72 is a common characteristic present in both subfamilies of Hymenophyllaceae. As previously mentioned, this number is present in 40% of the family's species and all genera except *Didymoglossum* and *Cephalomanes* (Fig. 3). This observation suggests the possibility of a plesiomorphic characteristic, *i.e.* a character from the common ancestor present in both lineages. The supposition of a plesiomorphic state for this characteristic has been previously inferred for the Trichomanoideae subfamily in Ebihara *et al.* (2007). Moreover, the chromosome number $2n = 72$ has previously been proposed as the ancestral character for the family based on phylogenetic reconstruction (Hennequin *et al.* 2010), adding evidence to this hypothesis.

Much like the previous cytogenetic feature, a considerable variation in the genome sizes of Hymenophyllaceae was verified, ranging from 2C = 21.47 pg for *Vandenboschia speciosa*, to 2C = 73.2 pg for *Vandenboschia subclathrata* K. Iwats. (Ebihara *et al*. 2005; Obermayer *et al*. 2002). On average the genome size for the available data of Hymenophyllaceae is 2C = 41.50±13.84 pg ($\overline{x} \pm s$). This value is superior to the mean genome size of monilophytes and lycophytes (2C = 24.22±27.68 pg) (Leitch *et al*. 2019; Pellicer & Leitch 2019). Concerning other embryophytes, bryophytes have the smallest genome size with a mean of 2C = 1.83±3.50 pg, followed by angiosperms with 2C = 10.26±17.88 pg (Leitch *et al*. 2019; Pellicer & Leitch 2019). On the other hand, gymnosperms show the largest mean with 2C = 36.70±14.63 pg (Leitch *et al*. 2019; Pellicer & Leitch 2019). The genome size variation for Hymenophyllaceae is illustrated in Figure 3.

There are 24 species with more than one cytotype associated (Tab. 1), including the genera *Hymenophyllum* (10 species), *Trichomanes* (four species), *Vandenboschia* (three species), *Abrodictyum* (three species), *Crepidomanes* (three species), and *Cephalomanes* (one species). Different cytotype values that are multiples of each other were found in 15 species, suggesting the possibility of intraspecific polyploidy (*e.g.*, in species from subgenus *Vandenboschia*). Some species had their counts differing by just a few chromosomes,

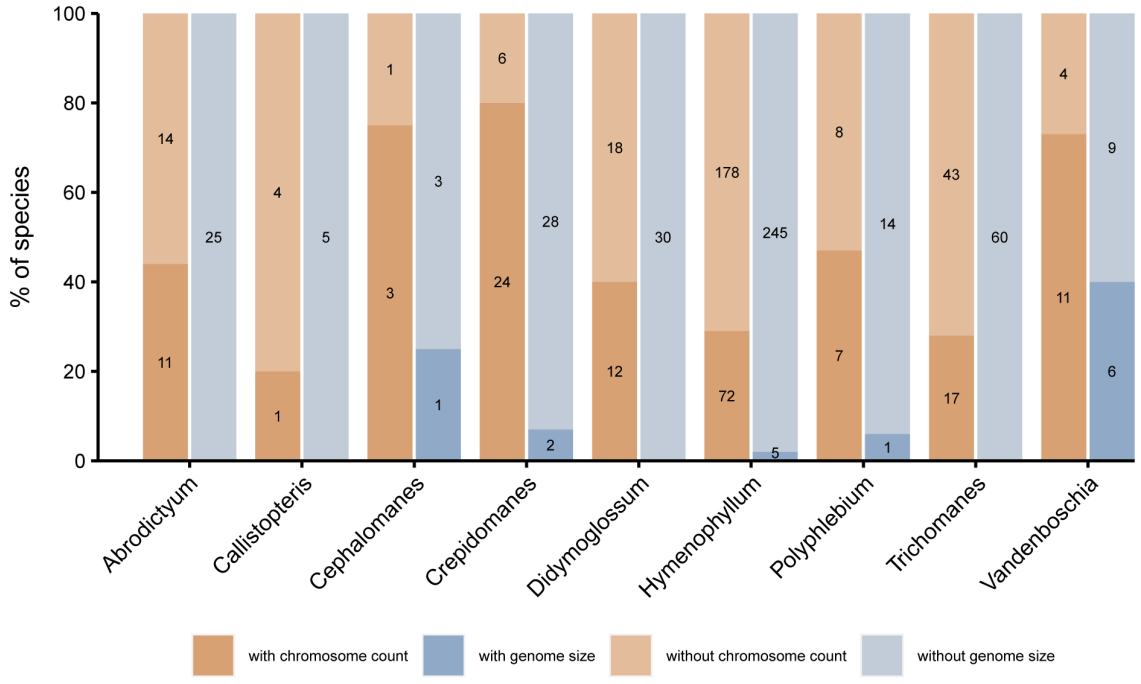

Figure 2 – Ratio of taxonomic groups for which chromosome number and genome size data is available.

in which case possible botanical classification problems may be suggested. One similar case is *Trichomanes elegans* Rich., for which populations sampled from Brazil and Trinidad display 2*n* = 64 (Tryon *et al*. 1975; Walker 1985) and populations from India are reported with 2*n* = 72 (Ammal & Bhavanandan 1992). It is important to mention that this species has a neotropical distribution (Pallos *et al*. 2017). Therefore, a misidentification of the Indian specimen is a plausible possibility to explain the difference in chromosome numbers.

Further analyzing species with more than one reported cytotype, *Hymenophyllum polyanthos* (Sw.) Sw. (2*n* = 54 and 56) has a particularly intriguing situation. This nomenclature was, indeed, applied to a highly polymorphic species complex with a wide geographic distribution (Braithwaite 1975; Hennequin *et al*. 2006; Vasques *et al*. 2019;

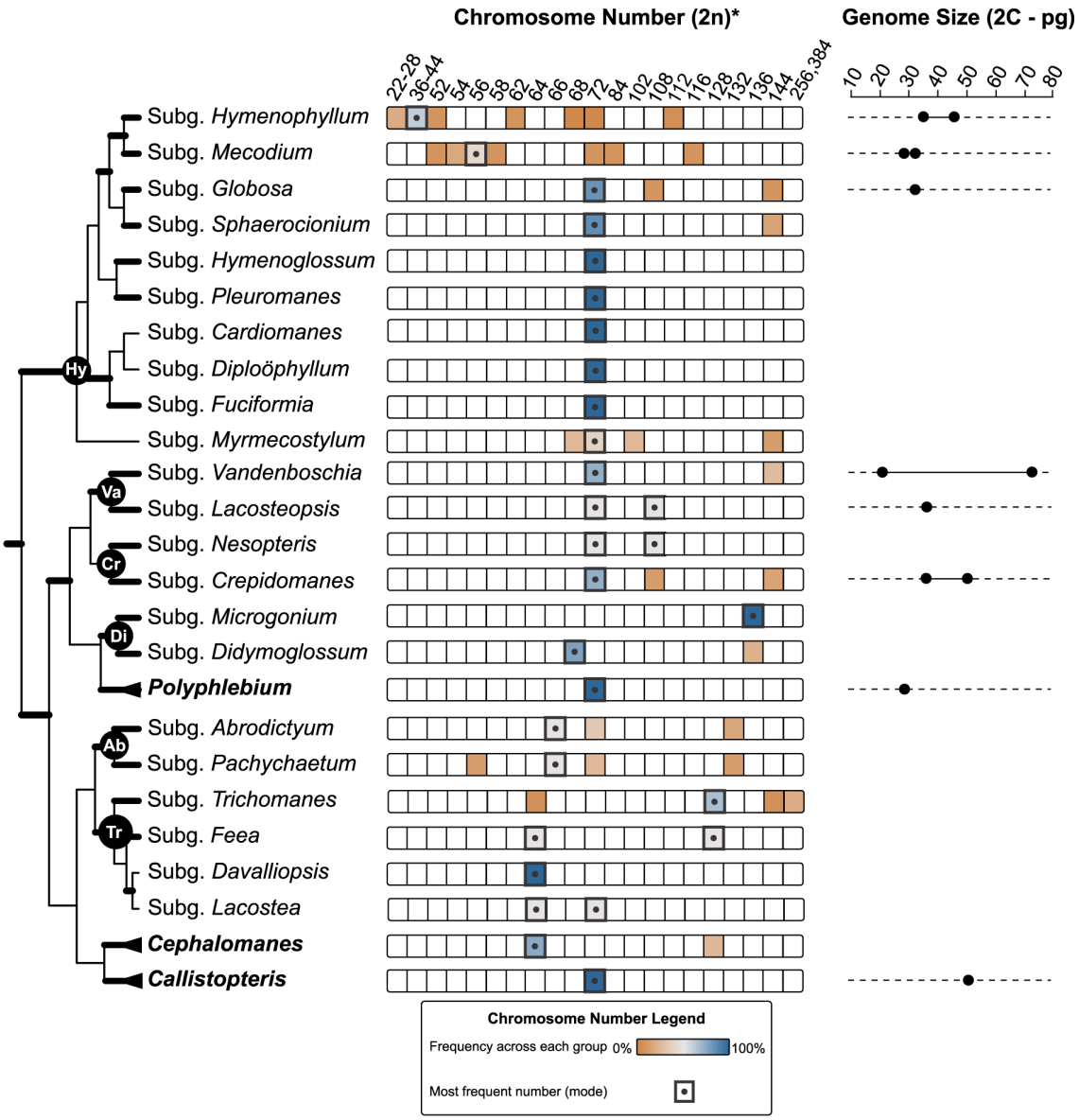

Figure 3 – Overview of chromosome number (2*n*) and genome size (2C in pg) for each genus and subgenus of Hymenophyllaceae. The phylogeny was reconstructed using a maximum-likelihood approach. Ab = *Abrodictyum*; Cr = *Crepidomanes*; Di = *Didymoglossum*; Hy = *Hymenophyllum*; Tr = *Trichomanes*; Va = *Vandenboschia*. Bootstrap values > 70% are shown by bold lines.

Vasques & Ebihara 2022; Gonzatti *et al*. 2023). However, the work of Vasques *et al*. (2019) revealed that *H. polyanthos* follows a less wide distribution than previously thought, and that the taxa from the neotropics is not genetically identical to the specimens from tropical and Old World regions. Therefore, the multiple chromosome counts are likely to derive from different species. A more comprehensive study is still needed to verify the aforementioned hypothesis and explain the cytogenetic variation encountered in this species complex.

There is a widespread disagreement among cytologists over the base chromosome numbers for Hymenophyllaceae (Tindale & Roy 2002). The base number refers to the haploid number derived from the initial population of a clade or monophyletic taxon and it may only be determined after a critical analysis of all chromosome numbers reported for the group (Guerra 2008). In light of this, our review provides essential data to contextualize and contribute towards a more informed and precise account surrounding base chromosome numbers in future research endeavours. This may, for sure, pave the way for significant implications on the evolution and taxonomy of Hymenophyllaceae. In this review, we adhere to the base chromosome numbers proposed by Ebihara *et al*. (2006). While a deeper discussion on this subject does not fit the scope of the current review, it is still an important topic yet to be fully explored.

The disagreements in base chromosome numbers for the genera within Hymenophyllaceae have important implications in determining the ploidy level of species. In this regard, populations of the same species and equal chromosome number counts have been attributed, in some instances, to different levels of ploidy by distinct authors. Two populations of *Vandenboschia radicans*, for example, were described as octoploid (Fabbri 1965) and 16-ploid (Mitui 1966), despite being attributed the same chromosome number ($2n$ = 144) in both publications. Similarly, *Abrodictyum rigidum* (Sw.) Ebihara & Dubuisson is recognized as diploid (Walker 1985) and hexaploid (Walker 1966) with $2n$ = 66 on both works.

The following sections will be dedicated to a descriptive analysis of the cytogenetic data (chromosome number and genome size) found for the Hymenophyllaceae genera. For access to the complete raw dataset detailed in the next sections refer to Supplementary Material 1 (available at <https://doi.org/10.6084/m9.figshare.22277602>).

Abrodictyum ($2n$ = 56, 66-132, 72)

The genus *Abrodictyum* has 11 species analyzed for chromosome number (44% of its species diversity) (Fig. 2) divided into the subgenera *Abrodictyum* and *Pachychaetum*. Four chromosome numbers were obtained in studies with this genus: $2n$ = 56, 66-132 and 72. The recognized base chromosome number for the *Abrodictyum* genus is x = 33 (Dubuisson *et al*. 2003; Ebihara *et al*. 2007). According to Dubuisson *et al*. (2003), species that have 36 pairs of chromosomes are considered doubtful or exceptional. The most frequent is $2n$ = 66 (64% of the sampled species) and the least common number is $2n$ = 56, registered only once (Fig. 3).

The *Abrodictyum* and *Pachychaetum* subgenera have registered chromosome numbers of $2n$ = 66-132 and 72, with only $2n$ = 56 exclusively present in *Pachychaetum*. Three species have shown more than one chromosome number (Tab. 1), including *Abrodictyum caudatum* (Brack.) Ebihara & K. Iwats, which displayed a polyploid series of $2n$ = 66-132 ($2x$-$3x$) (Walker 1966, 1985; Tilquin 1978). Additionally, there is no available data on genome size for the genus.

Most species of genus *Abrodictyum* have either the chromosome number 66 or a multiple of this value. This specific number pattern does not repeat in any other genera within Hymenophyllaceae, which renders the chromosome number circumstance for *Abrodictyum* rather unusual (Braithwaite 1969, 1975).

Callistopteris ($2n$ = 72) and *Cephalomanes* ($2n$ = 64-128)

The genus *Callistopteris* has one chromosome number registered (20% of its diversity) and no genome size data (Fig. 2). More specifically, the data available for this genus pertains to the species *Callistopteris apiifolia* (Presl) Copel., which has $2n = 2x = 72$ reported as its cytotype (Braithwaite 1969, 1975; Mitui 1976a).

The *Cephalomanes* genus has three species analysed for chromosome number, attaining the second highest percentage of species with cytogenetic data (75% of its diversity) (Fig. 2). The chromosome number $2n = 2x = 64$ was reported for all species included in this review (Fig. 3). Only the species *Cephalomanes atrovirens* C.Presl has two cytotypes ($2n$ = 64 and 128) (Tab. 1) (Braithwaite 1969, 1975). Additionally, genome size has been estimated for *Cephalomanes javanicum* (Blume) C. Presl, with a value of 2C = 51.61 pg (Fujiwara *et al*. 2023).

Table 1 – List of species with more than one cytotype reported.

Species	Chromosome number (ploidy level)	Location of the analyzed population	Reference
Abrodictyum caudatum (Brack.) Ebihara & K. Iwats.	*n* = 36 (2*x*)	Australia	Vessey & Barlow 1963
	n = 33 (2*x*)	New Caledonia	Braithwaite 1975
	n = 33 (2*x*)	Australia	Tindale & Roy 2002
Abrodictyum dentatum (Bosch) Ebihara & K. Iwats.	*n* = 36 (2*x*)	New Caledonia	Brownlie 1965
	n = 33 (2*x*)	Vanuatu, New Caledonia, Fiji	Braithwaite 1975
Abrodictyum rigidum (Sw.) Ebihara & Dubuisson	*n* = 33 (2*x*)	Jamaica, Trinidad	Walker 1966
	n = 33 (2*x*)	Nigeria	Walker 1985
	n = 66 (4*x*)	Trinidad	Tilquin 1978
Cephalomanes atrovirens C.Presl	*n* = 32 (2*x*)	Vanuatu	Braithwaite 1969
	n = 32, ca. 64 (2*x*, 4*x*)	Vanuatu, Fiji	Braithwaite 1975
Crepidomanes insigne (Bosch) Fu	*n* = 36 (2*x*) 2*n* = 72, 108 (2*x*, 3*x*)	India	Mehra & Singh 1957
	n = ca. 72 (4*x*)	-	Bir 1963 *apud* Fabbri 1965
Crepidomanes proliferum (Blume) Bostock	*n* = 72 (4*x*)	Malaysia	Braithwaite 1969
	2*n* = 108 (3*x*)	Solomon Island	Braithwaite 1975
	2*n* = 108 (3*x*)	Vanuatu	Bell 1960
Crepidomanes saxifragoides (C. Presl) Thapa	*n* = 36 (2*x*)	Solomon Islands	Braithwaite 1969
	n = 36, 72 (2*x*, 4*x*)	Vanuatu, New Caledonia	Braithwaite 1975
	n = 36, 72 (2*x*, 4*x*)	Australia	Tindale & Roy 2002
Hymenophyllum digitatum (Sw.) Fosberg	*n* = 36, 72 (2*x*, 4*x*)	Vanuatu	Braithwaite 1975
Hymenophyllum rarum R.Br.	*n* = 36 (?)	New Zealand	Brownlie 1954
	n = 56-58, 58 (4*x*)	Australia	Tindale & Roy 2002
Hymenophyllum wrightii Bosch	*n* = 27 (2*x*)	Japan	Mitui 1967
	2*n* = 84 (3*x*)	Japan	Tatuno & Takei 1969
	n = 28, 2*n* = 56 (2*x*)	Japan	Mitui 1986
Hymenophyllum australe Willd.	*n* = 36 (2*x*)	Australia	Vessey & Barlow 1963
	n = 36, 72 (2*x*, 4*x*)	Australia	Tindale & Roy 2002
Hymenophyllum javanicum Spreng.	2*n* = 72 (2*x*)	Sri Lanka	Manton & Sledge 1954
	2*n* = 108 (3*x*)	India	Mehra & Singh 1957
	n = 36 (2*x*)	Fiji	Braithwaite 1975
Hymenophyllum cupressiforme Labill.	*n* = 22 (2*x*)	Australia	Vessey & Barlow 1963
	n = 21; 2*n* = 42 (2*x*)	Australia	Tindale & Roy 2002

Species	Chromosome number (ploidy level)	Location of the analyzed population	Reference
Hymenophyllum peltatum (Poir.) Desv.	*n* = 11 (2*x*)	New Zealand	Brownlie 1958
	2*n* = 36 (?)	Tristan da Cunha	Manton & Vida 1968
	n = 11; 2*n* = 22 (2*x*)	Australia	Tindale & Roy 2002
Hymenophyllum wilsonii Hook.	*n* = 18, 31; 2*n* = 62 (2*x*, ?)	Madeira Island	Manton *et al.* 1986
	n = 18 (2*x*)	Madeira Island	Rasbach *et al.* 1990
	n = 18; 2*n* = 36 (2*x*)	Spain	Aguiar *et al.* 2006
Hymenophyllum polyanthos (Sw.) Sw.	*n* = 27 (2*x*)	India	Mehra & Singh 1957
	n = 28 (2*x*)	Jamaica, Trinidad	Walker 1966
	2*n* = 56 (2*x*)	Japan	Tatuno & Takei 1969
	n = 28 (2*x*)	Vanuatu, Fiji	Braithwaite 1975
	2*n* = ca. 28? (?)	Brazil	Löve 1976
	n = 28 (2*x*)	Trinidad	Walker 1985
Hymenophyllum sanguinolentum (Forst.) Sw.	*n* = 72 (4*x*)	New Zealand	Brownlie 1954
	n = 36, 72 (2*x*, 4*x*)	New Zealand	Brownlie 1961
	n = 34, 36, 66-70 (2*x*, 4*x*)	New Zealand	Daellenbach 1982 *apud* Dawson 2008
Trichomanes osmundoides DC. *ex* Poir.	*n* = 32 (2*x*)	Jamaica	Walker 1966
	n = 64 (4*x*)	Trinidad	Walker 1985
Trichomanes elegans Rich.	*n* = 32 (2*x*)	Brazil	Tryon *et al.* 1975
	n = 32 (2*x*)	Trinidad	Walker 1985
	n = 36 (?)	India	Ammal & Bhavanandan 1992
Trichomanes arbuscula Desv.	*n* = 64 (4*x*)	Jamaica	Walker 1966
	n = 128 (8*x*)	Brazil	Tryon *et al*. 1975
	n = 64 (4*x*)	Trinidad	Walker 1985
Trichomanes pinnatum Hedw.	*n* = 32 (2*x*)	Trinidad	Walker 1985
	n = 36 (?)	Brazil	Löve 1976
Vandenboschia auriculata (Blume) Copel.	*n* = 36 (2*x*) 2*n* = 108 (3*x*)	India	Mehra & Singh 1957
	n = 36 (2*x*)	Japan	Mitui 1966
	n = 36 (2*x*)	Japan	Mitui 1976a
Vandenboschia amabilis (Nakai) K.Iwats.	2*n* = 144 (4*x*)	Japan	Mitui 1976b
	n = 36 (2*x*)	Japan	Mitui 1986
Vandenboschia radicans (Sw.) Copel.	*n* = 72 (4*x*)	United Kingdom	Manton 1950
	n = 72 (4*x*)	India	Mehra & Singh 1957
	n = 72 (4*x*)	Japan	Mitui 1966
	n = 36 (2*x*)	Jamaica	Walker 1966

Crepidomanes (2*n* = 72-108-144)

The genus *Crepidomanes* has 24 analysed species, accounting for 80% of its diversity and making it the genus with the highest percentage of species for which there are reports of chromosome numbers (Fig. 2). The three registered numbers for the genus are 2*n* = 72-108-144 (2*x*-3*x*-4*x*), 2*n* = 72 being the most frequent and appearing in 75% of its species. The subgenus *Nesopteris* has chromosome counts for two species (50% of its diversity), which have 2*n* = 72 and 108. The subgenus *Crepidomanes* has data available for 22 species (85% of its diversity), registering 2*n* = 72, 108 and 144. There are four species in the genus with more than one reported cytotype (Tab. 1). In terms of genome size, there are estimates for two species, both from the *Crepidomanes* subgenus: *Crepidomanes latealatum* (Bosch) Copel. with 2C = 36.61 pg and *Crepidomanes minutum* (Blume) K.Iwats. with 2C = 51.2 pg (Nitta *et al*. 2011; Fujiwara *et al*. 2023).

Cytological records for the *Crepidomanes* genus include 2*n* = 2*x* = 72, a multiple of this value (2*n* = 4*x* = 144) and an intermediary between the two (2*n* = 3*x* = 108). The species *Crepidomanes proliferum* (Blume) Bostock has had two cases reported of populations with irregular meiotic behaviour (2*n* = 108) and indicatives of an apogamous life cycle (Bell 1960; Braithwaite 1975). The formation of unreduced spores through these meiotic irregularities offers a condition to possibly originate polyploids (Bell 1960; Braithwaite 1975). Apogamous reproduction has been associated with this genus by multiple authors (Mehra & Singh 1957; Bell 1960; Braithwaite 1975; Yoroi 1976; Nitta *et al*. 2011).

Didymoglossum (2*n* = 68-136)

The genus *Didymoglossum* has 14 analysed species (47% of its diversity) distributed across its two subgenera. Only 2*n* = 68-136 (2*x*-4*x*) are reported for the genus, with 2*n* = 2*x* = 68 being the most frequent chromosome number and appearing in 50% of its species (Fig. 3). The subgenus *Didymoglossum* has chromosome data for 9 species (45% of its diversity), registering 2*n* = 68 and 136; whereas *Microgonium* has data for three taxa (30% of its diversity), all of which register 2*n* = 136. No species displayed more than one cytotype and no data was found regarding genome size for this group.

All studied species of the genus *Didymoglossum* contain either 34 pairs of chromosomes or multiples of this number, a pattern that had previously been reported by cytologists (Walker 1966; Braithwaite 1969, 1975). In fact, the base chromosome number *x* = 34 is considered a synapomorphic character for this genus (Dubuisson *et al*. 2003). The taxonomic history of the group witnessed the union of *Microgonium* (previously regarded as a separate genus of Hymenophyllaceae) to the genus *Didymoglossum* due to their similar cytogenetic characteristics such as the uniformity in chromosome number and chromosome sizes. Moreover, the chromosome numbers of *Didymoglossum* seem to be distinct from other genera of the Trichomanoideae subfamily (Braithwaite 1969, 1975).

Hymenophyllum (2*n* = from 22 to 144)

The genus *Hymenophyllum* had 70 analysed species with representatives from all subgenera included: *Hymenophyllum* (33%), *Sphaerocionium* (17%), *Diploöphyllum* (100%), *Pleuromanes* (60%), *Cardiomanes* (100%), *Fuciformia* (50%), *Hymenoglossum* (33%), *Myrmecostylum* (50%), *Mecodium* (20%), and *Globosa* (36%). Out of these, *Sphaerocionium* and *Mecodium* had the lowest sampling relative to their species diversity.

A notable feature of this genus is the wide variety of counts reported, with a remarkable 20 different chromosome numbers (2*n* = 22, 24, 26, 28, 36, 41, 44, 52, 54, 56, 58, 62, 68, 72, 84, 102, 108, 112, 116 and 144). The most frequent sporophytic number is 2*n* = 72, appearing in 44% of its species, and the least frequent are 2*n* = 22, 28, 58, 84, 108, 116, each found for a single species (Fig. 3). The lowest value is 2*n* = 22 for *Hymenophyllum peltatum* (Poir.) Desv. from the subgenus *Hymenophyllum* (Brownlie 1958; Manton & Vida 1968; Tindale & Roy 2002) and the highest is 2*n* = 144 for *Hymenophyllum digitatum* (Sw.) Fosberg, *Sphaerocionium* × *tucuchense* Jermy & T.G.Walker, *Hymenophyllum australe* Willd., and *Hymenophyllum sanguinolentum* (Forst.) Sw, from the subgenera *Sphaerocionium*, *Globosa* and *Myrmecostylum* (Brownlie 1954, 1961; Braithwaite 1975; Walker 1985; Tindale & Roy 2002).

The chromosome number 2*n* = 72 is prevalent and conserved in different clades, namely the subgenera *Pleuromanes*, *Hymenoglossum*, *Cardiomanes*, *Fuciformia*, *Diploophyllum*,

Globosa, and *Sphaerocionium*. Conversely, a higher variation in chromosome numbers permeates the subgenera *Mecodium*, *Myrmecostylum*, but it is most extreme in *Hymenophyllum* (Fig. 3). The subgenus *Hymenophyllum* (2*n* = 22, 24, 26, 28, 36, 42, 44, 52, 56, 62, 68 and 72) displays the highest variation in chromosome number both within the genus *Hymenophyllum* as well as when comparing the entire Hymenophyllaceae. This subgenus also has the lowest chromosome number reported for homosporous ferns (Hennequin *et al*. 2010). Subgenus *Mecodium* also displays considerable chromosome number variation, although to a much lower extent compared to subgenus *Hymenophyllum*. The chromosome numbers previously reported for *Mecodium* are 2*n* = 52, 54, 58, 72, 56-84-116, which mostly vary around +/- 1 or 2 chromosome pairs. The species *Hymenophyllum rarum* R.Br. and *Hymenophyllum wrightii* Bosch have been reported as polyploids.

Further on the *Hymenophyllum* genus, the species *Hymenophyllum maderense* Gibby & Lovis (2*n* = 62) is reported as an allotetraploid, originating from the crossing of *Hymenophyllum tunbrigense* (L.) Sm. (2*n* = 26) and *H. wilsonii* Hook (2*n* = 36). The species *H. maderense* displays intermediary characteristics to those of its parentals and is able to backcross (Gibby & Lovis 1989; Aguiar *et al*. 2006). A shortcoming of older studies is the purely descriptive approach used to support polyploid origin, based only on chromosome behavior, fertility, segregation ratios and morphology, as exemplified by Grant (1981) and Soltis *et al*. (2004). An additional case that corroborates this idea is the description of the hybrid *Sphaerocionium* x *tucuchense* based on the observation of irregular meiosis, intermediary characteristics between the potential parentals, and sterility (Walker 1985). In order to form a better understanding of polyploid origin, the use of molecular approaches is recommended, including techniques such as chromosome painting methods (*e.g.*, GISH and FISH), genetic mapping and comparative genetics (Soltis *et al*. 2004).

When it comes to genome size, estimates were found for five species distributed across the subgenera *Hymenophyllum*, *Globosa*, and *Mecodium*. Values range from 2C = 29.7 pg in *Hymenophyllum polyanthos* (subgenus *Mecodium*) to 2C = 46.70 pg in *Hymenophyllum barbatum* Bosch (subgenus *Hymenophyllum*) (Kim & Kim 2020; Fujiwara *et al.* 2023). The mean genome size for the genus is 2C = 35.59 pg.

Genome size data has, in a study by Kim & Kim (2020), assisted the resolution of taxonomic dilemmas surrounding the *H. polyanthos* complex. Due to their morphological proximity, *Hymenophyllum coreanum* Nakai had previously been considered a synonym of *H. polyanthos*. Besides the differences found in genome size, the low plastome identity was also used as evidence for two distinct species (Kim & Kim 2020).

Polyphlebium (2n = 72)

The genus *Polyphlebium* has had seven species analysed for chromosome number (47% of its diversity). Every one of these has the same cytotype 2*n* = 2*x* = 72, which indicates stability in chromosome number for the genus. Genome size data has only been recorded for the species *Polyphlebium capillaceum* (L.) Ebihara & Dubuisson with a value of 2C = 29.46 pg (Clark *et al*. 2016).

Trichomanes (2*n* = 64-128-256-384 and 72-144)

Trichomanes is one of the largest genera in Hymenophyllaceae, but also one of the least studied having only 28% of its species diversity analysed for cytogenetic features (Fig. 2). The chromosome numbers 2*n* = 64-128-256-384 and 72-144 have been reported for 17 species distributed in the four subgenera: *Trichomanes*, *Feea*, *Davalliopsis*, and *Lacostea* (Fig. 3). The most frequent number is 2*n* = 128 appearing in 64% of species, while the values 2*n* = 144 and 384 appear only once. The subgenera *Feea*, *Davalliopsis* and *Lacostea* have data for one species each. The subgenus *Trichomanes* has chromosome counts for 14 species and showed a higher variation for this characteristic (2*n* = 64-128-256-384 and 144). There are four species in the genus with more than one cytotype (Tab. 1). Additionally, no genome size estimates are available.

Most of the *Trichomanes* species have 2*n* = 64 or multiples of this number (2*n* = 128-256-384). This uniformity in chromosome number coupled with distinguished morphological characteristics has been crucial in establishing the clade as a natural group (Walker 1966). Besides that, the genus also carries the highest chromosome number reported to date for Hymenophyllaceae (2*n* = 12*x* = 384). This value pertains to a hybrid between the species *Trichomanes crispum* L. (2*n* = 8*x* = 256) and *Trichomanes robustum* E. Fourn. (2*n* =

$2x$ = 128). The hybrid displays irregular meiosis and a morphology intermediary to its parentals (Walker 1985).

Taking into account the data compiled in this review it becomes apparent that *Cephalomanes* shares similar cytological features with the genus *Trichomanes*, both having either $2n$ = 64 or multiples of this chromosome number. This pattern has also been reported by Braithwaite (1969, 1975), whose work elaborates how the similarity in chromosome numbers could indicate a closer relationship between the taxa, although the two genera present differences in venation pattern and sori position. According to our phylogeny and the reconstructions in Dubuisson *et al*. (2003) and Ebihara *et al*. (2007), the two genera do not show a direct relationship, even though they share the same greater clade. In this case, *Cephalomanes* diverged earlier compared to *Trichomanes*. Although they are not supported by the molecular phylogeny, their relationship is still conceivable given they share the same base chromosome number (x = 32) (Dubuisson *et al*. 2003; Ebihara *et al*. 2007).

Vandenboschia ($2n$ = 72-108-144)

The genus *Vandenboschia* had eleven analysed species distributed across its two subgenera: *Vandenboschia* and *Lacosteopsis*. This genus has the third highest percentage of species with known chromosome numbers (73% of its diversity) (Fig. 2). The chromosome numbers reported are $2n$ = 72-108-144 ($2x$-$3x$-$4x$), being $2n$ = 72 the most frequent and appearing in 90% of its species. The subgenus *Lacosteopsis* only has this information for *Vandenboschia auriculata* (Blume) Copel, registering $2n$ = 72 and 108 (Mehra & Singh 1957; Mitui 1966, 1976a). As for the subgenus *Vandenboschia*, available chromosome data exists for ten species, which register $2n$ = 72 and 144. In the genus, there are three species with more than one cytotype reported (Tab. 1).

Genome size data is available for six species from the two subgenera, which makes the genus *Vandenboschia* the most thoroughly analysed regarding genome size (40% of its diversity). The subgenus *Lacosteopsis* contains data for *Vandenboschia auriculata* with 2C = 36.82 pg (Clark *et al*. 2016). The subgenus *Vandenboschia* varies from 2C = 21.47 pg for *Vandenboschia speciosa* to 2C = 73.2 pg for *Vandenboschia subclathrata* (Obermayer *et al*. 2002; Ebihara *et al*. 2005). The mean value for the whole genus is 2C = 47.97 pg.

A geographic perspective on cytogenetics data

As mentioned beforehand, cytogenetics is a relevant research subject for the Hymenophyllaceae. However, the utility of this information has been hindered by the lack of available data. One facet of this predicament that has not yet been introduced in our discussion is the geographic location of the populations studied. In this respect, Figure 4 provides the geographic regions encompassing the location of species with reported chromosome number counts, as well as instances of polyploid species occurrence.

The country with the highest number of species sampled for chromosome number was Jamaica, followed by New Zealand, Vanuatu and Japan (Supplementary Material 1, available at <https://doi.org/10.6084/m9.figshare.22277602>). All data for Jamaica was gathered by one cytotaxonomic survey, performed by Walker in 1966. In a broader sense, Oceania and Asia house the countries with the greatest number of studied species. A possible explanation for the expressive quantity of registers is the concentration of researchers that work with this group of ferns in these regions. Indeed, according to the data collected during this review, Asia comprises the highest number of research groups, having 24 first authors affiliated with institutions in the continent out of the 55 papers analysed. On the other hand, regions such as the Americas comprehend only four first authors from local institutions. This scenario is even more concerning when it comes to Africa, where studies with populations from this continent do not include any first author affiliated with local institutions. Accordingly, Africa and the Americas constitute research blind spots, reflected by the gap in cytogenetic knowledge available for these regions.

Interestingly, the best-sampled locations do not necessarily correspond to the geographic regions of higher species diversity. For Brazil, which comprises around 84 species of Hymenophyllaceae (Gonzatti & Windisch 2023), there are only eight species from Brazilian populations that were studied concerning chromosome numbers. Even if several of these species occur in various other regions, the lineages present in each place diverged a long time in the past. This renders it imprudent to extend the cytological characteristics of one population to another, regardless if they belong

to the same species. Therefore, we can state with certainty that the vast Brazilian flora has one of the most understudied fern populations regarding cytogenetics.

Concerning the distribution of polyploid species (Fig. 4), there are no apparent patterns to suggest preferential regions of occurrence. Given the currently available data, polyploids seem to display a relatively uniform distribution throughout the globe. However, a more robust sampling is still needed in order to make confident inferences of this nature. Nevertheless, a notable remark from the available data is that triploid presence is restricted to Asia and Oceania, while higher levels of ploidy (octaploids and 12-ploids) are exclusively found in Central America.

Methodological challenges

The deficit of cytogenetic research for Hymenophyllaceae may in part be attributed to difficulties in cultivation and the necessity of preparing material in the field (Brathwaite 1975). This group of ferns is not successfully grown for long periods under non-natural conditions. Therefore, fixation of filmy fern samples for posterior chromosome number analysis is recommended to be carried out in the native habitat of the species, at the moment of material collection (Manton 1950; Tindale & Roy 2002). Similarly, the collection and storage of samples for flow cytometry genome size estimation can turn into challenging tasks for Hymenophyllaceae, given the material needs to be fresh and well preserved for this analysis. In this regard, samples have to be maintained under low temperatures and are usually wrapped with a wet paper towel to conserve humidity (Doležel & Bartos 2005). These ferns are usually found in high humidity low light environments, such as cloud forests, waterfall splash zones or on boulders placed throughout bodies of water (Ebihara *et al.* 2007; Parra *et al.* 2009; Proctor 2012). This peculiar habitat aggravates the processes of sample collection and *in situ* material fixation for cytogenetic analyses.

The plant structures reserved for mitotic and meiotic analyses are the roots and sporangia, respectively (Manton 1950). However, mitotic chromosome counting in Hymenophyllaceae proves inconvenient on account of the challenges related to root morphology and harvesting. The thickness of rhizomes in Hymenophyllaceae is in the order of millimetres, hence the fragile nature of the root system. Some species of *Crepidomanes* and *Didymoglossum* are even rootless (Iwatsuki 1990; Schneider 2000, 2013; Ebihara *et al.* 2006). These characteristics make the use of

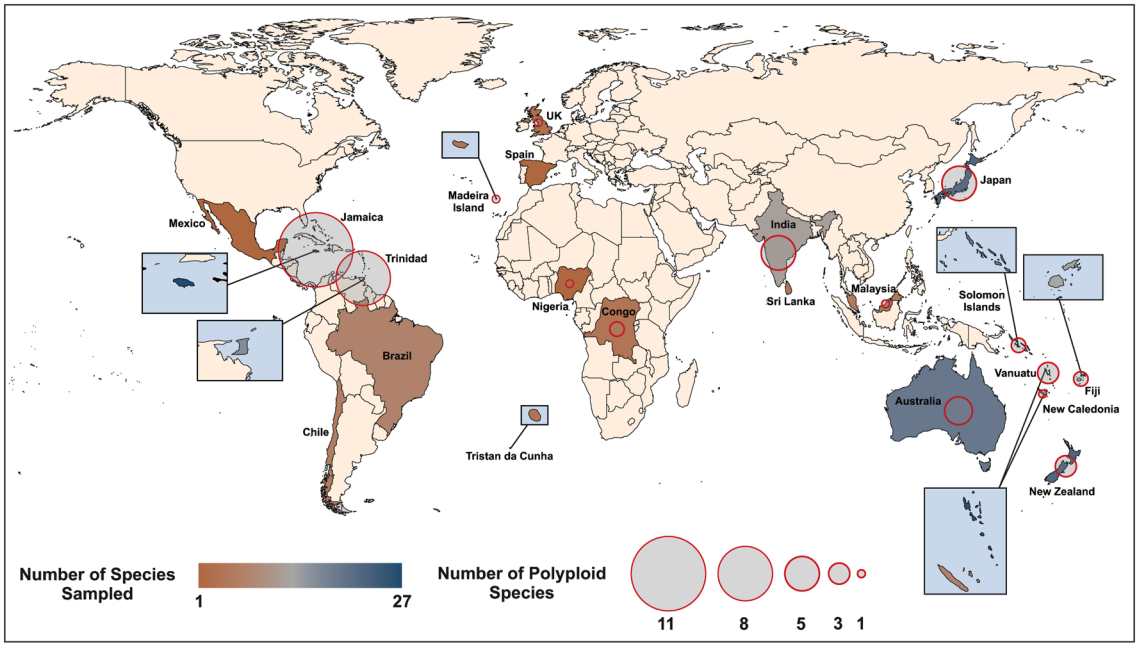

Figure 4 – Geographic distribution of Hymenophyllaceae species with reported chromosome numbers and polyploid species.

root tips for mitotic chromosome counting rather difficult, and at times impossible. Besides, there are species that tend to grow on fibrous substrates, such as on top of the chalice-like structures of *Dicksonia sellowiana* Hook. (Becker *et al*. 2015). Distinguishing between the roots of the epiphyte and the phorophyte host can be a challenging task, increasing the probability of error for mitotic chromosome counts. Therefore, this represents yet another factor that supports meiotic analyses as the best and most reliable method for obtaining Hymenophyllaceae chromosome number data.

Concerning sample acquisition for chromosome counting, meiotic analysis can be regarded as the most advantageous. In our field work, we were able to observe fertile material of Hymenophyllaceae species throughout the whole year, allowing sporangia collection for meiotic analysis regardless of season. Although this pattern facilitates the acquisition of biological material, little is known about Hymenophyllaceae phenology, a subject deserving further research (Lee *et al*. 2018). Additionally, species of *Trichomanes* and other genera of the Trichomanoideae subfamily adopt an arrangement of sporangia particularly favourable for meiotic analysis. Thereby, sporangia are gradually positioned along the receptacle, enabling reproductive structures in distinct stages of maturing (Tryon *et al*. 1975). It is important to note that this characteristic does not extend to the genus *Hymenophyllum*.

Another methodological difficulty lies in the acquisition of the chromosome number for certain species. In this regard, counting the chromosome number for organisms in which $2n \geq 100$ can be a challenging task and frequently induces errors and imprecision (Guerra 2008). Over 23% of counts have shown a high number (≥ 100) of chromosomes in the case of filmy fern species.

Furthermore, the presence of associations between Hymenophyllaceae and other organisms like bryophytes, fungi, small arthropods (Pócs 1982; Aptroot & Lücking 2001), and algae, demands extra attention when performing flow cytometry. Additional peaks ("ghost" peaks) appearing in the flow histogram that do not fall under an endopolyploid series suggest contamination of the sample. In this situation, it is imperative that the researcher repeats the analysis, checking beforehand whether the sample contains unwanted organisms (Pellicer *et al*. 2021; Sliwinska *et al*. 2022).

A panorama of existing research data on chromosome number and genome sizes for Hymenophyllaceae holds considerable significance since it brings to light the unsolved inconsistencies, reveals the unexplored perspectives and highlights the remaining gaps in our understanding. We therefore hope this review may stimulate and direct future cytogenetic investigations, as well as contribute towards insights regarding the evolution and taxonomy of Hymenophyllaceae. The following passages were formulated as short summaries of our findings and are formatted as answers to our initial research questions.

(*i*) Which species from the Hymenophyllaceae have previously reported data on chromosome number and genome size? Despite chromosome number and genome size information exhibiting great relevance in the context of Hymenophyllaceae research, data of this nature are restricted to 37% and 4% of taxa, respectively. We have compiled a dataset containing details with previously reported data (available in Supplementary Material 1 <https://doi.org/10.6084/m9.figshare.22277602>). This is, to our knowledge, the most complete and up-to-date compendium of Hymenophyllaceae cytogenetic trait records.

(*ii*) How are cytogenetic data distributed within taxonomic groups (genera and subgenera)? A pattern was identified for chromosome number data of Hymenophyllaceae. The species within the genera *Callistopteris*, *Polyphlebium*, *Vandenboschia*, *Crepidomanes* and *Hymenophyllum* predominantly displayed chromosome counts of $2n = 72$ or multiples of this number. In contrast to the previously mentioned genera, *Trichomanes* and *Cephalomanes* were mainly composed of species6 with $2n = 64$ or multiples of this number, while *Didymoglossum* mostly showed species with $2n = 68$ or multiples of this number. As discussed in detail previously, the patterns discovered for cytogenetic features are directly reflected in taxonomic and evolutionary aspects of Hymenophyllaceae. Regarding genome size, the lack of available data of this nature does not allow us to verify any clear patterns at this moment.

(*iii*) Which geographic regions have the least studied species diversity? Are there any areas with a notable prevalence of polyploid species? An uneven sampling distribution is observed when we consider the geographical location of specimens with associated cytogenetic data. Asia and Oceania are the geographic locations with the most well-studied species diversity in terms

of chromosome number. Moreover, some regions of high Hymenophyllaceae diversity fall short in this area of research; such as the case of South American countries. Polyploid species follow an apparently equal distribution throughout the globe, without preference for specific geographic regions. However, this scenario may shift as more counts are conducted.

(*iv*) What are the methodological challenges surrounding cytological data acquisition for Hymenophyllaceae? The difficulties in cultivation outside the natural habitat, morphological peculiarities and association with other nearby organisms were appointed as the main barriers for chromosome counting and genome size estimation in Hymenophyllaceae.

In order to gain richer insight into the mechanisms surrounding cytogenetic characteristics evolution, future research should focus on associating available cytogenetic data to the molecular phylogeny of the group using comparative phylogenetics approaches. Hennequin *et al*. (2010) has employed a successful framework for associating chromosome number data to the phylogeny of genus *Hymenophyllum* (with an emphasis on subgenus *Hymenophyllum*). Currently, more robust techniques have become available in this area [for further reference on this matter turn to Nunn (2011) and Harmon (2019)]. When it comes to genome size, the lack of available data poses a challenge for comparative phylogenetics, demanding a more thorough sampling.

A more complete and thorough sampling could contribute to a better understanding of the diverging evolutionary lineages within the group. Therefore, further research is needed and encouraged on species with a widespread geographic distribution, in the hope of shedding light on the evolutionary processes and local effects over cytogenetic parameters of Hymenophyllaceae.

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Data availability statement

In accordance with Open Science communication practices, the authors inform that the dataset originated from this study is available online: Supplementary Material 1 (<https://doi.org/10.6084/m9.figshare.22277602>); Supplementary Material 2 (<https://doi.org/10.6084/m9.figshare.24291094>); and Supplementary Material 3 (<https://doi.org/10.6084/m9.figshare.24291127>). The information gathered during this review can be found in those addresses, including chromosome numbers, genome sizes, taxonomic and geographic data, and bibliographic references.

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