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Finding Hidden Outliers to Promote the Consistency of Key Morphological Traits and Phylogeny in Dennstaedtiaceae

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Abstract: With the development of open science and technological innovation, using sharing data and molecular biology techniques in the study of taxonomy and systematics have become a crucial component of plants, which undoubtedly helps us discover more hidden outliers or deal with difficult taxa. In this paper, we take *Dennstaedtia smithii* as an example, based on sharing molecular database, virtual herbarium and plant photo bank, to clarify the outliers that have been hidden in *Dennstaedtia* and find the key morphological traits with consistent of molecular systematics. In molecular phylogenetic analyses, we used *rbcL*, *rps4*, *psbA-trnH* and *trnL-F* sequences from 5 new and 49 shared data; the results showed that *Dennstaedtia smithii* is nested within *Microlepia* rather than *Dennstaedtia*. We further studied the morphological characters based on the phylogeny result and found that *D. smithii* is distinguished from other species of *Dennstaedtia* by spore ornamentation and the unconnected of grooves between rachis and pinna rachis. According to morphological and molecular phylogenetic studies, our results supported that *D. smithii* should be a new member of *Microlepia* and renamed *Microlepia smithii* (Hook.) Y.H. Yan. Finding hidden outliers can promote the consistency of morphological and molecular phylogenetic results, and make the systematic classification more natural.

Keywords: taxonomy; palynology; revisions; ferns; *Dicksonia smithii*

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1. Introduction

From the evolutionary emergence of primitive organisms to today's broad variety of organisms, people have been constantly exploring how many species there are on the earth and what kind of evolutionary relationship among species. With the development of open science and technological innovation, methods of species identification range from using morphological characteristics to the integration of various methods (e.g., molecular biology, bioinformatics, bionomics) [1–5], which help us gain a more in-depth understanding of the evolutionary process between organisms and their accurate position in the tree of life. Due to the multi-disciplines combination and the improvement of sharing databases, many misclassifications hidden in the past have been gradually discovered, and their key morphological boundaries have also been redefined. For example, *Typhonium giganteum* Engler 1883 had long been recognized as a member of *Typhonium* Schott 1829 according

to the morphological characteristics, but the molecular phylogenetic evidence indicated that it should belong to the *Sauromatum* Schott 1832 and was renamed as *Sauromatum giganteum* (Engl.) Cusimano and Hetterscheid 2010 [1]. Ferns, an ancient group, also have similar examples, one of which is *Athyrium niponicum* (Mett.) Hance 1873. *A. niponicum* had been treated as a member of *Athyrium* Roth 1875, but was later confirmed to be within *Anisocampium* C. Presl 1851 based on *rbcL* and *trnL-F* region sequences [2]. At the end of the paper, the author revised the morphological boundaries of *Athyrium* and *Anisocampium* according to the results of systematics [2].

Microlepia C. Presl 1836, comprising about 60 species in the world, is mostly distributed in tropics and subtropics [6,7]. In the past, there had been much controversy over the relationships of *Microlepia* and *Dennstaedtia* Bernh. 1800. Some species of *Dennstaedtia*, including the type species *Dennstaedtia flaccida* (J.R. Forst.) Bernh. 1801, had been placed in *Microlepia* by Smith [8]. However, in previous molecular phylogenetic studies, *Microlepia* was monophyletic [6,9–11] with the type species of *Microlepia speluncae* (L.) T. Moore 1857 and was sister to the old-world clade of *Dennstaedtia* [10]. We can distinguish *Microlepia* and *Dennstaedtia* from the following characteristics: the abaxial condition of sori, the shallow costal grooves and the finely echinate spores [7,12].

Dennstaedtia smithii (Hook.) T. Moore 1861 was first described as *Dicksonia Smithii* Hooker 1846 because all ferns with bivalved indusia were originally united under *Dicksonia* L'Héritier 1789 [13]. Many genera were later segregated from *Dicksonia* (mostly by Smith 1875), and subse-quently *Dicksonia smithii* was treated as a synonym of *Dennstaedtia smithii* by Moore [14]. In 1904, Christ published a new species *Dennstaedtia formosae* Christ 1904 based on a Taiwan specimen [15], but was later renamed *Culcita formosae* (Christ) Maxon 1922 by Maxon [16]. In 1988, Richard and Melvin thought that *Culcita formosae* belonged to *Dennstaedtia* according to the morphological features, and treated it as a synonym of *Dennstaedtia smithii* [17]. Nowadays, *Dicksonia smithii*, *Dennstaedtia formosae*, *D. leptophylla* and *Culcita formosae* are merged as synonyms of *Dennstaedtia smithii* in *Flora of China* [7].

During our field investigation in Taiwan, we collected two population samples of *Dennstaedtia* and identified it as *Dennstaedtia smithii* based on the literature [7,8,13–18] and type specimens of virtual herbarium (e.g., CVH, GBIF, JSTOR). Using scanning electron microscopy (SEM) observation, we found that *D. smithii* resembled those of *Microlepia* rather than of *Dennstaedtia* based on the spore micro-morphological characteristics [8,9,12,18–24]. To further confirm the phylogenetic and taxonomical position of *D. smithii*, we collected 5 new and 49 shared data of Dennstaedtiaceae for morphological and systematic studies.

2. Materials and Methods

2.1. Morphological Observation

By means of JSZ-6 anatomical lens (Nanjing Jiangnan Novel Optica Co., Ltd., Nanjing, China), virtual herbarium (e.g., CVH, GBIF, JSTOR), plant photo bank (e.g., PPBC, CUGB, GBIF, Ferns) and the literature [6–8,10,12,18–27], we observed and compared the morphological characteristics (e.g., leaf shape, the position of sori, the grooves between rachis and pinna rachis) of all samples between *Microlepia* and *Dennstaedtia*.

The spores of *Dennstaedtia smithii* (*Yan 1706Y021*) were dispersed directly on stubs and observed using SEM (FEI, The United States of America) at 10 kV, and their sizes were measured using the ruler tool in Adobe Photoshop CS3's (Adobe Systems, San Jose, CA, USA). From the samples of the field of vision, a total of 109 spores were measured from the specimen. Spore terminology follows Wang and Dai [27] and Luo et al. [9].

2.2. DNA Extraction, Polymerase Chain Reaction and Sequencing

The total genomic DNA of five samples was extracted from silica gel-dried leaves with a DNA Secure Plant Kit (Tiangen Biotech, Beijing, China), according to the manufacturer's protocols. The *rbcL* gene, *rps4* gene, *psbA-trnH* intergenic spacer and *trnL-F* intergenic spacer were amplified using primers and PCR protocols designed in previous studies as follows: AF and 1379R for *rbcL* [28], rps4.5 [29] and trnS [30] for *rps4*, psbA and turH2 for *psbA-*

trnH [31], f and FernLr1 for *trnL-F* [32] and amplicons were sequenced with an ABI 3730xl DNA analyzer (Applied Biosystems, Foster City, CA, USA). Each of the four chloroplast DNA regions for 49 samples were downloaded from GenBank, and five samples were newly generated in this study. We have included a list of 54 samples; their voucher information and GenBank accession numbers are presented in Table 1.

Table 1. List of 54 Specimens information and GenBank accessions.

No.	Species	Voucher No.	Locality	Herbarium	GenBank Accession No.			
					rbcL	*rps4*	*trnL-F*	*psbA-trnH*
1	*Microlepia. strigosa*	SG272	Jiangxi, China	CSH	MK051745	MK051993	MK052534	MK052254
2	*M. strigosa*	YYH11609	Taiwan, China	CSH	MK051843	MK052104	MK052649	MK052373
3	*M. khasiyana*	ZXL5742	Yunnan, China	CSH	MK051616	MK052063	MK052601	MK052325
4	*M. khasiyana*	ZXL7194	Yunnan, China	CSH	MK051627	MK052087	MK052625	MK052349
5	*M. obtusiloba*	WYD098	Guangdong, China	CSH	MK051755	MK052006	MK052547	MK052267
6	*M. obtusiloba*	SG2854	Hainan, China	CSH	MK051664	MK051913	MK052443	MK052163
7	*M. lofoushanensis*	WYD642	Guangdong, China	CSH	MK051675	MK051924	MK052454	MK052174
8	*M. lofoushanensis*	WYD641	Guangdong, China	CSH	MK051674	MK051923	MK052453	MK052173
9	*M. trichosora*	WYD445	Guangdong, China	CSH	MK051855	MK052110	MK052662	MK052386
10	*M. trichosora*	WYD389	Guangdong, China	CSH	MK051829	MK052091	MK052635	MK052359
11	*M. marginata*	WZS006	Hainan, China	CSH	MK051696	MK051947	MK052477	MK052197
12	*M. marginata*	WYG156	Guizhou, China	CSH	MK051771	MK052024	MK052563	MK052286
13	*M. szechuanica*	WYG056	Guizhou, China	CSH	MK051677	MK051926	MK052456	MK052176
14	*M. szechuanica*	YanYH13825	Sichuan, China	CSH	MK051732	MK051980	MK052521	MK052241
15	*M. rhomboidea*	WYD529	Guangdong, China	CSH	MK051763	NA	MK052555	MK052278
16	*M. rhomboidea*	SG2641	Yunnan, China	CSH	MK051806	MK052059	MK052597	MK052321
17	*M. yaoshanica*	YYH12136	Yunnan, China	CSH	MK051834	MK052095	MK052640	MK052364
18	*M. yaoshanica*	WYD303	Guangdong, China	CSH	MK051667	MK051916	MK052446	MK052166
19	*M. firma*	ZXL6895	Yunnan, China	CSH	MK051813	MK052070	MK052608	MK052332
20	*M. firma*	ZXL6882	Yunnan, China	CSH	MK051812	MK052069	MK052607	MK052331
21	*M. kurzii*	ZXL7021	Yunnan, China	CSH	MK051815	MK052080	MK052618	MK052342
22	*M. kurzii*	YYH12098	Yunnan, China	CSH	MK051631	MK051874	MK052404	MK052124
23	*M. platyphylla*	WYD609	Guangdong, China	CSH	MK051831	MK052092	MK052637	MK052361
24	*M. platyphylla*	YYH12394	Yunnan, China	CSH	MK051634	MK051878	MK052408	MK052128
25	*M. hancei*	YanYH13703	Guangdong, China	CSH	MK051642	MK051886	MK052416	MK052136
26	*M. hancei*	SG258	Jiangxi, China	CSH	MK051661	MK051908	MK052438	MK052158
27	*M. todayensis*	INA-BL49	Bali, Indonesia	CSH	MK051733	MK051981	MK052242	MK052242
28	*M. todayensis*	INA-BL44	Bali, Indonesia	CSH	MK051646	MK051890	MK052420	MK052140
29	*M. speluncae*	ZXL09896	Chiang Mai, Thailand	CSH	MK051795	MK052048	MK052587	MK052310
30	*M. speluncae*	YYH12379	Yunnan, China	CSH	MK051712	MK051965	MK052501	MK052221
31	*M. hookeriana*	WYD218	Guangdong, China	CSH	MH289650	MH289714	MK052488	MK052208
32	*M. hookeriana*	ZXL5886	Yunnan, China	CSH	MK051810	MK052064	MK052326	MK052602
33	*M. tenera*	KY1426	Taiwan, China	NA	MK051802	MK052055	MK052593	MK052317
34	*M. tenera*	SG1026	Yunnan, China	CSH	MK051801	MK052054	MK052592	MK052316
35	*Dennstaedtiawilfordii*	JSL2982	Anhui, China	CSH	MK051796	MK052049	MK052588	MK052311
36	*D. smithii*	Yan 1706Y021	Taiwan, China	CSH	MZ959179	MZ983428	MZ959174	MZ983423
37	*D. smithii*	Yan 1706Y008	Taiwan, China	N/A	MZ959180	MZ983429	MZ959175	MZ983424
38	*D. appendicula*	ZhangXC5294	Tibet, China	PE	MK051807	MK052060	MK052598	MK052322
39	*D. scabra*	YYH12150	Yunnan, China	CSH	MH289649	MH289713	MK052490	MK052210
40	*D. scabra*	YYH11627	Hainan, China	CSH	MK051705	MK051958	MK052489	MK052209
41	*D. hirsuta*	SG159	Fujian, China	CSH	MK051800	MK052053	MK052591	MK052315
42	*D. punctilobula*	N/A	N/A	N/A	KP644118	AY459159	MT633781	N/A
43	*D. scandens*	YYH16230	Taiwan, China	CSH	MH289628	MH289707	N/A	N/A
44	*D. cornuta*	4374	N/A	N/A	MT416335	MT559747	MT633779	N/A
45	*D. spinosa*	5045	N/A	N/A	MT416337	MT593216	MT633782	N/A
46	*D. distenta*	4998	N/A	N/A	MT633748	MT559732	MT633780	N/A
47	*D. cicutaria*	3866	N/A	N/A	MT633747	MT593213	MT633776	N/A
48	*Leptolepia novae-zelandiae*	12400	New Zealand	DUKE	EF463168	N/A	N/A	N/A
49	*Leptolepia novae-zelandiae*	P027279	New Zealand	N/A	KT983829	N/A	N/A	N/A

Table 1. *Cont.*

No.	Species	Voucher No.	Locality	Herbarium	GenBank Accession No.			
					rbcL	*rps4*	*trnL-F*	*psbA-trnH*
50	*Leptolepia novae-zelandiae*	Wolf 682	New Zealand	UTC	U18639	N/A	N/A	N/A
51	*Oenotrichia maxima*	P026233	New Caledonia	N/A	KT983830	N/A	N/A	N/A
52	*Pteridium aquilinum*	BJZ003	Guangxi, China	CSH	MZ959183	MZ983432	MZ959178	MZ983427
53	*Hypolepis punctata*	MS067	Hunan, China	CSH	MZ959182	MZ983431	MZ959177	MZ983426
54	*Histiopteris incisa*	YYH11645	Hainan, China	CSH	MZ959181	MZ983430	MZ959176	MZ983425

N/A = not available.

2.3. Phylogenetic Analyses

Sequence assembly and editing were performed using SeqMan [33]. The four genes (*rbcL*, *psbA-trnH*, *rps4* and *trnL-F*) were aligned using ClustalW and manually edited using BioEdit v.7.1.11 [34]. Phylogenetic trees of the combined cpDNA data set were constructed using the Maximum likelihood (ML) and Bayesian inference (BI) methods. The ML tree was constructed using IQ-TREE 2 [35] with the K3Pu + F + I + G4 model by ModelFinder based on Akaike information criterion (AIC) [36]. To calculate maximum likelihood bootstrap values (BSML), 1000 replicates were run under the same criteria. BI analysis was performed with MrBayes 3.1.2 [37] and the K3Pu + F + G4 model recommended by ModelFinder based on Bayesian information criterion (BIC) [36]. Two simultaneous runs were performed with four chains. Each chain had 1,000,000 generations and was sampled every 100 generations. The first 25% of the samples were discarded as burn-in and the others were used for the calculation of the majority-rule consensus tree. Then, Tracer ver.1.4 was used to make a convergence test.

3. Results

3.1. Morphological Observation

The spores of the specimen (*Yan 1706Y021*) are trilete and tetrahedral-globose (Figure 1). The equatorial and polar diameters are 35.2 ± 0.9 and 25.9 ± 1.7 μm, respectively. The perispore shows the following two distinct layers: the inner layer, which is irregularly reticulated with small areolae, and the outer layer, which is granular (Figure 1g,h).

By comparing the costal grooves between rachis and pinna rachis of *Microlepia* and the other clade of *Dennstaedtia*, we found that the rachis of *Microlepia* were unconnected with the pinna rachis (Figure 2B). This morphological characteristic can be used as one of the species boundaries between *Microlepia* and *Dennstaedtia*.

3.2. Molecular Phylogenetic Analyses

We used the four-gene (*rbcL*, *trnL-F*, *psbA-trnH* and *rps4*) combined matrix to reconstruct the phylogenetic tree. The combined matrix was 3320 bp long and included 1043 variable sites, 753 of which were parsimony-informative. The topologies of the ML and BI trees were consistent with one another, but some branches had different statistical values (Figure 2A). The results showed high support for the nesting of the two specimens (*Yan 1706Y008*, *Yan 1706Y021*) in *Microlepia* (BSML = 100%; Bayesian posterior probabilities (PPBI) = 1.0). The position of this species is not well-resolved, and it is found to be sister to *M. todayensis*, *M. hancei* and *M. speluncae* with weak support values (BSML = 78%; PPBI = 0.79).

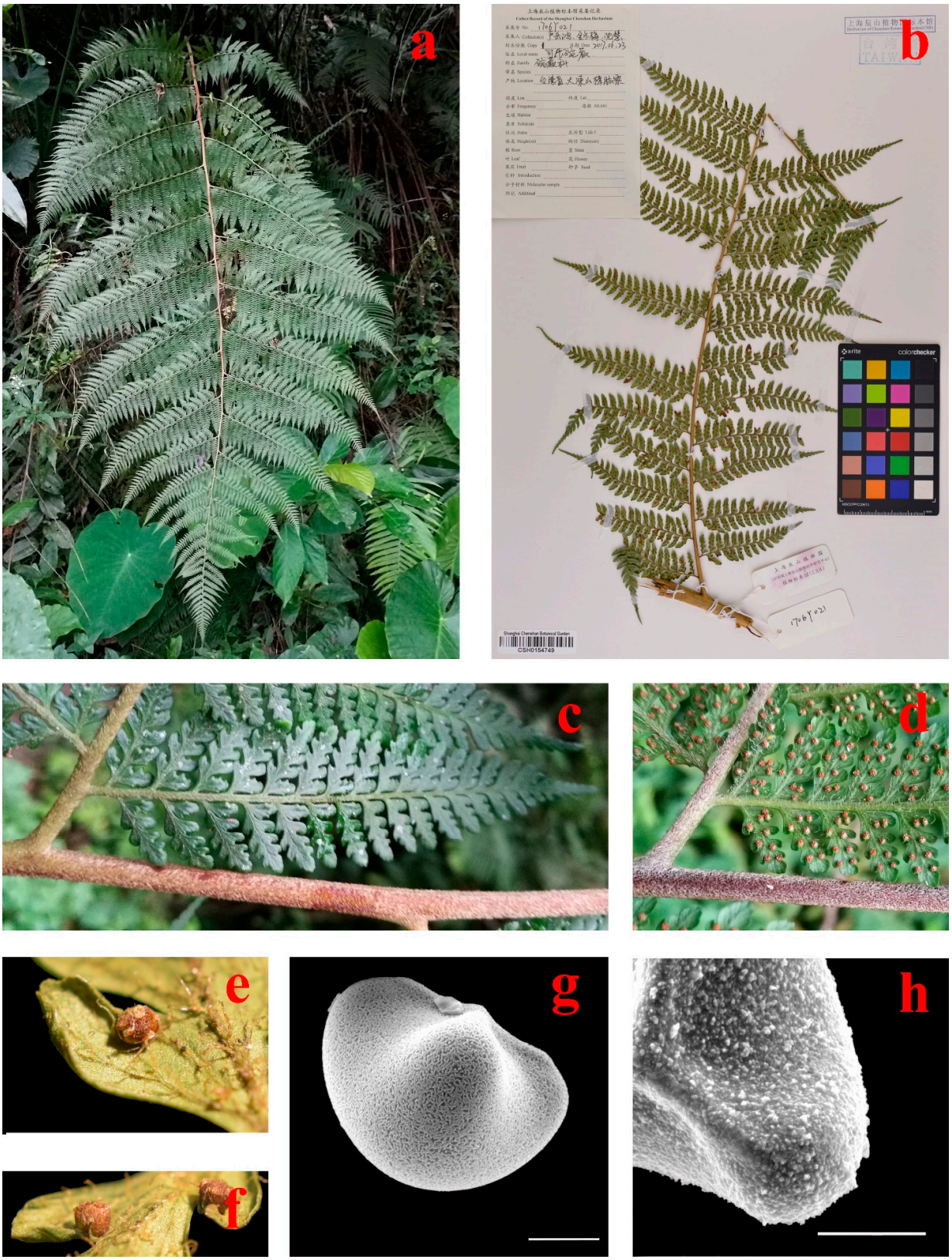

Figure 1. Morphological characters in *Microlepia smithii*. (**a**): *M. smithii* in the forest; (**b**): Herbarium specimen of *M. smithii*; (**c**): Ultimate-pinnule in live specimen; (**d**): Position and shape of sori in life specimen; (**e**,**f**): Indusia in dried herbarium specimen; (**g**): SEM of equatorial view of spore showing inner perispore reticulation with small areolae; (**h**): SEM of outer perispore showing granular. Scale bar in (**g**,**h**) = 10 μm.

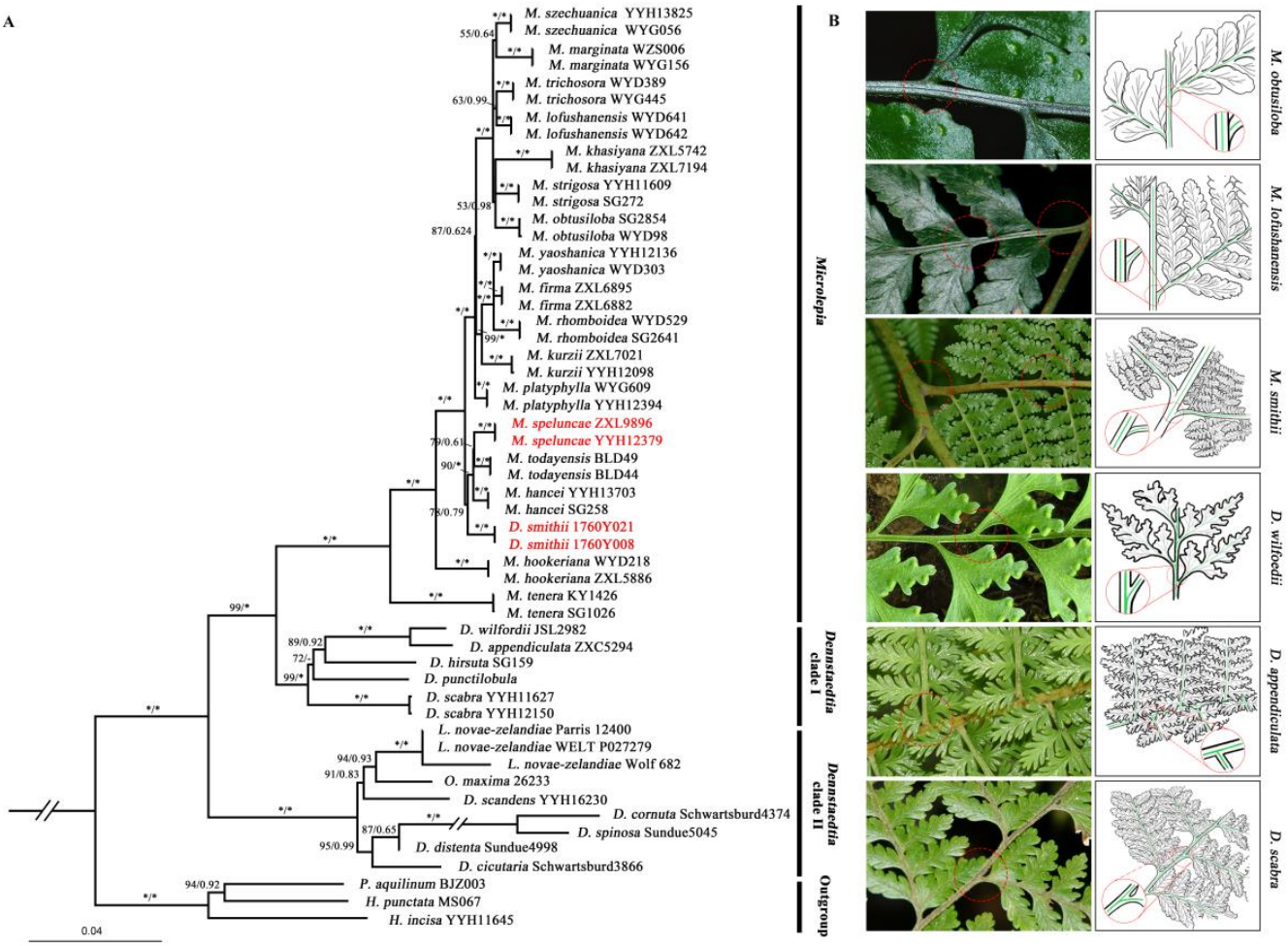

Figure 2. (**A**): Phylogenetic tree from ML and BI analyses of combined data from four chloroplast regions (*rbcL*, *rps4*, *psbA-trnH* and *trnL-F*). Both analyses have the same topology. *Dennstaedtia smithii* and *Microlepia speluncae* (the type of *Microlepia*) are marked in red text. Support values beside each node represent bootstrap support for ML (BSML) followed by posterior probabilities for BI (PPBI). Asterisks (*) indicate BSML = 100% and PPBI = 1.0. (**B**): The connection of costal grooves between rachis and pinna rachis of Microlepia and Dennstaedtia.

3.3. Taxonomy

Microlepia smithii (Hook.) Y.H. Yan, comb. nov.

Dicksonia smithii Hooker: Species Filicum 1: 80. 1846

Dennstaedtia smithii (Hooker) T. Moore: Index Filicum 308. 1861

Dennstaedtia formosae Christ: Bulletin de l' Herbier Boissier, sér. 2, 4(7): 617. 1904

Culcita formosae (Christ) Maxon: Journal of the Washington Academy of Sciences 12: 456. 1922 Basionym

Type. Luzon, Manilla, *Cuming*, n. 108, 145 and 222 (Isosyntype: RBGE-E00348832!, RBGE-E00348833!, NBC-L0051505!, NBC-L0051509!).

Distribution: China (Taiwan), Indonesia (Sulawesi), Philippines (Mindanao, Calabarzon)

Additional specimens examined: China, Taiwan, Chiayi, 23 June 2017, *Yan 1706Y021*, *Yan 1706Y008* (CSH); Taitung, ChengKung, 4 March 2002, 172660 (TAIF). **Indonesia**, Sulawesi, 16 May 1979, 3101538 (US National Herbarium); **Philippines**, Mindanao, Zamboanga, San Ramon, 12 February 1905, 1190334 (University of Michigan Herbarium); Calabarzon, Rizal, Luzon, 1 January 1907, 2987818 (US National Herbarium).

4. Discussion

4.1. Molecular Systematics and Morphological Analysis Support Dennstaedtia smithii Belongs to Microlepia

According to previous studies, *Microlepia* and *Dennstaedtia* differ in perispore characteristics: the perispore of *Microlepia* shows distinct two layers, the inner layer is irregular reticulation, while the outer layer is capillate, sericate [9,18,19,21] or verrucae [20]; the perispore of *Dennstaedtia* is composed of one or two layers, which are often verrucae or tuberculate and sometimes coarsely ridged to reticulate [12,18]. Observing the spore micromorphological characteristics of *D. smithii*, we found that the perispore has two layers, and the inner-perispore exhibits irregular reticulation (Figure 1g,h). By comparison of the spores' ornamentation (Figure 1g,h, [8,9,12,18–24]) and the observation result of the connection of costal grooves between rachis and pinna rachis (Figure 2B), it can be seen that *D. smithii* is similar to those in *Microlepia* rather than in *Dennstaedtia*. Moreover, the molecular systematics results also supported that *D. smithii* (*Yan 1706Y008*, *Yan 1706Y021*) was included in the *Microlepia* clade (Figure 2). Thus, based on the results of morphology and molecular systematics, we transferred *D. smithii* from *Dennstaedtia* to *Microlepia* and renamed *Microlepia smithii* (Hook.) Y.H. Yan.

4.2. Redefining the Distinguishing Morphological Characteristics of Dennstaedtia and Microlepia

In previous studies, the position of sori or the indusium shape of *Dennstaedtia* have been used to distinguish the genus from *Microlepia*. In *Microlepia*, the cup-shaped or half-cup-shaped indusium usually attaches to the base or on the side, and only the outer edge is free; in *Dennstaedtia*, the bowl-shaped indusium attaches to the base only and usually reflexes at maturity [25,38]. This is why *M. smithii* was generally regarded as a member of *Dennstaedtia* [7,26,38,39]. In fact, the sorus position and indusium form tend to blur the classification boundaries between genera and may be not applicable to some species of *Microlepia*, such as *M. smithii*. Therefore, we redefine the distinguishing morphological characteristics of *Microlepia* and *Dennstaedtia*.

Spore characteristics, such as spore ornamentation, are relatively conserved traits in ferns [12,24,27]. A two-layered perispore and a reticulate inner-perispore are the common traits in *Microlepia* (Figure 1g, h, [8,9,12,18–24]), and it was inferred as a synapomorphy for this genus [9,20,21]. According to the above morphological analysis results, we found that spore ornamentation and the connection of grooves between rachis and pinna rachis were relatively reliable distinguishing character between *Microlepia* and *Dennstaedtia*.

4.3. Finding Key Morphological Traits with Consistent of Molecular Systematics

For hundreds of years, botanists used morphology, or overall appearance, to identify and classify species [40]. However, due to subjectivity or artifact, it was easy to produce wrong reports, or the selected morphological feature was not the critical dividing line. With the development of open science and technological innovation, using molecular biology techniques and shared data in the study of taxonomy and systematics have become a crucial component of plants. Having genetic characterization at the disposal of researchers has produced mostly useful, and arguably more objective, conclusions than those only based on morphological characters [41]. The advantage of this method is that it can reduce the error caused by subjectivity or artifact and establish a more natural classification framework.

In the past, people thought that sporangium and indusium were the key traits for the division of genera; therefore, the taxonomic status of *Microlepia smithii* had been classified in *Dennstaedtia* [13–16]. However, with the help of molecular systematics, we found that *M. smithii* belongs to *Microlepia* not to *Dennstaedtia*. According to this result, we searched again for key traits between *Microlepia* and *Dennstaedtia*, in order to make the morphological classification of *Dennstaedtia* more natural. For the taxa whose morphology is difficult to define or whose genera relationships are complex, we encourage the use of stable phylogenetic results for detecting key characteristics of the study group, thus reducing erroneous revision.

4.4. Open Science and Technological Innovation Are Accelerating the Discovery of Hidden Outliers in Taxonomy

Open science and technological innovation have promoted the co-development of different disciplines, including taxonomy. We can obtain global specimens and data from virtual herbarium (e.g., CVH, GBIF, JSTOR), plant photo bank (e.g., PPBC, CUGB, GBIF, Ferns) and obtain genetic data of different species from molecular databases (e.g., NCBI, CNGBdb), which greatly facilitates the taxonomic processing of target taxa. However, among the tens of thousands of species on Earth, how to quickly find the hidden outliers requires more technology and standards. For example, to make digital specimens truly digital, the standard of species description and corresponding detailed data should be unified, such as the morphology, size and proportion of plants, leaves, pinnae, scales, sporangia, spores, pollen and fruit. We can use this digitized information to initially identify the 'outliers' of a taxa by programming language (e.g., python, perl, java, C++) and verify them through the material and molecular biological technique. At the same time, we can also use the shared molecular data and computer language to automatically search for the groups with obvious conflicts or low support in the phylogenetic structure, and re-expand the sample according to the results to find the natural taxonomic boundaries that are consistent with the phylogeny and morphology.

Technological advances allow for unprecedented taxonomic approaches [42], and the integration of artificial intelligence methods to guide species delimitation analyses will enable the faster implementation of natural systems of taxonomy, which may be the trend of the taxonomy of the future.

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