Article

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, pbsA-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

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* [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 | SC272 | Jiangxi, China | CSH | MK051745 | MK051993 | MK052254 | MK052254 |
| 2   | M. strigosa | YHH11609 | Taiwan, China | CSH | MK051843 | MK052010 | 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 | WYD98 | 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 | MK051835 | MK052110 | MK052662 | MK052386 |
| 10  | M. trichosora | WYD389 | Guangdong, China | CSH | MK051829 | MK052091 | MK052635 | MK052359 |
| 11  | M. marginata | WYD098 | Guangdong, China | CSH | MK051771 | MK052024 | MK052563 | MK052286 |
| 12  | M. marginata | WYD303 | Guangdong, China | CSH | MK051677 | MK051926 | MK052456 | MK052176 |
| 13  | M. szechuanica | ZXL5742 | Yunnan, China | CSH | MK051616 | MK052063 | MK052601 | MK052325 |
| 14  | M. szechuanica | ZXL7194 | Yunnan, China | CSH | MK051627 | MK052087 | MK052625 | MK052349 |
| 15  | M. rhomboidea | YXY11609 | Taiwan, China | CSH | MK051843 | MK052010 | MK052649 | MK052373 |
| 16  | M. rhomboidea | YXY11609 | Taiwan, China | CSH | MK051843 | MK052010 | MK052649 | MK052373 |
| 17  | M. raouliana | ZXL5742 | Yunnan, China | CSH | MK051616 | MK052063 | MK052601 | MK052325 |
| 18  | M. raouliana | ZXL7194 | Yunnan, China | CSH | MK051627 | MK052087 | MK052625 | MK052349 |
| 19  | M. firma | ZXL5742 | Yunnan, China | CSH | MK051616 | MK052063 | MK052601 | MK052325 |
| 20  | M. firma | ZXL7194 | Yunnan, China | CSH | MK051627 | MK052087 | MK052625 | MK052349 |
| 21  | M. kurzii | ZXL5742 | Yunnan, China | CSH | MK051616 | MK052063 | MK052601 | MK052325 |
| 22  | M. kurzii | ZXL7194 | Yunnan, China | CSH | MK051627 | MK052087 | MK052625 | MK052349 |
| 23  | M. speluncae | ZXL09896 | Chiang Mai, Thailand | CSH | MK051763 | NA | MK052278 | MK052278 |
| 24  | M. speluncae | ZXL09896 | Chiang Mai, Thailand | CSH | MK051763 | NA | MK052278 | MK052278 |
| 25  | D. appendicula | ZXL5742 | Yunnan, China | CSH | MK051616 | MK052063 | MK052601 | MK052325 |
| 26  | D. appendicula | ZXL7194 | Yunnan, China | CSH | MK051627 | MK052087 | MK052625 | MK052349 |
| 27  | D. appendicula | ZXL5742 | Yunnan, China | CSH | MK051616 | MK052063 | MK052601 | MK052325 |
| 28  | D. appendicula | ZXL7194 | Yunnan, China | CSH | MK051627 | MK052087 | MK052625 | MK052349 |
| 29  | D. appendicula | ZXL5742 | Yunnan, China | CSH | MK051616 | MK052063 | MK052601 | MK052325 |
| 30  | D. appendicula | ZXL7194 | Yunnan, China | CSH | MK051627 | MK052087 | MK052625 | MK052349 |
| 31  | D. appendicula | ZXL5742 | Yunnan, China | CSH | MK051616 | MK052063 | MK052601 | MK052325 |
| 32  | D. appendicula | ZXL7194 | Yunnan, China | CSH | MK051627 | MK052087 | MK052625 | MK052349 |
| 33  | D. appendicula | ZXL5742 | Yunnan, China | CSH | MK051616 | MK052063 | MK052601 | MK052325 |
| 34  | D. appendicula | ZXL7194 | Yunnan, China | CSH | MK051627 | MK052087 | MK052625 | MK052349 |
| 35  | D. appendicula | ZXL5742 | Yunnan, China | CSH | MK051616 | MK052063 | MK052601 | MK052325 |
| 36  | D. appendicula | ZXL7194 | Yunnan, China | CSH | MK051627 | MK052087 | MK052625 | MK052349 |
| 37  | D. appendicula | ZXL5742 | Yunnan, China | CSH | MK051616 | MK052063 | MK052601 | MK052325 |
| 38  | D. appendicula | ZXL7194 | Yunnan, China | CSH | MK051627 | MK052087 | MK052625 | MK052349 |
| 39  | D. appendicula | ZXL5742 | Yunnan, China | CSH | MK051616 | MK052063 | MK052601 | MK052325 |
| 40  | D. appendicula | ZXL7194 | Yunnan, China | CSH | MK051627 | MK052087 | MK052625 | MK052349 |
| 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 N/A N/A |
| 53  | Hypolepis punctata        | MS067       | Hunan, China  | CSH       | MZ959182 MZ983431 MZ959178 MZ983427 |
| 54  | Histiopteris incisa      | YHY11645    | Hainan, China | CSH       | MZ959181 MZ983430 MZ959176 MZ983426 |

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’ Herbarie 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 morphological 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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