



# Phylogeny, morphology and distribution of the *Hymenophyllum polyanthos* (Sw.) Sw. species complex (Hymenophyllaceae, Polypodiidae) in Japan

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(Manuscript received 21 June 2022; Accepted 20 September 2022; Online published 29 September 2022)

**ABSTRACT:** The taxonomy of the filmy fern *Hymenophyllum polyanthos* species complex has been unclear for a long time. A recent study clarified the phylogenetic and morphological relationships among Taiwanese plants of the complex. This raised questions about which lineages occurred in the surrounding geographic regions. Hence, we investigated whether the Taiwanese phylotypes of *H. polyanthos* also occurred in Japan. To test this we gathered 63 new samples of *H. polyanthos* from Japan, and generated 126 new plastid DNA sequences that were phylogenetically compared with sequences available from previous studies. We also analyzed herbarium specimens, assessing the ranges of morphological and distribution variation of the groups identified in the phylogeny. As a result, we confirmed that subgenus *Mecodium* is represented in Japan by *H. coreanum*, *H. mikawanum*, and *H. wrightii*, plus three cryptic lineages of *H. polyanthos*, namely the 'fujisanense', 'parallelocarpum', and 'punctisorum' phylotypes. We prefer to use informal names for these three lineages because of unresolved taxonomic and nomenclatural issues. Among the *H. polyanthos* phylotypes, the 'punctisorum' form is very distinct from the other two, both in morphology and distribution. Although the 'fujisanense' and 'parallelocarpum' forms show considerable overlap in morphology and distribution, this study brings enough evidence to distinguish them with significant accuracy.

**KEY WORDS:** Cryptic species, *Hymenophyllum polyanthos*, Japan ferns, *Mecodium*, sympatric distribution.

## INTRODUCTION

The genus of filmy ferns *Hymenophyllum* Sm., included in the subcosmopolitan family Hymenophyllaceae (PPGI, 2016), is represented by mostly epiphytic plants with long-creeping, nearly glabrous and filiform rhizomes, and with bivalvate soral involucre (Mickel and Smith, 2004; Ebihara *et al.*, 2006; Ebihara, 2016). By using plastid DNA markers, Ebihara *et al.* (2006) circumscribed the genus *Hymenophyllum* as the largest within Hymenophyllaceae, including some taxa formerly assigned to *Trichomanes* L. s.l. and *Sphaerocionium* C.Presl., the latter now recognized as a subgenus of *Hymenophyllum*. Under the circumscription of Ebihara *et al.* (2006), the genus *Hymenophyllum* includes ca. 250 species, which are further categorized in 10 subgenera. However, for some groups within the genus *Hymenophyllum*, the identification and circumscription of species can still be challenging due to the limited number of diagnostic traits in these plants.

An example of this is *Hymenophyllum* subg. *Mecodium* Copel., recognized as monophyletic by Ebihara *et al.* (2006), and further supported by subsequent analyses (Hennequin *et al.*, 2010; Hsu *et al.*, 2019; Vasques *et al.*, 2019). The taxonomic history of subg. *Mecodium* starts with the first use of the name *Mecodium* by Presl (Presl, 1849) to describe *H. sanguinolentum* (G.Forst.) Sw., a species now included in *Hymenophyllum* subg. *Myrmecostylum* (C.Presl) Ebihara

& K.Iwats. (Ebihara *et al.*, 2006). Despite this, the name *Mecodium* was only legitimized much later by Copeland (Copeland, 1937), who selected *M. polyanthos* (Sw.) Copel., which was described from a specimen collected from Jamaica (Swartz, 1788), as the lectotype of the genus.

Recent studies based on molecular data recognize the circumscription of subg. *Mecodium* including *H. polyanthos* and its closely related species (Larsen *et al.*, 2013; Del Rio *et al.*, 2017; Hsu *et al.*, 2019; Vasques *et al.*, 2019). *H. polyanthos* s.l. corresponds to a complex of species including plants from three phylogenetic lineages geographically distributed in the Neotropics, the Pacific Islands, and Asian and African regions (Vasques *et al.*, 2019). Moreover, although Neotropical plants have not yet been sufficiently sampled, our previous study (Vasques *et al.*, 2019) brought evidence that *H. polyanthos* s.s. (the type lineage for subg. *Mecodium*) is possibly only distributed in the Neotropical region, leading to the interpretation that lineages in other geographical regions of the globe may represent separate species within the subgenus. Current efforts are now aimed at improving the phylogenetic understanding of these geographically distinct lineages within a global framework, as well as looking for ways to morphologically circumscribe separate species that closely resembles phylogenetic patterns.

Hsu *et al.* (2019) combined molecular phylogenetic analysis based on plastid DNA markers and an extensive morphological investigation of *in situ* and herbarium



specimens from *Mecodium* plants in Taiwan to establish a phylogenetic and morphological hypothesis that may explain the diversity of these plants in the region. Those authors recognized the existence of five phylogenetic lineages, which they associated with names previously described for *Mecodium*, by confirming the occurrence of *H. paniculiflorum* C.Presl in the island, reinstating *H. fujisanense* Nakai, *H. parallelocarpum* Hayata and *H. punctisorum* Rosenst. lineages, and delimiting a new species named *H. exquisitum* T.C.Hsu & Y.S.Chao. Additionally, the authors excluded *H. blumeanum* Spreng., *H. integrum* Bosch, *H. polyanthos*, *H. tenellum* D.Don, and *H. wrightii* Bosch from the Taiwanese flora, and considered *H. exquisitum* and *H. parallelocarpum* as endemic to the island.

The conclusions drawn by Hsu *et al.* (2019) offer new insights on how to approach the diversity of species of subg. *Mecodium*, especially considering how Taiwanese species are distributed overseas, though the precise range of morphological variation within and among cryptic lineages of *H. polyanthos* outside of Taiwan remains unclear. Geographically close to Taiwan is the Japanese archipelago, with many phytogeographical similarities, particularly in Japan's Ryukyu Islands (Hsieh, 2002). In Japan, the distribution of subg. *Mecodium* ranges from Hokkaido to the Ryukyu Islands, close to Taiwan. Besides *H. polyanthos*, names such as *H. coreanum* Nakai, *H. mikawanum* (Seriz.) Seriz., *H. paniculiflorum*, and *H. wrightii* are commonly used to describe the diversity observed. However, a wide morphological variation is observed in *H. polyanthos* samples from Japan, which, combined with recent phylogenetic evidence (Vasques *et al.*, 2019), suggests the possible existence of several cryptic groups within the archipelago.

In light of the recent findings by Hsu *et al.* (2019), and considering the geographic proximity of Japan and Taiwan, we investigated how closely related Japanese and Taiwanese plants are, and whether the phylogenetic groups recovered by Hsu *et al.* (2019) also occur in Japan. First, we combined our previous phylogenetic results with those presented by Hsu *et al.* (2019) to guide the collection of new samples in Japan for a novel phylogenetic analysis. Based on the results, we then tested the range of morphological and distribution variation within Japan using an extensive survey of specimens deposited in the TI and TNS herbaria. Here, we discuss the phylogenetic position of these lineages, while describing each lineage's morphological and biogeographic characteristics.

## MATERIALS AND METHODS

### *Taxa sampling, identification and voucher deposit* –

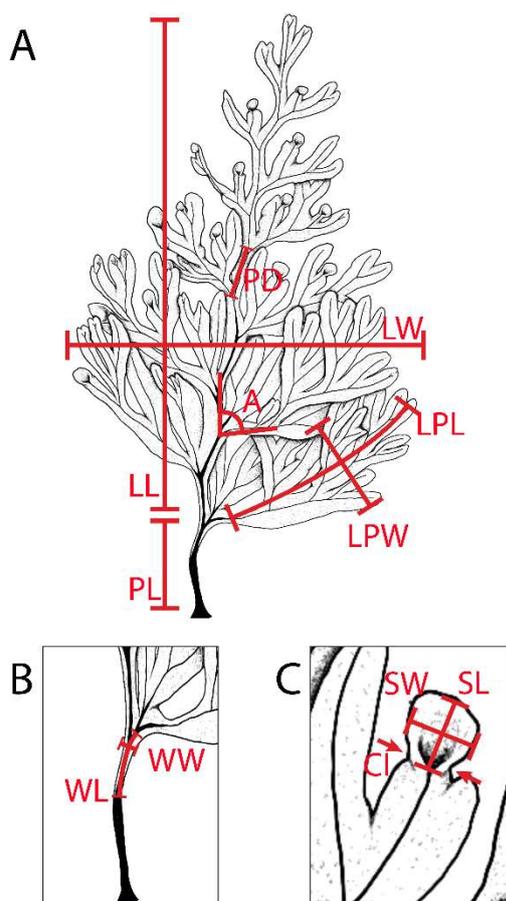
Our analysis combined data from 126 newly obtained sequences and 241 DNA sequences available on GenBank of specimens within *Hymenophyllum* subg.

*Mecodium*. Samples newly collected for this study were obtained through field trips between 2018 and 2021 in Japan. Plants collected were identified and sampled for DNA analysis, and subsequently pressed as vouchers deposited at the TI and TNS herbaria in Japan. For means of comparison, we used the taxa suggested by Hsu *et al.* (2019) to name cryptic lineages within *H. polyanthos*. Because of unresolved taxonomic and nomenclatural issues, we used the names 'fujisanense', 'parallelocarpum', and 'punctisorum' (presented by Hsu *et al.*, 2019) to label the phylotypes within the *H. polyanthos* complex. A list of samples and their collection data are available as Supplementary Table S1 and S2.

**DNA extraction and marker amplification** – Fresh leaf samples were collected and wrapped in nonwoven bags and dehydrated with silica gel. For DNA extraction, we pulverized about 20mg of dehydrated leaf tissue using TissueLyser II by QIAGEN, followed by DNA extraction using the DNeasy plant mini kit (QIAGEN). Extracted DNA samples were then stored at -30°C and used for amplification reactions. In order to compare with previous studies (Hsu *et al.* 2019; Vasques *et al.* 2019), we amplified coding regions from the *rbcL* gene and regions between *rps4* – *trnS*, including part of the genes and the intergenic spacer. For the amplification reaction, 0.4µl (ca. 100ng DNA) of template were mixed over ice with 2µl of ExTaq Buffer (Takara), 1.6µl of DNTP mix (Takara), 0.4µl of 10M forward primer (*rbcL* 30F: 5'GTGTTGGATTCAAAGCTGGTG 3'; *rps4* F1: 5'GCCGCTAGACAATTAGTCAATC3'; primer sequence sources: Hasebe *et al.* 1994; Hennequin *et al.* 2003), 0.4µl of 10M reverse primer (*rbcL* 1300R: 5'ACCTTCACGAGCAAGATCACG 3'; *trnS*: 5'TACCGAGGGTTCGAATC 3'; primer sequence sources: Hasebe *et al.* 1994; Souza-Chies *et al.* 1997), 0.1µl of ExTaq polymerase HotStart (Takara), and 15.1µl of deionized water. The amplification reactions were performed according to the following parameters: *rbcL* – 1 cycle of 94°C for 3 min, 3 cycles of 10 loops of 94°C for 45 sec, 52°C + 0.2°C/loop for 45 sec, and 72°C for 75 sec, and one finalizing cycle of 72°C for 10 min ; *rps4-trnS* – 1 cycle of 95°C for 5 min, 3 cycles of 10 loops of 95°C for 30 sec, 50°C + 0.5°C/loop for 30 sec, and 72°C for 1 min, and one finalizing cycle of 72°C for 5 min. Amplified products were purified using ExoSap-IT (Affymetrix) and submitted to Sanger sequencing using ThermoFisher's Applied Biosystems 3730xl DNA Analyzer.

### *Sequence alignment and Phylogenetic analysis* –

Obtained forward and reverse sequences were first reassembled using ATGC v. 4.3.5, and then aligned and trimmed with MEGA v. 10.2.6 (Kumar *et al.*, 2018) and Mesquite v. 3.7 (Maddison and Maddison, 2021). The final alignment included 1696 bp (1175 bp for *rbcL* and 521 bp for *rps4-trnS*) for 11 outgroup terminals and 173 ingroup terminals. 126 new DNA sequences were generated for this study and added to GenBank. All the



**Fig. 1.** Visual representation of a *H. polyanthos* leaf, with descriptions of the morphometrics used in this study. **A.** overall leaf morphometry: LL – Lamina length; LW – Lamina width; PL – Petiole length; PD – Pinnae distance (i.e., distance between adjacent pinnae junction points to the rachis); LPL – Pinnae length; LPW – Pinnae width; A – Angle between the costa and the pinnae. **B.** Petiole morphometry: WL – Petiole wing length; WW – Petiole wing width. **C.** Sori morphometry: SL – Sori involucre length; SW – Sori involucre width; CI – Sori constriction (calculated as a constriction index).

GenBank assessment numbers, and the final alignment file are reported as Supplementary Table S2.

The best fit model for the analysis was calculated using jModelTest 2 (Darriba *et al.*, 2012) and phylogenetic analyses were run under Bayesian Inference (BI) using MrBayes v. 3.2.5 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003), and under Maximum Likelihood (ML) analysis using RAxML (Stamatakis, 2006). For the BI analysis, four Markov Chain Monte Carlo (MCMC) were set for 2 runs for 10 million generations, with invgamma rates, a burn in fraction of 0.25, and trees sampled every 1000 generations. For the ML analysis, the GTRCATI model was used under 1000 bootstrap replicates. MCMC chain convergence was assessed using Tracer v. 1.7.2. The final trees were visualized using FigTree v. 1.4.2 (Rambaut, 2012) and edited using Illustrator v. 21.1.0.

526

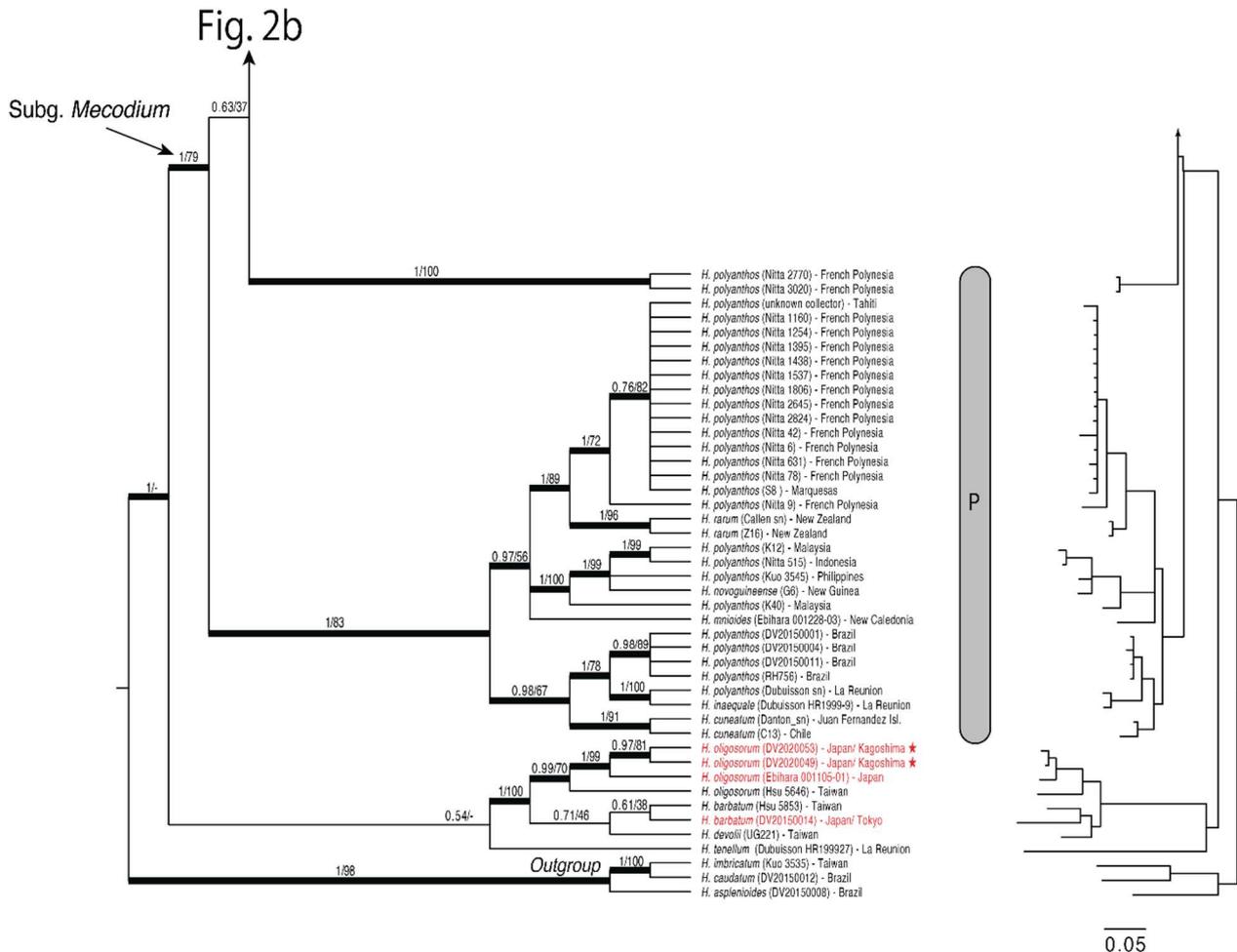
**Morphological analyses** – The new samples obtained for this study and used for DNA analysis were also used for morphological analyses, looking for correlations with the phylotypes obtained by the phylogenetic analysis. To assess the morphology of samples, three fertile leaves per specimen were measured for the following parameters (Figure 1): lamina length (LL), lamina width (LW), petiole length (PL), petiole wing length (WL), petiole wing width (WW), pinna length (LPL), pinna width (LPW), pinna distance (PD), and the angle between the lamina costa and the pinna (A). Traits used for the analysis were chosen because they (1) are easy to measure in a reproducible manner; (2) cover both vegetative and reproductive aspects of the morphology; (3) can be measured as numerical continuous factors. We also assessed morphological differences regarding the architecture of the sori, by selecting three sori from each sample and measuring the sori involucre length (SL), and sori involucre width (SW). Additionally, some samples within the subg. *Mecodium* show a constriction of the leaf tissue at the basis of the sori. To assess this constriction, we measured a constriction index (CI) by calculating the ratio between the width of the basis of the sori and the width of the lamina below the sori insertion region. Morphological trait differences observed between groups were assessed through inferential statistics using Rstudio v. 1.4.1717 (R Core Team, 2020). In total, 138 measurements were used for the morphological inferences herein (Supplementary Table S3).

**Herbarium specimens' identification and determination** – In addition to the phylogenetic analyses, specimens in the TNS herbarium were carefully observed and used to determine distribution patterns. First, we sorted the DNA sampled specimens into one of the three phylotypes 'fujisanense', 'parallelocarpum' and 'punctisorum', and applied this data on a Linear Discriminant Analysis (LDA) using Rstudio v. 1.4.1717 (R Core Team, 2020), with the 'mda' package. The LDA generated a comparison model of clustering that could be used to sort additional samples if they are measured for the same traits. This was applied to 410 specimens from Japan, identified as *H. polyanthos*, and deposited in the TNS herbarium. These measurements were compared to the LDA model, giving an estimate of which phylotype each specimen best resembled. The specimens grouped with each phylotype were then mapped using the 'ggplot2' package in RStudio v. 1.4.1717 (R Core Team, 2020). Data used for these analyses are available as Supplementary Table S4.

## RESULTS

### Phylogenetic inferences and Japanese taxa

Our phylogenetic analysis recovered the tree observed in figure 2, which includes subg. *Mecodium* as a monophyletic group (PP = 1, ML = 79%). Within the



**Fig.2a.** Phylogenetic tree for *Mecodium*, with focus on the *H. polyanthos* complex species in Japan. The tree to the left is a dendrogram showing the relationships between the OTUs, while the tree to the right represents the phylogram with the respective branch lengths for the clades. Numbers above branches represent, respectively, the Bayesian inference and the maximum likelihood support values. Branches with enough support from either analysis method (i.e. BI>0.9; ML>80) are highlighted in bold lines. Samples from Japan are highlighted in red and samples newly sequenced for this study are highlighted with a ★ mark. Legend for the main groups of subg. *Mecodium*: N – Neotropics; A – Asia/ Africa; P – Pacifics.

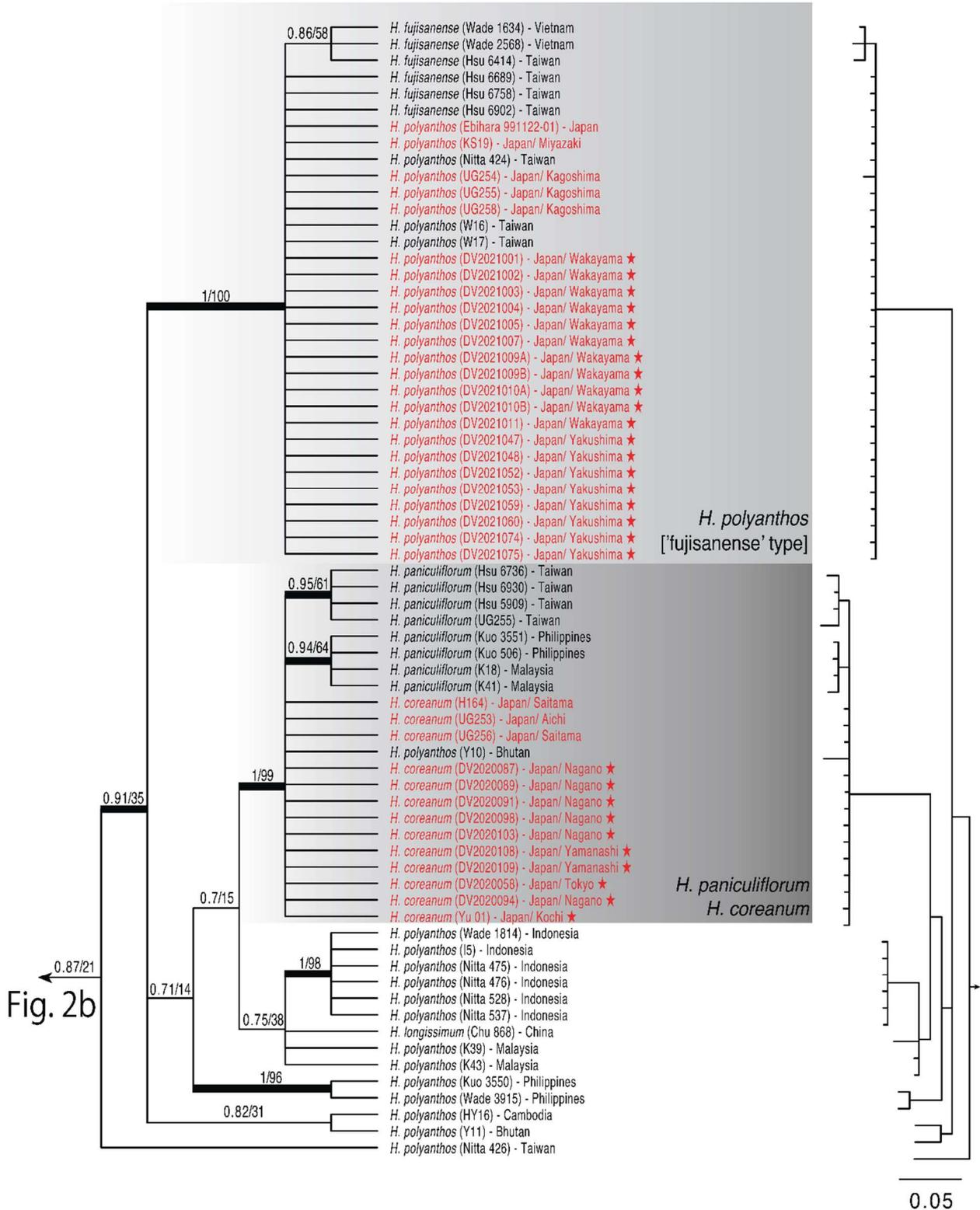
subgenus, we recovered clades that were largely consistent with the three geographical clades obtained by Vasques *et al.* (2019), namely the Neotropics clade (PP = 1, ML = 86%), the Asia/ Africa clade (PP = 0.94, ML = 53%) and the Pacific Islands clades (recovered as two clades not grouped together due to a weak support from our analysis, but nevertheless well supported around each other: PP = 1, ML = 100%, and PP = 1, ML = 83%). As this study centers on the Japanese specimens, further descriptions of the phylogeny focus on the groups obtained in the Asia/ Africa clade.

Within the Asia/ Africa clade, Japanese samples were placed in six distinct and well supported groups. Sister to the rest is a group (PP = 1, ML = 100%), previously recovered by Vasques *et al.* (2019), represented by plants identified as *H. wrightii*, including samples from Japan, Canada and the United States. This clade also includes DNA sequences a specimen from Hsu *et al.* (2019)

labelled as *H. szechuanense* (unvalidated name for *Mecodium szechuanense* Ching & Chiu). We have not assessed this specimen, and therefore cannot draw further conclusions about its identification. The new insight related to this clade regards its sister position to the remaining groups within the Asia/ Africa clade. The next earliest divergence separates a clade of Malaysian and Chinese samples, including specimens of *H. corrugatum* Christ and *H. polyanthos* (PP = 0.88, ML = 53%). Sister to this we recovered several lineages that help reveal the phylogenetic history of the *H. polyanthos* complex in Asian regions, particularly in Japan.

Japanese samples (excluding *H. wrightii*, described above) were found in five distinct clades, well supported by the analyses. *H. coreanum* and *H. paniculiflorum*, collected from several localities in Japan, Taiwan, the Philippines and Malaysia, appeared as a monophyletic group (PP = 1, ML = 99%), in accordance with previous studies (Hsu *et al.* 2019;

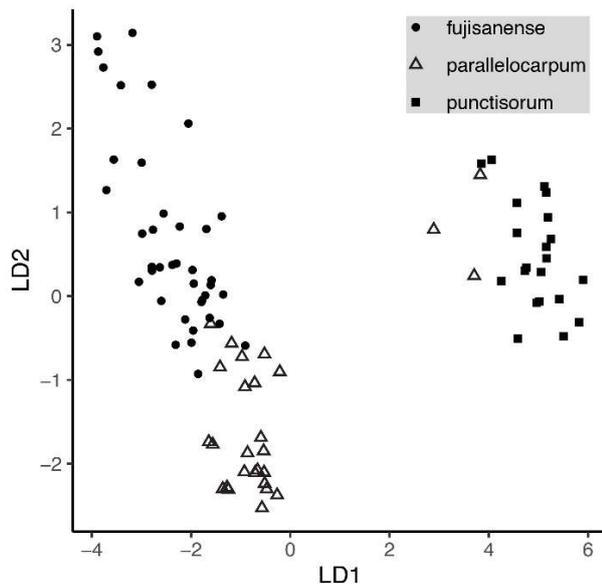




**Fig. 2c.** Phylogenetic tree for *Mecodium*, with focus on the *H. polyanthos* complex species in Japan. The tree to the left is a dendrogram showing the relationships between the OTUs, while the tree to the right represents the phylogram with the respective branch lengths for the clades. Numbers above branches represent, respectively, the Bayesian inference and the maximum likelihood support values. Branches with enough support from either analysis method (i.e. BI>0.9; ML>80) are highlighted in bold lines. Samples from Japan are highlighted in red and samples newly sequenced for this study are highlighted with a ★ mark. Legend for the main groups of subg. *Mecodium*: N – Neotropics; A – Asia/ Africa; P – Pacifics.

**Table 1.** Coefficients of linear discriminant for the traits used in the linear discriminant analysis.

Trait	LD1	LD2
Lamina Length (cm)	-0.22291929	0.2668655
Lamina Width (cm)	-0.49207548	-0.1970654
Petiole Length (cm)	-0.03494573	-0.2730410
Pinnae Length (cm)	-0.01683873	1.1609914
Pinnae Width (cm)	0.77450141	0.2236988
Pinnae distance (cm)	-0.75654811	2.4840244
Sori Length (mm)	0.06826162	0.5405652
sori width (mm)	-0.85320887	-1.4266921
Constriction Index (CI)	-1.46001575	0.4629518
Wing (presence/ absence)	-5.04271949	-2.4805139

**Fig. 3.** Linear Discriminant Analysis of the morphological traits' measurements. The three morphotypes of *H. polyanthos* are distinguished by different shapes. ● – 'fujisanense' form; △ – 'parallelocarpum' form; ■ – 'punctisorum' form.

['parallelocarpum' form] and *H. mikawanum* (a species endemic to Japan, PP = 1, ML = 99%) is not strongly resolved in our analysis. Finally, a group identified here as *H. polyanthos* ['punctisorum' form] (PP = 1, ML = 83%, figure 2) also occurs in Japan, as well as China and Taiwan, as previously reported (Hsu *et al.*, 2019).

### Morphology

The results of our phylogenetic analysis suggest the existence of three cryptic lineages within *H. polyanthos* in Japan, and that those lineages are very closely related to 'fujisanense', 'parallelocarpum', and 'punctisorum' forms, recognized as species by Hsu *et al.* (2019) for Taiwan. Based on these findings, we conducted morphometric analysis of 11 traits of specimens included in the phylogenetic analysis (not including vouchers from Hsu *et al.* 2019). We focused the morphometric analyses on the three cryptic lineages of *H. polyanthos* (i.e., 'fujisanense', 'parallelocarpum', and 'punctisorum'

lineages), aiming to describe morphological differences between the putative species. For this reason, the analyses here do not include samples of the remaining *Mecodium* species distributed in Japan (i.e., *H. coreanum*, *H. mikawanum*, and *H. wrightii*).

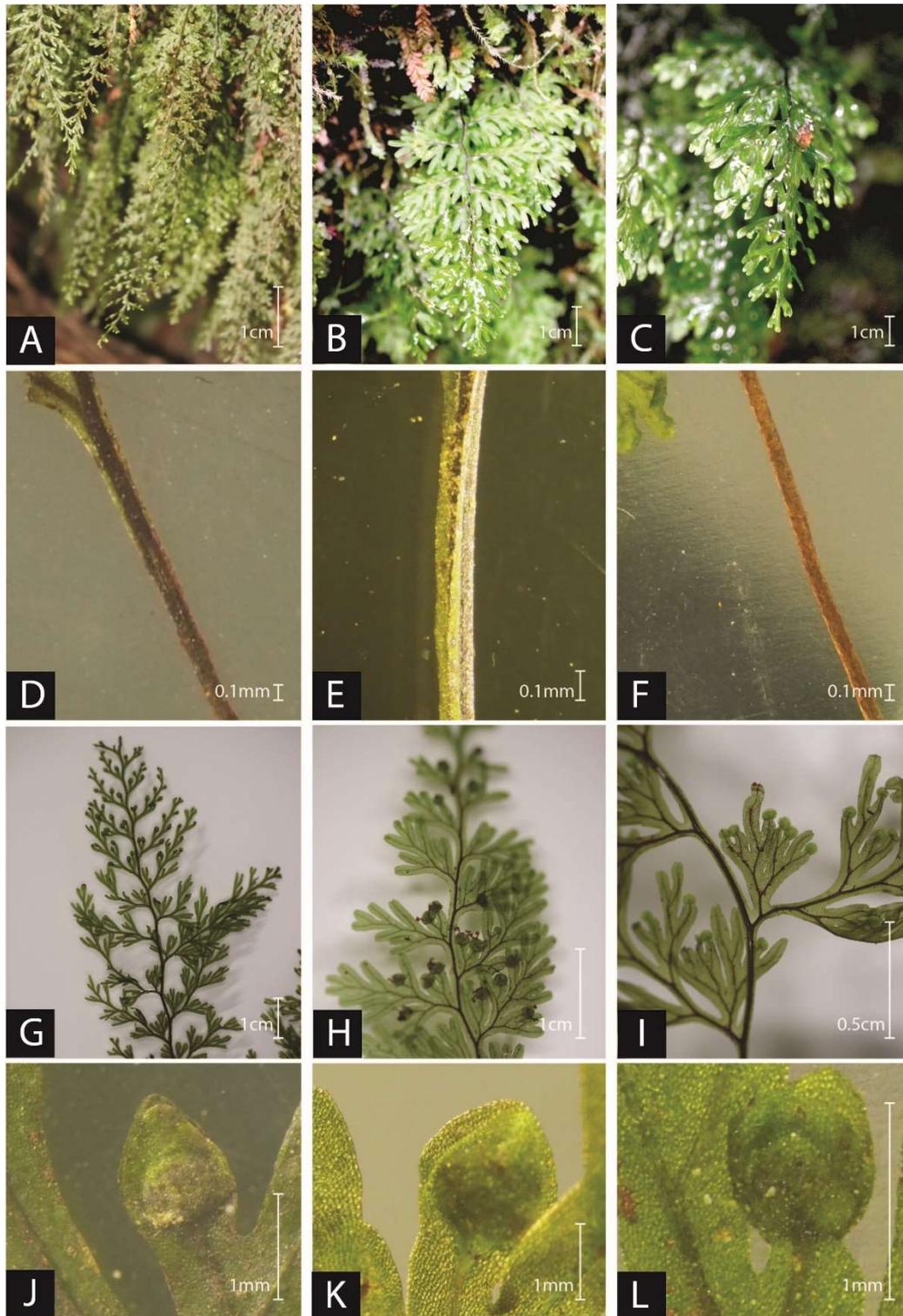
Figure 3 shows the results of the LDA analysis (respective coefficients of linear discriminant are shown in Table 1). Overall, the 'punctisorum' form is more clearly distinguished from the other two phylotypes, and that is due to the absence of the petiole wing, observed in the other two forms. Further comparisons of the morphological traits included in this analysis show that traits like petiole length, leaf lamina dimension, pinnae distance, sori size, and sori format can still be useful for distinguishing between the three phylotypes (Figures 4 and 5). Out of all morphological traits investigated here, only the sori length and sori width datasets showed a parametric distribution.

The 'fujisanense' form (Figure 4) morphological variation shows that this lineage includes the largest plants in terms of leaf dimensions, including plants with petioles varying from ca. 1 to 8 cm in length, and leaf blades varying from ca. 4 to 16 cm in length and ca. 1 to 7 cm in width. Pinnae are connected to the rachis at a mean angle of ca. 60 degrees, and their dimensions vary from ca. 1 to 4 cm in length, and ca. 0.5 to 2 cm in width (Figure 5). Pinnae are also comparatively more separated from each other, with the rachis junctions at intervals of ca. 0.5 cm. The sori of the 'fujisanense' form are ovate in shape, with an acute apex. The 'fujisanense' form dimensions, however, overlap considerably with the dimensions observed in the 'parallelocarpum' form (Figures 4 and 5). The 'parallelocarpum' form has relatively long petioles (ca. 1 to 5 cm in length), and the observed samples showed ovate to lanceolate leaf blades, varying from ca. 3 to 10 cm in length, and ca. 1 to 5 cm in width. Pinnae vary from ca. 0.5 to 4 cm in length, and ca. 0.5 to 1.6 cm in width, and the point of junction to the rachis is less than 0.5 cm. Sori are wider than longer (length = ca. 0.5 to 1.5 mm; width = ca. 0.7 to 1.2 mm), with a round to acute apex.

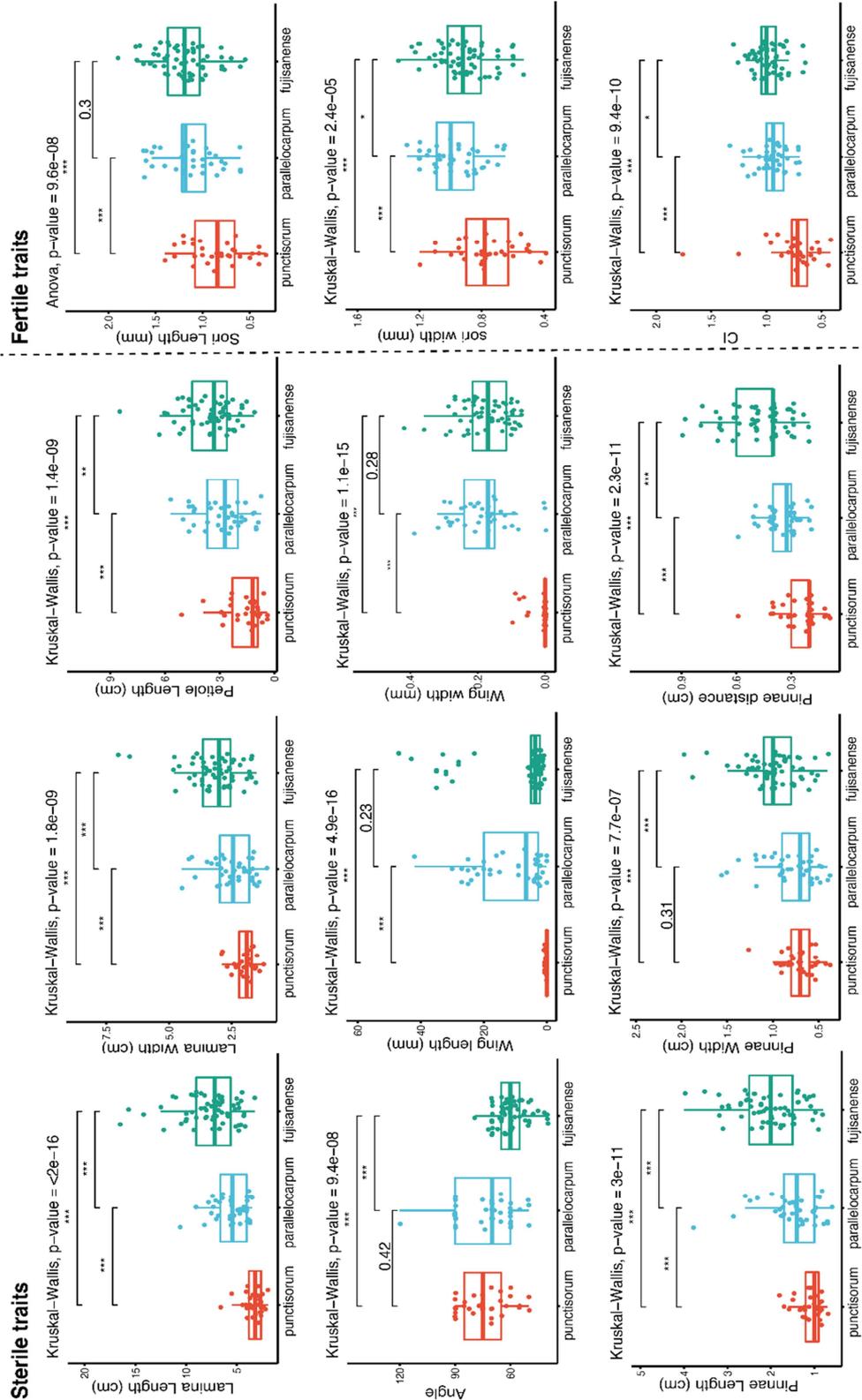
The 'punctisorum' form (Figure 4) is represented by smaller plants, with shorter petiole (length = ca. 0.2 to 5 cm), elliptic leaf lamina (length = ca. 1 to 7 cm; width = ca. 1 to 3 cm), and smaller pinnae (length = ca. 0.5 to 2 cm; width = ca. 0.5 to 1.2 cm). The group can be easily distinguished by the inconspicuous petiole wing and by the small and orbicular sori (Figure 5). Additionally, a prominent contraction at the basis of the sori involucre can be observed.

### Horizontal and vertical distribution ranges

In addition to the morphological differences, we also investigated possible differences in the geographic distribution of these three cryptic lineages of *H. polyanthos*. The analysis was inferred from the LDA model, which we



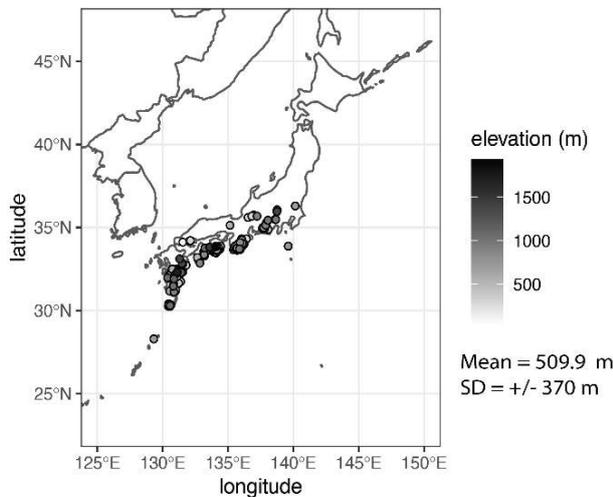
**Fig. 4.** Morphology of Japanese specimens of the *Hymenophyllum polyanthos* complex. **A:** habit of *H. polyanthos* ['fujisanense' form] (DV2021002, Wakayama prefecture); **B:** habit of *H. polyanthos* ['parallelocarpum' form] (DV2021049, Kagoshima prefecture); **C:** habit of *H. polyanthos* ['punctisorum' form] (DV2021077, Kagoshima prefecture); **D:** petiole wing of *H. polyanthos* ['fujisanense' form] (DV2020111); **E:** petiole wing of *H. polyanthos* ['parallelocarpum' form] (DV2021005); **F:** wingless petiole of *H. polyanthos* ['punctisorum' form] (DV2020090); **G:** sori position of *H. polyanthos* ['fujisanense' form] (DV2021002); **H:** sori position of *H. polyanthos* ['parallelocarpum' form] (DV2020095); **I:** sori position of *H. polyanthos* ['punctisorum' form] (DV2020056); **J:** sori detail of *H. polyanthos* ['fujisanense' form] (DV2021011); **K:** sori detail of *H. polyanthos* ['parallelocarpum' form] (DV2020117); **L:** sori detail of *H. polyanthos* ['punctisorum' form] (DV2020088).



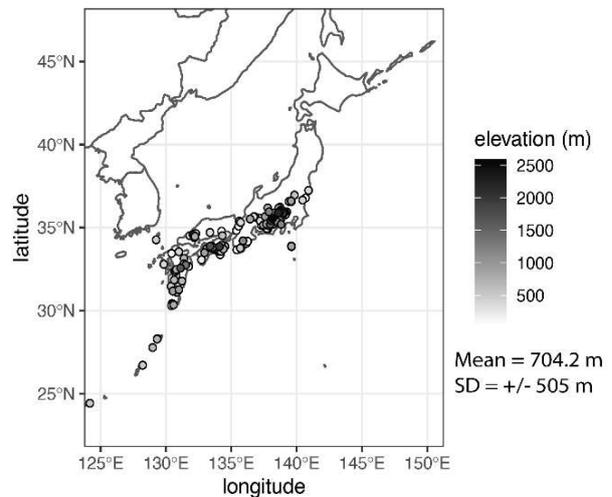
**Fig. 5.** Box plots describing the morphological variation between morphotypes of *H. polyanthos*. The y axis of each graph describes, respectively: petiole length (cm); lamina length (cm); lamina width (cm); pinnae distance (cm); pinnae length (cm); pinnae width (cm); petiole wing length (mm); petiole wing width (mm); sori length (mm); sori width (mm); sori base constriction index (CI); and the pinnae inception angle (degrees). Pairwise comparisons between groups were assessed through a t-test, and the p-values are shown above the boxplots for every 2-groups (\* =  $p < 0.1$ ; \*\* =  $p < 0.05$ ; \*\*\* =  $p < 0.01$ ).



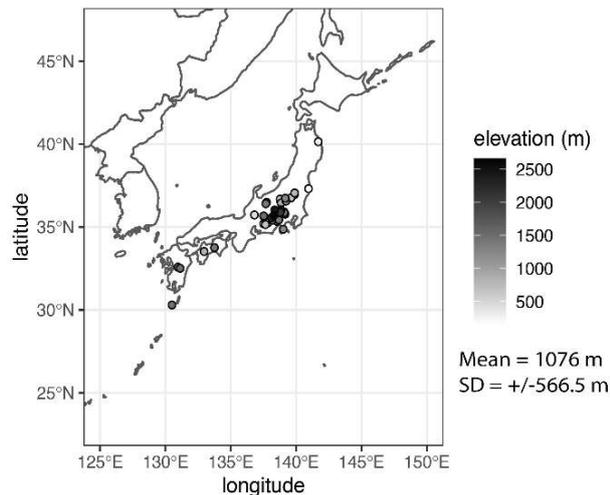
*H. polyanthos*  
['fujisanense' form]



*H. polyanthos*  
['parallelocarpum' form]



*H. polyanthos*  
['punctisorum' form]



**Fig. 6.** Distribution maps for the three forms of *H. polyanthos* in Japan. Black and white gradients represent the elevation position of the collection points.

used to compare herbarium specimens that we could not sample for DNA. Observing the coefficients of linear discriminant obtained from our model, it becomes clear that the petiole wing presence/absence, the sori constriction index, the sori involucre dimensions and the pinnae distance on the rachis were very relevant for the groups clustering (Table 1). The 'fujisanense' form is apparently distributed from the Kanto region towards the south of Japan, with a relatively concentrated distribution around the east part of the country. Although sympatrically distributed in many regions with the other two cryptic lineages of Japanese *H. polyanthos*, the 'fujisanense' form seems to be most predominant phylotype in the Kinki region (Figure 6). The

'parallelocarpum' form is also distributed from the Kinki region to the south of Japan, occurring sympatrically with 'fujisanense' form populations in many localities, but with a more conspicuous distribution towards the Chubu and Kanto regions. In terms of habitat, 'fujisanense' form was predominantly found growing over rocks, in relatively well illuminated open ravines, while 'parallelocarpum' form was found in darker woods (personal observation). Both phylotypes have relatively long petioles, so it was common to observe individuals growing towards the light. The 'punctisorum' form, on the other hand, mostly occurs in the Kanto region in the central part of Japan (Figure 6), but some individuals were also found in the Shikoku region, and at the



Yakushima Island, in Kagoshima prefecture (located in the south of the archipelago). This phylotype is morphologically the smallest of the three and was found predominantly growing over rocks near rivers or waterfalls, around shaded areas.

Besides the distribution patterns described above between the three cryptic phylotypes of *H. polyanthos*, an elevation gradient is also observed (figure 6). Although some overlap is observed, ‘punctisorum’ form samples seem to be distributed at higher elevation (mean = 1076 m, SD = ±566.5 m), followed by the ‘parallelocarpum’ form (mean = 704.2 m, SD = ±505 m), with the ‘fujisanense’ form occurring at lower elevation (mean = 509.9 m, SD = ±370 m). Regarding the latitude range, ‘punctisorum’ is mainly distributed at higher latitudes (≥ 35°N), when compared to the other two phylotypes.

## DISCUSSION

### Biogeography of *Hymenophyllum polyanthos* in Japan

This study is the first attempt to uncover the complicated taxonomic history of the *H. polyanthos* complex in Japan, after our first global phylogeny for the subgenus *Mecodium* (Vasques *et al.* 2019). Our phylogenetic results, together with the biogeographical and morphological patterns observed, support the hypothesis that three of the phylotypes recognized in Taiwan by Hsu *et al.* (2019) also occur in Japan. Therefore, we conclude that subgenus *Mecodium* is represented in Japan by six independent lineages: *H. coreanum*, *H. mikawanum*, *H. wrightii* and three cryptic lineages within the *H. polyanthos* complex, namely ‘fujisanense’, ‘parallelocarpum’, and ‘punctisorum’. This is the first record for the ‘punctisorum’ and ‘parallelocarpum’ forms in Japan, as these two phylotypes were described as occurring exclusively in China and Taiwan (with *H. parallelocarpum* being endemic to Taiwan, type locality: Alishan ‘Arisan’) by Hsu *et al.* (2019).

Our extensive investigation of herbarium specimens revealed that, among the three cryptic lineages of *H. polyanthos*, ‘punctisorum’ occurs mostly at higher latitudes in Japan, and also at higher elevation. These observations corroborate the hypothesis by Hsu *et al.* (2019), who described plants growing on rocks of cloudy forests at elevation levels higher than 1700 m. The remaining two phylotypes, ‘fujisanense’ and ‘parallelocarpum’ forms, are mostly distributed at lower latitudes in Japan, from the Kanto region towards the south of the archipelago, and in characteristically warmer environments. It is notable that the distribution gradient between these three lineages seems to somewhat reflect the Fossa Magna region, an important floristic region located on Honshu Island in Japan. The Fossa Magna is a back-arc rift formed by movements of the mantle beneath the Eurasian Plate during the opening of the Japan Sea in

the Miocene (Okamura *et al.*, 2016) and several studies of plants and insects have obtained phylogeographic patterns correlated with the Fossa Magna topography (Saito and Tojo, 2016; Tojo *et al.*, 2017; Gamboa *et al.*, 2019; Watanabe *et al.*, 2021).

The pattern of distribution of *H. polyanthos* cryptic species seems also similar to patterns described by Ebihara and Nitta (2019), which showed that species richness and phylogenetic diversity in ferns becomes progressively higher from Honshu Island to the south of the archipelago. Additionally, the investigation by Ebihara and Nitta (2019) highlighted the existence of fern diversity hotspots at mountainous islands at the southernmost part of the country, in particular in Yakushima Island. Yakushima Island’s landscape is characterized by high-elevation mountains, abundant rainfall throughout the year, and by a variety of climate zones due to it being located in a biogeographical boundary between tropical and temperate zones (Okano and Matsuda, 2013). Furthermore, our observations and DNA analysis demonstrated that all three *H. polyanthos* cryptic lineages also occur in Yakushima Island, and this information was confirmed by our phylogenetic analysis (Figure 2).

### Cryptic species of *Hymenophyllum polyanthos*

The ‘parallelocarpum’ and ‘fujisanense’ phylotypes are the most widespread in Japan, and occur sympatrically in many locations, while ‘punctisorum’ phylotype is predominantly distributed at higher elevation and latitudes. The ‘parallelocarpum’ forms is probably the most widespread lineage of *H. polyanthos* in Japan. Although the specimens from Japan are clustered together with the specimens from Taiwan in our phylogeny, the Japanese samples show some morphological differences when compared to the descriptions for plants in Taiwan. Specifically, Japanese samples show a wing of tissue running along  $\frac{3}{4}$  of the petiole, and the sori involucre sometimes display an acute apex, while the Taiwanese specimens’ descriptions presented by Hsu *et al.* (2019) show no wing tissue on the petiole, and round to orbiculate sori involucre. These traits make field identification significantly challenging, as the ‘fujisanense’ phylotype displays similar characteristics. Plants of the ‘parallelocarpum’ form in Japan are found growing over rocks, and their long petioles allow the leaves to reach toward the light in the shaded forest meadows where they are found.

The ‘fujisanense’ form is represented by plants with long petioles, longer distances between pinnae, and broad leaf blades when compared to the other cryptic phylotypes. Besides its distribution in Japan, highlighted by this study, this morphospecies seems to also occur in China, Taiwan and Vietnam (Figure 2; Hsu *et al.*, 2019), whilst further studies might reveal its true biogeographical range. In Japan, plants were found growing over fallen tree trunks and over rocks, in



locations relatively exposed to sunlight. The lectotype for *H. fujisanense* designated by Hsu *et al.* (2019) is one specimen deposited at the TI herbarium (barcode: TI00001424 [lecto]; TI00001425 [isolecto]) and were collected in the Aokigahara forest, at the base of Mount Fuji in Yamanashi Prefecture, Japan. Upon investigation of the area, however, we only managed to find specimens of ‘parallelocarpum’, as confirmed by our phylogenetic analysis. Upon measurement of the type specimens in TI, the comparison of the morphology of leaves to our LDA model also pointed to the ‘parallelocarpum’ form. It thus seems that the lectotype designated by Hsu *et al.* (2019) for *H. fujisanense* are specimens of the ‘parallelocarpum’ form. There are other names (*i.e.*, *Mecodium hainanense* Ching, or *Hymenophyllum tenellum* D. Don.) that could be applied to this group, but final conclusions cannot be drawn without a global analysis, including samples from all type localities related.

Finally, ‘punctisorum’ is represented by plants with shorter petioles and by its smaller blade dimensions (blade length and width), when compared to the other cryptic phylotypes. Distinctive traits for this lineage include the ovoid to orbicular leaf format, the wingless petioles, and the clear contraction of the leaf lamina width at the base of the sori. The lectotype for *H. punctisorum* was also designated by Hsu *et al.* (2019) and was collected in Mt. Alishan (‘Arisan’), Taiwan.

### Taxonomic considerations

Through this study, we reinforced the conclusions presented previously (Vasques *et al.*, 2019) showing that *H. polyanthos* s.s. is not present in Japan, but instead represented by cryptic lineages within the complex. Cryptic species are described as sexually isolated lineages that are poorly distinguishable by morphological traits, and thus are historically misinterpreted as belonging to the same species (Stebbins, 1950; Grant, 1981; Wiley, 1981; Paris *et al.*, 1989). Even though we assume an evolutionary or phylogenetic concept of species as a premise for biological studies, in practice morphological differences are still very important for taxonomic inferences, and thus the search for relevant diagnostic traits to aid the identification work is of critical importance. Cryptic species are particularly common in ferns, because of many cases of asexual and apomitic lineages (Walker, 1984; Haufler, 1996; Haufler *et al.*, 2016), reticulate evolution, resulting in allopolyploid lineages (Adjie *et al.*, 2007; Ebihara *et al.*, 2005; Chen *et al.*, 2014; Fujiwara *et al.*, 2017), and sexual diploid cryptic species (Dauphin *et al.*, 2014; Yatabe *et al.*, 2002). Besides that, autopolyploid organisms, taken as not so frequent in polyploidy scenarios (Spoelhof *et al.*, 2017), are also known and reported in ferns, highlighting specific evolutionary scenarios that still lack attention from more studies (Beck *et al.*, 2010; Metzgar *et al.*, 2016; Schuettpelez *et al.*, 2008).

We were able to confirm that three of the Taiwanese lineages identified by Hsu *et al.* (2019) also occur in Japan. The Taiwan endemic *H. exquisitum*, described by Hsu *et al.* (2019) was recovered in a polytomy together with the ‘parallelocarpum’ samples, indicating that significant differences were not observed among the DNA sequences investigated here. *H. exquisitum* was described by Hsu *et al.* (2019) as a new species endemic to Taiwan, and phylogenetically close to *H. parallelocarpum*. In fact, Hsu *et al.* (2019) found no genetic difference between the two species, although differences in the stipe wing, degree of lamina crispation, and lamina size may be used to distinguish the two species. We recognized the Japanese lineages in this clade as [‘parallelocarpum’ form] since none of the sampled Japanese ‘parallelocarpum’ show the strongly crisped leaves, described as a diagnostic trait for the Taiwanese *H. exquisitum*. Nevertheless, Hsu *et al.* (2019) report *H. parallelocarpum* as bearing wingless stipes, while winged stipes were observed for the Japanese samples, which may suggest a broader morphological range for these plants. The distinctiveness of *H. exquisitum* and *H. parallelocarpum* requires further study.

The type specimens for the species names corresponding to the three phylotypes recovered in Japan (*i.e.*, ‘fujisanense’, ‘parallelocarpum’, and ‘punctisorum’) were designated by Hsu *et al.* (2019), with *H. fujisanense* lectotype described for a specimen collected at the Aokigahara forest, in Yamanashi prefecture, Japan. However, upon in situ investigation, and after DNA analysis and voucher comparisons, we could only find specimens of the ‘parallelocarpum’ form in the aforementioned forest. The best course of action to resolve this matter is to sequence the relevant gene markers for the type specimen of *H. fujisanense*, which was not feasible during this study. For the time being, we have decided to keep the *fujisanense* name to describe this phylotype within the cryptic lineage.

Apart from the three cryptic lineages described above, *H. coreanum*, *H. mikawanum*, and *H. wrightii* also occur in Japan. *H. mikawanum* is a species endemic to the Japanese archipelago, only known from a few localities, including Aichi, Gifu, Nagano and Shizuoka prefectures. The plants are small, grow over rocks in dark environments and have a characteristic dentation on the margins of the sori involucres (Serizawa 1983; Ebihara 2016; personal observations). *Hymenophyllum coreanum* is represented by small plants, with ovate to lanceolate fronds, and protuberant round sori involucres. *Hymenophyllum coreanum* is widely distributed in Japan, growing over rocks or fallen trunks, and usually in close proximity to specimens of *H. polyanthos* s.l. and *H. barbatum*. *H. coreanum* is often confused with *H. paniculiflorum*, a morphologically similar species occurring in the Philippines and Taiwan. Finally, *H. wrightii* is a species that displays pinnatifid fronds, with



few (1–3) pinnae. This species occurs at higher elevation levels, and its frond architecture is significantly distinct from the other subg. *Mecodium* species. The species seems to be phylogenetically closely related to *H. szechuanense*, but further sampling should be performed before additional conclusions are taken.

## CONCLUSION

This study is the first modern investigation of subg. *Mecodium*, particularly the *H. polyanthos* species complex, in Japan. By combining molecular, morphological and distribution data, we have demonstrated that subg. *Mecodium* is represented in Japan by the *H. polyanthos* complex, plus three additional species, namely *H. coreanum*, *H. mikawanum* and *H. wrightii*. Furthermore, by making comparisons with the adjacent Taiwan region, we have found support for the hypothesis that the *H. polyanthos* complex in Japan comprises three phylotypes: ‘fujisanense’, ‘parallelocarpum’ and ‘punctisorum’. This is the first record of the ‘parallelocarpum’ and ‘punctisorum’ forms in Japan. Additionally, we have found morphological and distribution evidence that allows the identification of the three phylotypes with reasonable accuracy. Finally, we found an altitudinal and latitudinal pattern in the distribution of the phylotypes of *H. polyanthos* in Japan, and we also report the occurrence of all three types in the South Island of Yakushima. The results and conclusions drawn here are an important step towards resolving the global taxonomy for the *H. polyanthos* complex.

### Identification key for Japanese species of *Hymenophyllum* subg. *Mecodium*

- 1a. Sori involucre margins dentate ..... *H. mikawanum*
- 1b. Sori involucre margins entire ..... 2
- 2a. Lamina pinnatifid, with 1–3 pinnae pairs, pinna insertion angle from 30° to 45° ..... *H. wrightii*
- 2b. Lamina pinnatifid to pinnatisect, with 3 or more pinnae pairs, pinna insertion angle more than 45° ..... 3
- 3a. Lamina length less than 6 cm ..... 4
- 3b. Lamina length more than 6 cm ..... 5
- 4a. Laminae ovate to lanceolate, with sori gathered at the distal portion of the lamina; sori usually big (>1mm length), with no constriction of the lamina at their base ..... *H. coreanum*
- 4b. Laminae elliptic to ovate; sori usually small (<1mm length), with a constriction of the lamina at their base ..... *H. polyanthos* [‘punctisorum’ form]
- 5a. Lamina length less than 8 cm, lamina width less than 3 cm, petiole length less than 3 cm, pinnae angle insertion to the rachis wider than 60°, pinnae length of less than 2cm, pinnae width less than 1cm width, distance between pinnae less than 0.5 cm, sori concentrated on the point of insertion of the pinnae to the rachis ..... *H. polyanthos* [‘parallelocarpum’ form]
- 5b. Lamina length more than 8 cm, lamina width more than 3 cm, petiole length more than 3 cm, pinnae angle insertion to the rachis of 60° or narrower, pinnae length of more than 2cm, pinnae width more than 1cm width, distance between pinnae more than 0.5 cm, sori concentrated near the apex of the lamina ..... *H. polyanthos* [‘fujisanense’ form]

## FUNDING

This research was partly funded by the Japan Science Society (JSS) Sasagawa early-career scientist grant (Grant number: 2021-5015), and by the Environment Research and Technology Development Fund (JPMEERF20204001) of the Ministry of the Environment, Japan.

## AUTHORS CONTRIBUTIONS

All authors contributed to the study conception and design. Sample collection and specimen observation were performed by DTV and AE. Data collection and analysis were performed by DTV. The first draft of the manuscript was written by DTV and all authors commented on subsequent versions of the manuscript. All authors read and approved the final manuscript.

## ACKNOWLEDGMENTS

We thank Seikan Kurata, Toshihiro Saito and Katsunori Ueno for their support with collection trips and sampling, and Motomi Ito and Shigeto Dobata for their support with laboratory facilities. We also thank Marcelo Valpeteris for checking the English of the manuscript.

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