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REGULAR PAPER

Integrated taxonomy of the *Asplenium normale* complex (Aspleniaceae) in China and adjacent areas

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Abstract

The *Asplenium normale* D. Don complex comprises several taxa that are either diploid or tetraploid. The tetraploids are assumed to have originated from diploid ancestors by relatively recent autopolyploidization or allopolyploidization. Some of the diploids are readily recognized morphologically but most of the taxa have until now been placed into a single species. However, phylogenetic studies have challenged this treatment and emphasized the notion that the taxonomic treatment of this complex needs to be revised. An integrative taxonomic approach was employed to delimit species in the complex using cytological, morphological, and DNA sequence data. Initially, we employed a diploid first approach to establish a robust taxonomic framework. Special efforts were made to collect and identify the diploid progenitors of each polyploid lineage identified in the plastid DNA based phylogenetic hypothesis. A total of six distinct diploid species were identified. The distinctive nature of the six diploids is strongly supported by sequence differences in plastid DNA and nuclear loci, as well as by the results of morphometric analysis. Diagnostic morphological characters were identified to distinguish the six diploid species, resulting in their revised taxonomy, which includes two novel species, namely, *Asplenium normaloides* and *A. guangdongense*. Further studies to strengthen the taxonomic classification of all of the tetraploid taxa are warranted.

Keywords Aspleniaceae · *Asplenium* · Species complex · Morphometric · Taxonomy

Introduction

In vascular plants, species complexes are frequently generated as a consequence of cladogenesis events, resulting in an increase in the number of closely related diploid species and the establishment of polyploid species by allo- and autopolyploid speciation (Linder and Rieseberg 2004; Wood et al. 2009). These complexes arguably contribute a substantial number of species to some lineages of vascular plants, such as derived ferns (Barrington et al. 1989; Lovis 1978). However, the contribution to species diversity of polyploidy species is widely underestimated because these complexes often exhibit continuous or semi-continuous distribution of morphological variations that hinder species delimitation using morphological diagnostics. Thus, a lot of species complexes currently comprise a few morphologically distinct units and one or more morphologically highly variable species that each includes entities with different cytotypes. The taxonomic challenge of these species complexes may be best overcome by the application of an integrative approach (Riedel et al. 2013; Schlick-Steiner et al. 2010; Will et al. 2005) that combines morphological evidence

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(phenotype), cytological evidence (cytotype), and molecular evidence (genotype) in a conceptual framework aiming to identify each of the independent evolutionary units as a species (de Queiroz 2007). The application of this concept is theoretically straightforward but requires the assembly of large datasets to characterize all of the putative evolutionary units to the required degree. Thus, unnatural morphospecies continue to be used due to its unambiguously identifiable nature (Véla et al. 2015). Here, we justify the need to identify the diploid evolutionary units originating from cladogenesis events in a so-called "diploid first approach" (Beck et al. 2010) to obtain a framework that may be subsequently employed in establishing polyploid lineages.

The nearly globally distributed spleenwort genus *Asplenium* L., which consists approximately 700 species, is a species-rich fern genus (PPG1 2016; Schneider et al. 2017) that consists of various species originating via reticulate evolution involving hybridization and polyploidization (e.g., Chang et al. 2013; Schneider et al. 2004, 2005, 2017). Reticulate evolution has been documented mainly for spleenwort species in temperate climate zones, such as the Appalachian mountains in North America (e.g., Wagner 1954), Central and Mediterranean Europe (e.g., Bennert and Fischer 1993; Pinter et al. 2002), and New Zealand (Perrie and Brownsey 2005; Perrie et al. 2010; Shepherd et al. 2008), although studies have also shown evidence for reticulate evolution in Australia (Ohlsen et al. 2014a, b), Southeast Asia (Chang et al. 2013, 2014; Matsumoto 1975; Iwashina and Matsumoto 1994; Matsumoto et al. 2003), and the Neotropics (Dyer et al. 2012). These studies have shown not only the high frequency of reticulate evolution in spleenworts but they have also established methods and concepts to uncover the core processes that have shaped the diversity of spleenworts and the global distribution of polyploidy in these ferns (Schneider et al. 2017).

Here, we focus on the *Asplenium normale* complex (Chang et al. 2013, 2014; Fujiwara et al. 2017) that is nested in the black-stemmed rock spleenwort clade besides the *A. monanthes* complex, the *A. trichomanes* complex, the Hawaiian endemic *A. diellia* complex, and the highly isolated *A. viride* Huds. (Dyer et al. 2012; Schneider et al. 2004, 2005, 2017). The distribution of the *A. normale* complex extends from tropical East Africa, Madagascar, and the Mascarene Islands in northeastern Australia, New Guinea, and Solomon Islands in the south, Hawai'i of the tropical Pacific islands in the east, and central China and Japan in the north. Most recent floristic treatments (e.g., Lin 1999; Lin and Viane 2012; Roux 2009) recognize a broadly defined *A. normale* D. Don besides a few small segregates such as the Chinese endemic *A. kiangsuense* Ching & Y. X. Jin (Ching and Jin 1977; Wu 1989), the Japanese endemic *A. oligophlebium* Baker (Matsumoto et al. 2003), and the tetraploid Hawaiian endemic *A. hobdyi* W. H. Wagner (Wagner 1993). Other putative segregates are treated either as synonyms without further rank, such as *A. pseudonormale* W. M. Chu and X. C. Zhang (Zhu 1992), or accepted as subspecies, e.g., *A. boreale* (Ohwi ex Sa. Kurata) Nakaike as *A. normale* var. *boreale* Ohwi ex Sa. Kurata and *A. shimurae* (H. Ito) Nakaike as *A. normale* var. *shimurae* H. Ito. However, Chang et al. (2013) showed that this treatment does not coincide with the evolutionary history of the complex. This notion was supported by a recent study on Japanese occurrences that provide evidence for "cryptic speciation" (Fujiwara et al. 2017), but that study was challenged by the need to propose unknown diploid taxa. However, the hypothesis of existing but as yet undocumented diploid taxa is supported by the recent discovery of a diploid species endemic to Taiwan Island, *A. pifongiae* L.-Y. Kuo, F.-W. Li & Y.-H. Chang (Li et al. 2016).

The plastid DNA-based phylogenetic hypothesis supports not only the recognition of the morphologically distinct Japanese endemic *Asplenium oligophlebium* and the Chinese endemic *A. kiangsuense*, but also provides evidence for the recognition of *A. boreale* and *A. shimurae* as distinct from *A. normale* (Chang et al. 2013; Fujiwara et al. 2017; Li et al. 2016; Ohlsen et al. 2014a), which agrees with the concept established for the occurrence of this complex in Japan (Ito 1972; Iwatsuki 1995; Kurata 1963; Matsumoto 1975; Matsumoto et al. 2003; Nakaike 1992). The establishment of at least three distinct taxa, namely, *A. boreale, A. normale*, and *A. shimurae*, is supported by flavonoid composition and DNA sequencing studies (Chang et al. 2013; Ebihara et al. 2010; Fujiwara et al. 2017; Iwashina 2000; Iwashina and Matsumoto 1994; Murakami et al. 1999; Matsumoto et al. 2003). This three-species concept has not been widely adopted because of the perceived lack of unambiguous diagnostic morphological characteristics separating them, despite differences in pinnae shape and the presence and number of leaf buds. The broadly defined *A. normale* (e.g., Lin and Viane 2012) is also challenged by the observation of several different cytotypes; at least diploid and tetraploid have been recorded (Ghatak 1977; Matsumoto and Nakaike 1988; Wagner 1993; Wang 1988; Weng and Qiu 1988). Most recently, plastid DNA analyses have provided evidence not only for a previously overlooked species in Taiwan, namely, *A. pifongiae* (Li et al. 2016), but also for one still undescribed species distributed in SW China and NW Vietnam (Chang et al. 2013).

Here, we apply the diploid first approach to facilitate in the establishment of a natural classification for the *Asplenium normale* complex. This approach aims to determine the diploid species that are expected to be recognizable not only by their unique plastid DNA sequence variations, but also by morphological diagnostic characters. The approach assumes that the challenges in morphological identification are mostly caused by polyploids that show overlapping

morphological features with their diploid parents. Thus, morphometric analyses should allow differentiation of the diploid species, whereas the segregation of diploids and tetraploids may be achieved in morphometric analyses by incorporating characters distinguishing cytotypes, such as spore size (Erkt and Stech 2008). Another challenge to the diploid first approach is the prediction that none of the distinct plastid DNA clusters is unique to tetraploids. However, some major genotypes were reported only in tetraploids of the *A. normale* complex (Chang et al. 2013, 2014). To test these predictions, we expanded our existing dataset (Chang et al. 2013) by integrating relevant published data (Fujiwara et al. 2017; Li et al. 2016; Ohlsen et al. 2014a) and sampling novel crucial specimens, such as accessions obtained from the type locality of *A. pseudonormale*. We also obtained the DNA sequences of three regions of the likely maternally inherited plastid genome of these specimens (Vogel et al. 1998) and of two biparentally inherited single copy genes, namely, *LEAFY* and *pgiC*. Cytotypes were determined by spore size measurements, and for some specimens, by DNA C value estimation. Finally, we conducted morphometric analyses of the specimens. By integrating these data with the proposed species segregates, we establish a taxonomic framework for the *A. normale* complex for China and adjacent regions, particularly focusing on diploid taxa. Here, we follow the concept that cytotypes representing different ploidy levels are recognized as separate species (Beck et al. 2010).

Materials and methods

Plant materials

This study expanded the previously sampled accessions (Chang et al. 2013, 2014), particularly focusing on putative distinct taxa and undersampled areas in China and adjacent regions. Special efforts were taken to obtain accessions growing at the type locality of *Asplenium pseudonormale* at Dulongjiang, Yunnan Province of China. We also surveyed the south and southeast regions of China, such as Zhejiang, Guangdong, Guangxi, Guizhou and Yunnan Provinces, and Japan in the hope of discovering previously undetected diploid progenitors of tetraploid lineages, such as clades II and IV (Chang et al. 2013). In order to obtain a comprehensive geographical representation, we also included accessions of the *A. normale* complex from previous studies of Chang et al. (2013, 2014), Ohlsen et al. (2014a), Li et al. (2016) and Fujiwara et al. (2017). A total of 293 accessions representing 250 samples were incorporated into the DNA-based analyses (see Table S1 for further details). The selection of outgroup taxa reflects the reported phylogenetic relationships of the *A. normale* complex and closely related species (Schneider et al. 2004, 2005). All of the specimens that were available for morphological assessment were included in morphometric analyses (see Table S1 for further details).

Cytotype determination

Spores of all of the new accessions were examined with an Olympus BX-51 light microscope to check for aborted spores, determine the number of spores per sporangia, and measure spore size. Mature sporangia from each specimen were removed and ruptured with a needle tip. The number of spores per sporangia was counted, and the length and width of 25 randomly selected spores of each new accession were measured. Spore measurements were performed by focusing on the outer border of the exine (exospore) instead of the perine (perispore) because the latter showed irregular ornamentation. The presence of 64 spores per sporangium was considered as an indicator for the absence of apomictic reproduction (Dyer et al. 2012; Wagner and Chen 1965). The measurements were then assessed using standard statistical procedures. For a selected group of specimens, DNA ploidy levels (Suda et al. 2006) were determined by measuring DNA C values with an Accuri C6 Flow Cytometer (Accuri Cytometers, Inc., Ann Arbor, MI, USA) using standard protocols (Dolezel et al. 2007), and *Glycine max* (L.) Merr. Was employed as the internal standard, with a DNA C value of ca. 1.13 pg/C (http://data.kew.org/cvalues/). The obtained data were then compared to the previously recorded genome size of this complex (Chang et al. 2013; Clark et al. 2016; Li et al. 2016).

Chloroplast and nuclear-DNA sequencing

DNA was extracted from all of the samples and was then used for sequencing following the previous procedure (Chang et al. 2013). The plastid DNA markers *trnL-trnF* (*trnL* intron and *trnL-trnF* intergenic spacer), *trnG-trnR* (*trnG* intron and the *trnG-trnR* intergenic spacer), and *rps4-trnS* (partial *rps4* gene and *rps4-trnS* intergenic spacer) of the new collections were sequenced. These chloroplast regions were selected because these are useful in detecting relationships among diploid taxa belonging to the *Asplenium normale* complex. Fern-specific amplification primers were developed for low-copy nuclear regions, including *gapCp* (Schuettpelz et al. 2008), *pgiC* (Ishikawa et al. 2002), and *LEAFY* (*LFY*) (Shepherd et al. 2008). However, the currently available primer set for *gapCp* did not successfully amplify some samples of the *A. normale* complex (Chang et al. 2013; Schneider et al. 2013); therefore, *trnL-trnF, trnG-trnR*, and *rps4-trnS*, as well as nuclear *pgiC* and *LFY*, were selected for this study. The plastid genomes of spleenworts are likely maternally inherited (Vogel et al. 1998), and nuclear genome markers are biparentally inherited. Given the complexities

of data generation, not all of the samples used in chloroplast phylogenetic analyses were included in the nuclear dataset.

All of the loci were PCR amplified on a MyCycler thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). PCR reaction mixtures and primers for chloroplast and nuclear *pgiC* were as described by Chang et al. (2013). The nuclear region of *LFY*-encompassing part of Exon 2 followed by a region of intron 2, was amplified with the primers leafyF and leafyR (Shepherd et al. 2008). The PCR thermocycling conditions for *LFY* were as described by Shepherd et al. (2008). The PCR products were electrophoresed on a 1.0% agarose gel in TBE buffer and then excised and purified using a DNA Purification Kit (TianGen Biotech, Beijing, China) following the manufacturer's protocols, eluted with 30 µl of elution buffer. The purified PCR products were either used for direct sequencing (for the cpDNA fragments) or ligated into a pGEM-T Vector (for nuclear genes) with a Promega Kit (Promega Corp., Madison, WI, USA). For each of the three chloroplast regions, identical sequences of specimens from the same location were reduced to a single accession per population and deposited in GenBank (see Table S1 for accession numbers). To sequence nuclear regions, direct sequencing was first conducted using samples of the *A. normale* complex. Samples with low or no *pgiC* or *LFY* allelic variations as indicated by direct sequencing were not subjected to cloning; otherwise, all of the nuclear PCR products were cloned. Ten positive clones for each individual were randomly selected for sequencing. The plasmid was extracted with an Axyprep Kit (Axygene Biotechnology, Hangzhou, China), and the universal vector primer T7 was used in the sequencing reactions. Cycle sequencing was conducted with BigDye Terminator version 3.1 (Applied Biosystems, Foster City, CA, USA). The sequenced products were run on an ABI 310 genetic analyzer (Applied Biosystems).

For some accessions, up to five PCR products per individual were cloned for sequencing in an attempt to detect all of the homologous sequences. To account for false sequence variations attributable to PCR- and cloning-based sequencing errors and chimeras, unique substitutions found in only a single clone were ignored and consensus sequences for each individual were compiled. In this way, the unique alleles present in each individual were determined. Consensus sequences were used in all of the subsequent analyses of the nuclear dataset and deposited in GenBank (see Table S1 for accession numbers).

Sequence alignment and phylogenetic analysis

Sequences were edited using the Staden Package (Staden et al. 2000), automatically aligned with Clustal X (Thompson et al. 1997), and manually corrected in BioEdit v.7.0.1 (Hall 2004). Special attention was given to the detection of ambiguously aligned regions and putative sequencing errors. Three separate alignments were constructed, namely, a combined *trnL-trnF, trnG-trnR* and *rps4-trnS* chloroplast alignment, a *pgiC* allele alignment, and a *LFY* allele alignment. The allelic datasets of the two nuclear genes, namely, *pgiC* and *LFY*, were analyzed separately, whereas sequences of the three cpDNA fragments were combined because these are inherited together. Ambiguous indels were excluded, and unambiguous indels were coded and scored using GapCoder (Young and Healy 2003). Maximum parsimony (MP) analyses, maximum likelihood (ML) analyses and Bayesian inference (BI) were run for the three datasets using PAUP* v.4.0b10 (Swofford 2002), PhyML v.3.0 (Guindon et al. 2010) and MrBayes v.3.1.2 (Huelsenbeck and Ronquist 2001), respectively. The best-fit models for parameter-based analyses were selected using jModelTest (Posada 2008) with the Akaike information criterion (Akaike 1974).

Morphometric analyses

A total of 223 accessions were included in the morphometric analyses (Table S1). Four operational taxonomic units (OTUs), corresponding to the four plastid DNA lineages previously reported in the *Asplenium normale* complex (Chang et al. 2013) that have been commonly treated together under the name *A. normale*, were included in the morphometric analyses to test their morphological distinctiveness. Quantitative and qualitative characters considered to be of diagnostic value were scored and analyzed. A total of 30 characters, including 10 continuous and 20 binary, were documented and scored (Table S2). Macro- and micromorphological characters were studied using an Olympus BX-51 light microscope, whereas spore ornamentation was examined using a tabletop scanning electron microscope (ZEISS EVO LS 10) with spores sputter-coated with gold particles.

Cluster analysis (CA) for qualitative variables (for all of the specimens) was conducted using the cluster package in R (R Core Team 2017) based on a similarity matrix obtained with the simple matching coefficient and using the UPGMA clustering algorithm. Log-transformed quantitative characters were analyzed in the R package, and the relationships among samples were illustrated with a scatter-plot showing vectors estimates in principal components analysis, with the first and second principal components (PCA1 and PCA2, respectively) plotted on axes *x* and *y*.

Results

Ploidy analyses

The spores of all of the studied specimens were well-formed, and intact sporangia contained 64 spores that were then inferred as sexual reproduction. Specimens with spores

checked were sorted into two groups based on spore size. One group had smaller spore sizes (mean sizes 27–34 µm, Fig. 1). Another group had larger spore sizes (mean sizes 35–37 µm, Fig. 1). The spore size range of both groups was mostly consistent with the former estimation of diploid and tetraploid taxa in the *Asplenium normale* complex. DNA C values were determined for two specimens that were assumed to be diploids, namely, "Dulongjiang Yunnan, Chang1089" from the type locality of *A. pseudonormale* and "Ruyuan Guangdong, 390", which had spores with a mean size of 29 and 31 µm, respectively. The genome sizes of these two specimens were 8.05 and 8.26 times that of the internal standard. Because the genome size of the internal standard (*Glycine max*) is ca. 1.13 pg/C, the genome sizes of the two specimens with small spores were estimated as 9.1 and 9.34 pg/C, respectively, which is close to the value previously estimated for diploid taxa in the *A. normale* complex.

Chloroplast DNA phylogeny

The total length of the concatenated *trnL-trnF, rps4-trnS* and *trnG-trnR* alignment was 2,644 bp, and it contained 151 variable characters, of which 116 were parsimony informative. No significant conflict (bootstrap value > 70%) was detected among the topologies obtained by separate phylogenetic analyses of each region. The three phylogenetic analyses, Maximum parsimony, Maximum likelihood and Bayesian inference, of the combined chloroplast dataset recovered the same topology, including the monophyly of the *Asplenium normale* complex and five distinct clades, i.e., clades I (including two subclades: Ia and Ib), II, III, IV (including four subclades: IVa, IVb, IVc and IVd), and V (Fig. 2). Clades I, II, III and two subclades in clade IV (IVa and IVc) corresponded to the broadly defined *A. normale*, whereas subclades IVb and IVd corresponded to *A. oligophlebium* and *A. kiangsuense*, respectively. Clade V corresponded to *A. pifongiae*.

Samples from tropical Asia, such as the Philippines, Thailand, and Myanmar, Australia, Papua New Guinea, various parts of southern and southeastern China, Taiwan, and Japan were nested in clade I Samples from southeastern China, Japan, Hawai'i, Kenya, and Madagascar from Africa belonged to clade II Accessions from the type locality (Dulongjiang, Yunnan Province, China) of *Asplenium pseudonormale* were also classified under clades I and II. Samples from southeastern China (including Guangxi and Yunnan) and northern Vietnam were clustered in clade III, whereas those from southern China, Taiwan, and Japan were grouped as clade IV. Two sequences representing two samples from Taiwan formed clade V. Samples inferred as diploids were found in each of the five clades.

Nuclear DNA phylogeny

Analyses of the *pgiC* phylogeny included representatives of all of the taxa with different putative cytotypes in the *Asplenium normale* complex. No additive sites were detected in the *pgiC* sequences of the diploid samples. The three phylogenetic analyses, Maximum parsimony, Maximum likelihood and Bayesian inference, of the *pgiC* sequences recovered three main clades (clades A–C), with one comprising three subclasses originating from a polytomy (subclades C1–C3) (Fig. 3). Diploids in chloroplast clade IV, including specimens identified as *A. oligophlebium* (Japan, 102404, 029, 153, 164, 342) and a currently undescribed diploid species (Ruyuan Guangdong, 390) were nested in clade A. This clade comprised tetraploid specimens with chloroplast genomes belonging to chloroplast clades I and IV. Diploids in chloroplast clade III were nested in clade B together with tetraploids from chloroplast clade I. Clade C comprised three subclades (subclades C1–C3), with

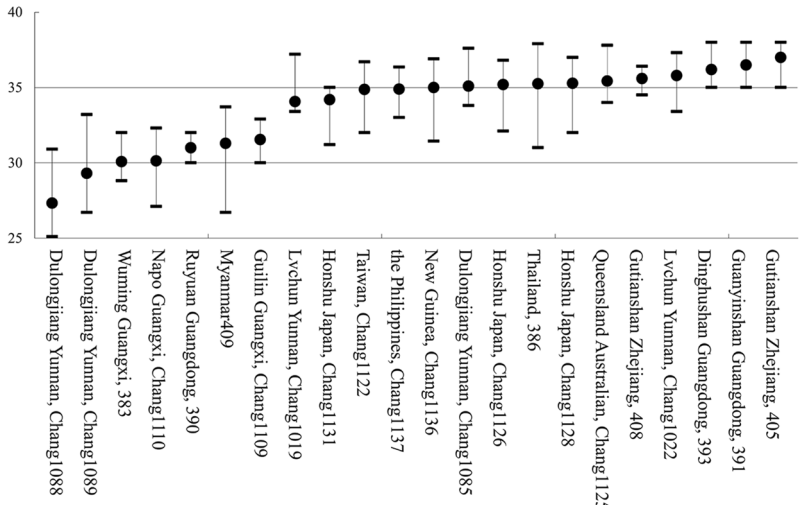
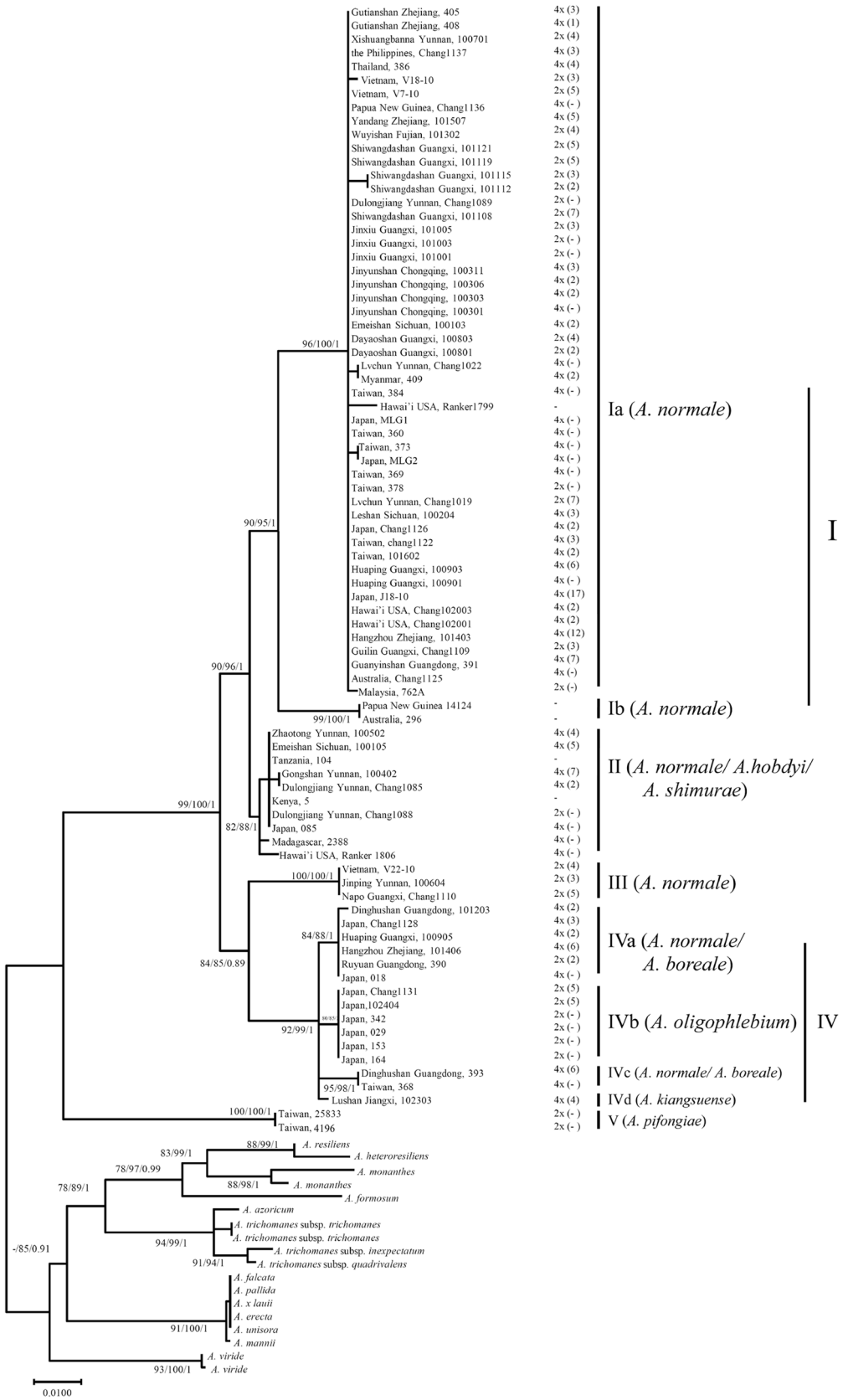


Fig. 1 Spore measurements taken from the new collection. Spore size arranged from the smallest to the largest. Dot = mean value and interval = maximum and minimum values measured


Gutianshan Zhejiang, 405
Gutianshan Zhejiang, 408
Xishuangbanna Yunnan, 100701
the Philippines, Chang1137
Thailand, 386
Vietnam, V18-10
Vietnam, V7-10
Papua New Guinea, Chang1136
Yandang Zhejiang, 101507
Wuyishan Fujian, 101302
Shiwangdashan Guangxi, 101121
Shiwangdashan Guangxi, 101119
Shiwangdashan Guangxi, 101115
Shiwangdashan Guangxi, 101112
Dulongjiang Yunnan, Chang1089
Shiwangdashan Guangxi, 101108
Jinxiu Guangxi, 101005
Jinxiu Guangxi, 101003
Jinxiu Guangxi, 101001
Jinyunshan Chongqing, 100311
Jinyunshan Chongqing, 100306
Jinyunshan Chongqing, 100303
Jinyunshan Chongqing, 100301
Emeishan Sichuan, 100103
Dayaoshan Guangxi, 100803
Dayaoshan Guangxi, 100801
Lvchun Yunnan, Chang1022
Myanmar, 409
Taiwan, 384
Hawai'i USA, Ranker1799
Japan, MLG1
Taiwan, 360
Taiwan, 373
Japan, MLG2
Taiwan, 369
Taiwan, 378
Lvchun Yunnan, Chang1019
Leshan Sichuan, 100204
Japan, Chang1126
Taiwan, chang1122
Taiwan, 101602
Huaping Guangxi, 100903
Huaping Guangxi, 100901
Japan, J18-10
Hawai'i USA, Chang102003
Hawai'i USA, Chang102001
Hangzhou Zhejiang, 101403
Guilin Guangxi, Chang1109
Guanyinshan Guangdong, 391
Australia, Chang1125
Malaysia, 762A
Papua New Guinea 14124
Australia, 296
Zhaotong Yunnan, 100502
Emeishan Sichuan, 100105
Tanzania, 104
Gongshan Yunnan, 100402
Dulongjiang Yunnan, Chang1085
Kenya, 5
Dulongjiang Yunnan, Chang1088
Japan, 085
Madagascar, 2388
Hawai'i USA, Ranker 1806
Vietnam, V22-10
Jinping Yunnan, 100604
Napo Guangxi, Chang1110
Dinghushan Guangdong, 101203
Japan, Chang1128
Huaping Guangxi, 100905
Hangzhou Zhejiang, 101406
Ruyuan Guangdong, 390
Japan, 018
Japan, Chang1131
Japan,102404
Japan, 342
Japan, 029
Japan, 153
Japan, 164
Dinghushan Guangdong, 393
Taiwan, 368
Lushan Jiangxi, 102303
Taiwan, 25833
Taiwan, 4196
A. resiliens
A. heteroresiliens
A. monanthes
A. monanthes
A. formosum
A. azoricum
A. trichomanes subsp. trichomanes
A. trichomanes subsp. trichomanes
A. trichomanes subsp. inexpectatum
A. trichomanes subsp. quadrivalens
A. falcata
A. pallida
A. x lauii
A. erecta
A. unisora
A. mannii
A. viride
A. viride
96/100/1
90/95/1
90/96/1
99/100/1
82/88/1
99/100/1
84/85/0.89
100/100/1
84/88/1
92/99/1
95/98/1
100/100/1
83/99/1
88/99/1
78/97/0.99
88/98/1
78/89/1
94/99/1
-/85/0.91
91/94/1
91/100/1
93/100/1
0.0100
4x (3)
4x (1)
2x (4)
4x (3)
4x (4)
2x (3)
2x (5)
4x (-)
4x (5)
2x (4)
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2x (7)
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4x (4)
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4x (7)
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2x (5)
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4x (2)
4x (6)
2x (2)
4x (-)
2x (5)
2x (5)
2x (-)
2x (-)
2x (-)
2x (-)
4x (6)
4x (-)
4x (4)
2x (-)
2x (-)
Ia (A. normale)
Ib (A. normale)
II (A. normale/ A.hobdyi/ A. shimurae)
III (A. normale)
IVa (A. normale/ A. boreale)
IVb (A. oligophlebium)
IVc (A. normale/ A. boreale)
IVd (A. kiangsuense)
V (A. pifongiae)
I
IV

◂**Fig. 2** Maximum likelihood phylogeny based on the concatenated plastid DNA sequence dataset. Maximum parsimony and Bayesian analyses recovered identical topologies with respect to the relationships among the main lineages of the *Asplenium normale* complex. For each node, the following values are provided: maximum parsimony bootstrap (%)/maximum likelihood bootstrap (%)/and posterior confidence (p value). Columns on the right refer to inferred ploidy level (2×, 4×), numbers of individuals with identical sequences in parentheses, clade abbreviation (I–V) and species names commonly accepted for each lineage. Outgroup taxa are shown as sister to the *Asplenium normale* complex

subclade C1 comprising both diploid and tetraploids from chloroplast clade II, subclade C2 consisting both diploids and tetraploids only from chloroplast clade I, and subclade C3 including diploids and tetraploids from chloroplast clade I and tetraploids from chloroplast clade II. The alignment of *LFY* sequences from the *A. normale* complex was 337 bp in length. Amplification and sequencing of inferred diploid samples produced DNA sequences with no additive sites. Additivity was observed with direct sequencing tetraploid samples, but only in a few sites. Inference of relationships among species of the *A. normale* complex based on *LFY* was limited by poor resolution of the phylogenies of the *LFY* cloned sequences. Thus, *LFY* was not informative in resolving relationships within the *A. normale* complex compared to the other DNA markers used in the present study, and thus it was excluded from further analysis and discussion (the sequences are deposited in GenBank, see Table S1).

Morphometric analyses

UPGMA cluster analysis of qualitative characters of samples from four chloroplast clades, including two subclades, namely, chloroplast clades I–IV (including subclades IVa/IVc), resulting in a dendrogram consisting of four distinct clusters (Fig. S1). All of the samples included in the analysis were previously treated as *Asplenium normale* s.l. The four clusters corresponded to chloroplast clades I to IV as long as only diploid samples were considered. However, several tetraploids nested in chloroplast clade I were also observed in clusters II–IV besides cluster I. The principal coordinates analysis (PCoA) of quantitative characters of samples from the four chloroplast clades and subclade (chloroplast clades I–III and subclades IVa/IVc) in the *A. normale* complex yielded similar results. The specimens formed four groups corresponding to chloroplast clades I–III–IVa/IVc as long as only diploids nested in chloroplast clade II–IV were considered (Fig. 4). Tetraploids nested in chloroplast clade I were located in different groups. The eigenvalues of the first three components represented 86.4% of the observed total variation; the ratios of pinnae length to width with the highest eigenvector 0.405, followed by the number of sori per pinnae with an eigenvector 0.268, and plant size with an eigenvector 0.191.

Discussion

Recognizable species in the *Asplenium normale* complex

According to the previously reported and the newly generated evidence, the *Asplenium normale* complex clearly includes several species (Chang et al. 2013; Fujiwara et al. 2017; Li et al. 2016). However, only three segregates have been widely recognized, namely the morphologically highly distinct *A. oligophlebium* endemic to Japan, *A. kiangsuense* endemic to southeastern China, and *A. pifongiae* endemic to Taiwan. Several other segregates have been earlier proposed before, such as *A. boreale, A. hobdyi, A. shimurae*, and the poorly understood *A. pseudonormale*. With the exception of the Hawai'ian *A. hobdyi*, these species have been recently treated as synonyms of *A. normale* (e.g., Lin 1999; Lin and Viane 2012; Roux 2009), although previously published evidence described these as distinguished units (see Chang et al. 2013; Fujiwara et al. 2017). The taxonomy of the *A. normale* complex is considered to be difficult because of the existence of not only diploid taxa, but also tetraploid taxa that originated from their diploid ancestors by either autopolyploidization or allopolyploidization, following the formation of hybrids between distinct diploids. In this study, we employed a diploid first approach to identify the evolutionary units of diploid taxa that can be reliably recognized using morphological characters before addressing the complicated classification of tetraploid taxa.

Up to six chloroplast lineages were identified in the *Asplenium normale* complex by Chang et al. (2013), including clades I–IV, and two distinct species, *A. oligophlebium* and *A. kiangsuense*, but only three of these included previously described diploids. Recently, Li et al. (2016) recognized a new diploid species, *A. pifongiae* from Taiwan. In this study, we also report diploids in lineages that exclusively contain tetraploids that were described in a previous study (Chang et al. 2013). A total of six diploids in five clades, including seven lineages recognized by chloroplast DNA-based phylogeny, are now documented as part of the *A. normale* complex (Fig. 2). The distinctiveness of the six diploids is strongly supported by chloroplast phylogeny and nuclear *pgiC* sequencing analyses as well as by the results of morphometric analysis (Figs. 2, 3, 4, S1). However, only two of the six diploids are widely accepted as species, namely, *A. oligophlebium* in a subclade in chloroplast clade IV (clade IVb) and *A. pifongiae* in chloroplast clade V. The other four diploids in chloroplast clades Ia, II, III, and IVa are all commonly designated as *A. normale*. This classification does

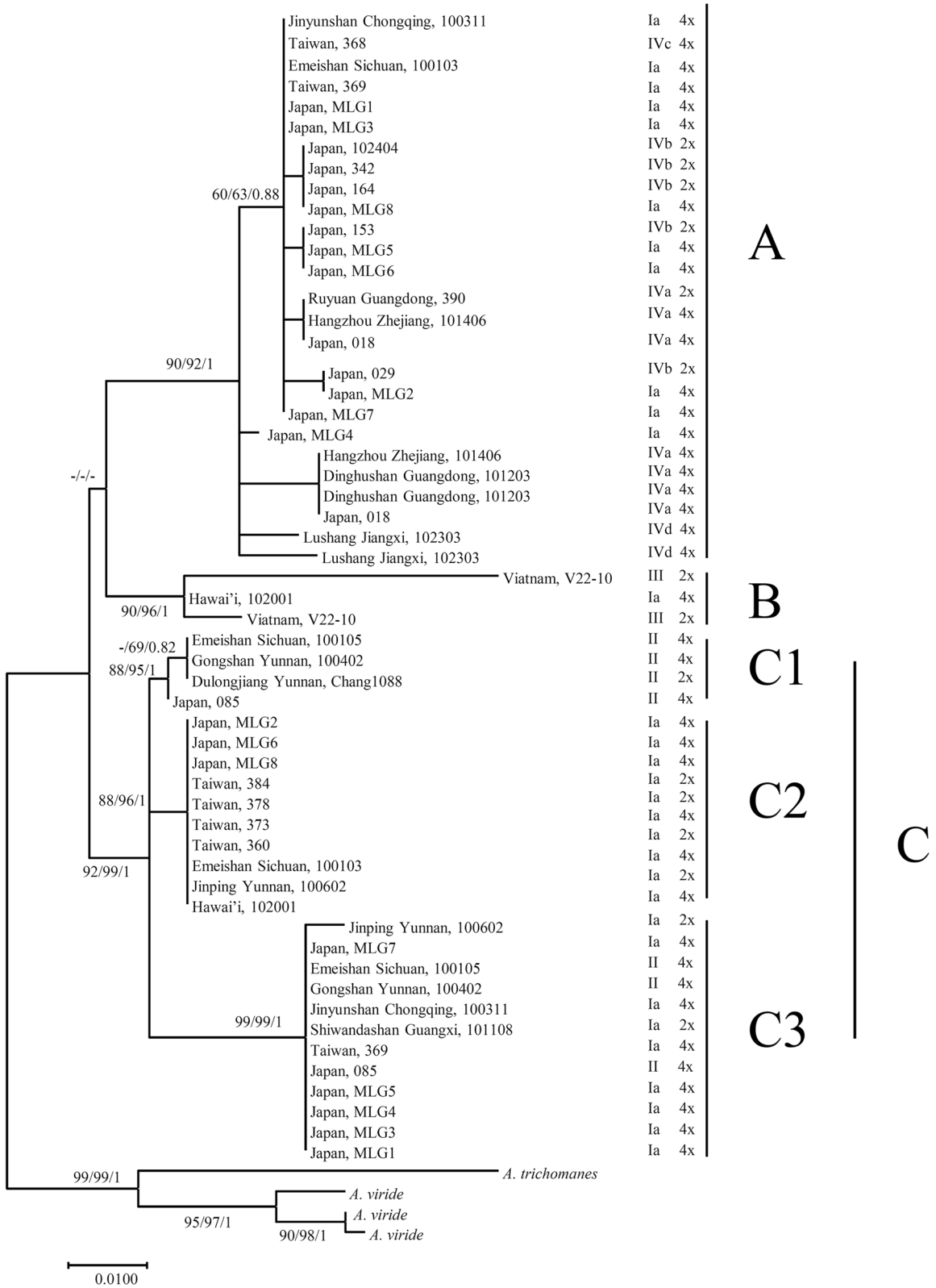

Fig. 3 Maximum likelihood phylogeny of the nuclear gene *pgiC* dataset. Maximum parsimony and Bayesian analyses recovered identical topologies. For each node, the following values are provided: maximum parsimony bootstrap (%)/maximum likelihood bootstrap (%)/ and posterior confidence (p value). Columns on the right refer to clade abbreviations obtained from the chloroplast phylogeny (Fig. 2) and inferred ploidy level (2×, 4×). Terminals with the same OTU name represent different sequences at the duplicated gene loci of the same accession

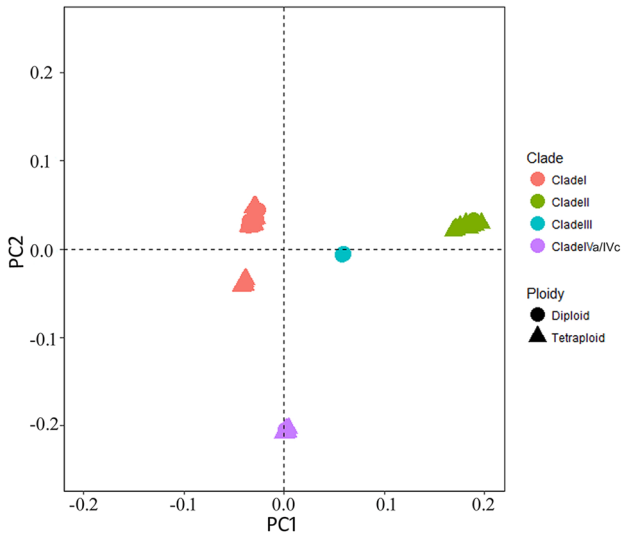

Fig. 4 Principal coordinates analysis (PCoA) results based on quantitative characters of four chloroplast clades commonly treated as *Asplenium normale*. Clade I, II and IVa/IVc include both diploids and tetraploids, whereas, clade III includes only diploids

not agree with the phylogenetic evidence and morphological distinctiveness of these diploid taxa. These six diploids found in the *A. normale* complex are thus proposed to be accepted as species in this study.

It appears premature to introduce new classifications for tetraploid taxa in the *A. normale* complex because these represent an assemblage of cytologically distinct units but with limited morphological and genetic separations. Multiple origins of tetraploids in the *A. normale* complex are supported by the observation of tetraploid individuals containing more than one divergent nuclear copy of each characteristic of diploids in different cpDNA lineages (Fig. 3). The available evidence supports the recognition of three distinct tetraploids that are nested within chloroplast clade IV. Tetraploids from chloroplast subclade IVd are identified as *A. kiangsuense*, which is morphologically distinct from the other members of the *A. normale* complex. Tetraploids from chloroplast subclades IVa and IVc are morphologically identical and are identified as *A. normale* or *A. boreale*. Currently available evidence is insufficient to erect robust working hypotheses concerning the origin of these three tetraploids that are nested in chloroplast clade IV. Chloroplast clade II is comprised at least two tetraploid species, *A. hobdyi* and *A. shimurae*, of distinct origin as indicated by the *pgiC* evidence. Chloroplast clade I comprises multiple tetraploids that exhibit distinct nuclear genotypes and phenotypes. Some of these may be interpreted as originating from hybrids between diploids of chloroplast clade I with diploids of chloroplast clade IV (Jinyunshan Chongqing, 100311; Emeishan Sichuan, 100103; Taiwan, 369; Japan, MLGs1-8) or diploids of chloroplast clade III (Hawai'i, 102001).

Taxonomic revision of recognized species in the *Asplenium normale* complex

Six diploids and at least four tetraploids in the five clades of the *Asplenium normale* complex may be recognized, but further studies are needed to determine the number of tetraploid evolutionary units. To assign the correct names and construct synonymies for these taxa in the *A. normale* complex, the morphology of each finally accepted taxon was compared to the protologue of the original species description and we extensively studied the type specimens of each species (Table 1).

Asplenium normale was assigned to diploids in clade I because the morphology of the lectotype of *A. normale* D. Don (Fraser-Jenkins 2008) is most similar to that of the specimens in clade I, i.e., one bud occurring on the rachis and similarities in pinnae shape and sori position (Fig. 5). However, both diploids and tetraploids coexist in this group. Thus, further evidence to confirm the ploidy level of the holotype specimen is warranted. Furthermore, the status of several names related to or synonyms of *A. normale* remain unclear, such as *A. minus* Blume from Java and *A. multijugum* Wall. Ex Mett. from Nepal. Additional studies on other varieties that are related to materials from Madagascar, such as *A. normale* var. *angustum* C. Chr. and its synonyms, e.g., *A. monanthes* L. var. *triangularipinnatum* Bonap. and *A. normale* var. *grandidentatum* Bonap., are thus necessary because we have observed that some of these are morphologically distinct from the type specimen of *A. normale*. For the moment, we recommend that the name *A. normale* be applied to both diploids and tetraploids with the plastid DNA of chloroplast clade I.

Asplenium pseudonormale was assigned to diploids in clade II, which have more than one bud occurring on the rachis. The number of sori is generally less than 5 (Fig. 5). These characteristics match the types and descriptions of *A. hobdyi, A. shimurae* and *A. pseudonormale*. However, we observed that the materials identified as *A. shimurae* and *A. hobdyi* are all tetraploids (Wagner 1993; Fujiwara et al. 2017). Spores from both type and topotype of *A. pseudonormale* are smaller (29 μm), which indicates that these are likely diploids. Thus, we interpret *A. pseudonormale* as the diploid species of chloroplast clade II. Currently, clade II includes tetraploids that are distributed in Afromadagascar, Japan, Hawai'i, and southwestern China. Additional studies are needed to clarify the status of the Hawaiian endemic *A. hobdyi* and *A. shimurae*, as well as the distribution of the latter.

Clade III is a semicryptic diploid lineage found in the *Asplenium normale* complex (Chang et al. 2013), with similarities to other members of the complex (Fig. 5). No previously described taxon has been associated with this clade. Thus, a new diploid species, *Asplenium normaloides* Yan

Table 1 Taxonomy of the *Asplenium normale* complex

Taxon	Synonyms	Type	Published by
A. boreale (Ohwi ex Sa. Kurata) Nakaike	*A. normale* var. *boreale* Ohwi ex Sa. Kurata	H. Sekimoto s. n., Japan, Tochigi, Mt. Kogashi. HT: TNS	Kurata (1963)
A. guangdongense Yan Fen Chang & Schneider		Zhang 390, China, Guangdong, Ruyuan, Nanling, Nature Reserve. HT: HITBC	
A. gulingense Ching & S. H. Wu		C. E. Devol s. n., China, Jiangxi, Lu Shan, Guling. HT: PE	Wu (1989)
A. hangzhouense Ching & C. F. Zhang		C.F.Zhang 7250, China, Zhejiang, Hangzhou, Jiuqi. HT: PE	Ching and Zhang (1983)
A. hobdyi W. H. Wagner		W. H. Wagner 87164, Hawaii. HT: MICH	Wagner (1993)
A. normaloides Yan Fen Chang & Schneider		Chang 1110, China, Yunnan, Jinping, Maandi. HT: HITBC	
A. kiangsuense Ching & Y. X. Jin	*A. gulingense* Ching & S. H. Wu, *A. parviusculum* Ching, *A. hangzhouense* Ching & C. F. Zhang	C. Q. Yuan et al. 75,014, China, Jiangsu, Yixing. HT: JSBI	Ching and Jin (1977)
A. multijugum Wall. ex Mett		Wallich N. 207, Nepal. HT: K	Mettenius (1859)
A. normale D. Don	*A. multijugum* Wall. ex Mett., *A. normale* var. *angustum* C. Chr	F. B. Hamilton s. n., Nepal. HT: BM	Don (1825)
A. normale var. *angustum* C. Chr.*		H. Perrier de la Bathie 15635, Madagascal. HT: P	Christensen (1932)
A. oligophlebium Baker		Mabies s. n., Japan. HT: K	Baker (1880)
A. pavonicum Brack.*		G. E. Davenport 01, Hawaii. HT: MICH	Brackenridge (1854)
A. parviusculum Ching		C. C. Wu s.n., China, Zhejiang, Hangzhou, Qingfong Shan. HT: PE	Ching and Zhang (1983)
A. pifongiae L.Y. Kuo, F.W. Li & Y.H. Chang		P. F. Lu 25833, Taiwan, Chiayi, Alishan Twonship, Alishan Forest Receeation Area. HT: TAIF	Li et al. (2016)
A. pseudonormale W. M. Chu & X. C. Zhang ex W. M. Chu		W. M. Chu & S. G. Lu 19,071, China, Yunnan, Gongshan, Dulongjiang, Qinlangdang. HT: PYU	Zhu (1992)
A. shimurae (H. Ito) Nakaike	*A. normale* var. *shimurae* H.Ito	Y. Shimura s. n., Japan, Shizuoka. Sakuma-machi, Kozuma. HT: TI	Ito (1972)

The list include all of the species names introduced for members of the complex with the following information: taxon name; common synonyms; type information, including collector, collector number, place of origin, and herbarium (herbarium acronym) deposited with focus on the holotype (HT); and finally the protologue publication. Species accepted in this study are printed in bold. Taxa names of uncertain status are marked by asterisks (*)

Fen Chang & H. Schneider, sp. nov., is introduced here. This taxon has only been reported in southwestern China, including SE Yunnan, SW Guangxi Provinces, and northeastern Vietnam (Sapa region). Future investigations on the distribution of this previously overlooked taxon, particularly in Laos and Vietnam, are thus warranted.

Two diploid species were classified under clade IV. *Asplenium oligophlebium* corresponds to the diploid in clade IVb. The specimens of this subclade are all from Japan and match the type and description of *A. oligophlebium* (Nakaike 1992). Its distribution is restricted to Japan. This species can be easily recognized based on its dissected pinnae shape (Fig. 5). A new species, *Asplenium guangdongense* Yan Fen Chang & H. Schneider sp. nov., is introduced here, which comprises diploids in clade IVa. The only previously described species associated with this clade, *A. boreale*, is considered to be a tetraploid. The distribution of this new species is currently known only from one locality in northern Guangdong, and further research is required to determine its distribution.

Asplenium pifongiae corresponds to clade V. It is a diploid species that was discovered in Taiwan and found to be a sister species of all of the other members of the *A. normale* complex (Li et al. 2016). We include this taxon as a member of the *A. normale* complex not only based on phylogenetic reconstruction, but also because of similarities in morphology, including pinnae shape, number of sori, and lack of buds attached to the rachis, which may be utilized

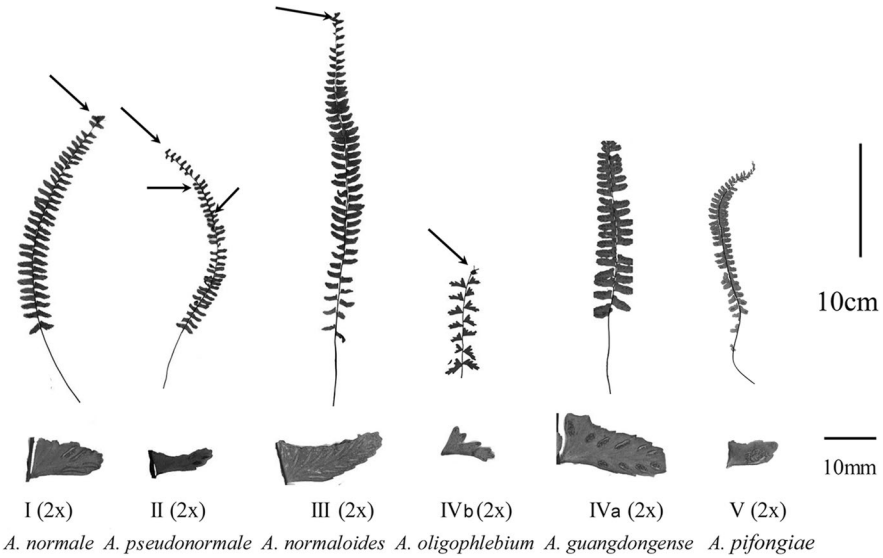

Fig. 5 Comparison of frond and pinnae sketches of representative specimens of the six recognized diploid species. Arrows indicate the position of the buds

as distinguishing features of this species and those nested in clade II (Fig. 5). This species has not been reported in any other region except for Taiwan.

Several tetraploid taxa are now distinguished from other closely related species. We hereby recognize four distinct species, namely, *Asplenium shimurae, A. hobdyi, A. boreale*, and *A. kiangsuense*. Several names related to *A. kiangsuense*, such as *A. gulingense* Ching & S. H. Wu, *A. parviusculum* Ching, and *A. hangzhouense* Ching & C. F. Zhang, were reduced to synonyms of *A. kiangsuense* because of their highly similar morphological features and distribution in eastern China (Lin 1999; Lin and Viane 2012). However, the taxonomic revision of tetraploid taxa in the *A. normale* complex requires further investigation, not only because the *A. shimurae* and *A. hobdyi* cluster in the same morphotype of chloroplast clade, namely, clade II, and the *A. boreale* and *A. kiangsuense* cluster in the morphotype of chloroplast clade IV, but also because tetraploids of the chloroplast clade I type likely represent distinct evolutionary units that were formed either by the hybridization of diploid *A. normale* with other diploid species or by autopolyploidization. Furthermore, we may also need to consider the formation of taxa by hybridization between sexual tetraploid species and the occurrence of triploid sterile crosses between diploids and tetraploids.

Taxonomic treatment

Asplenium normaloides Yan Fen Chang & Schneider, **sp. nov**.

The new species is most similar to *A. normale* D. Don, and differs by spores with lophate-perforate perispore and more but shorter sori on the pinnae.

Type: China. Yunnan Province, Jinping Xian, Maandi, Wutaishan Waterfall: Chang 1111. 2016. (holotype, HITBC; isotype, KUN) (Fig. 6).

Plants 20–45 cm tall. Rhizome erect, 3–4 mm in diameter, densely covered with scales; scales ovate-lanceolate, blackish brown, ca 2.5 mm × 0.7 mm, with median opaque zone, apex filiform. Fronds caespitose; stipe castaneous-brown, shiny, 10–20 cm long, 1–2 mm in diameter, terete to tri- or tetragonous, rachis adaxially with a deep furrow, always gemmiferous near apex. lamina linear-lanceolate, 20–30 cm × 2–4 cm, 1-pinnate; pinnae 20–30 pairs, alternate, sessile, middle pinnae trapeziform 10–18 mm × 4–6 mm, base asymmetric, acroscopic side truncate and subauriculate, basiscopic side narrowly cuneate, margin serrate, apex obtuse; basal pinnae often somewhat reflexed. Venation pinnate, single or forked. Sori 10–14 per pinna, linear, 1.2–2.5 mm long, median on subtending vein; indusium brown, semi-elliptic, membranous, entire, opening toward costa. Spore ornamentation with perforated cristae. Sporangia with 64 spores. Plants diploid.

Distribution and habitat: *A. normaloides* is only known in southeastern China and northeastern Vietnam. The new species grows in soil or on mossy rocks in dense forests at alt. 1000–2000 m. Collected specimens are often close but not within waterfalls.

Specimens examined (Paratypes): China. Yunnan Province, Jinping County, Fenshuiling Nature Reserve, 2010, Shugang Lu, 100604, 100605, 100606 (HITBC). Yunnan Province, Jinping Maandi, Wutaishan Waterfall, 2016, Yanfen Chang, Chang 1111, Chang 1112, Chang 1114, Chang 1115 (HITBC). Guangxi Province, Napo County, Baiyunshan Nature Reserve, 2015, Yanfen Chang, Chang 1110 (HITBC). Vietnam, Lao Cai Province, Ngan Lu Thien & Harald Schneider, V22-10 (BM).

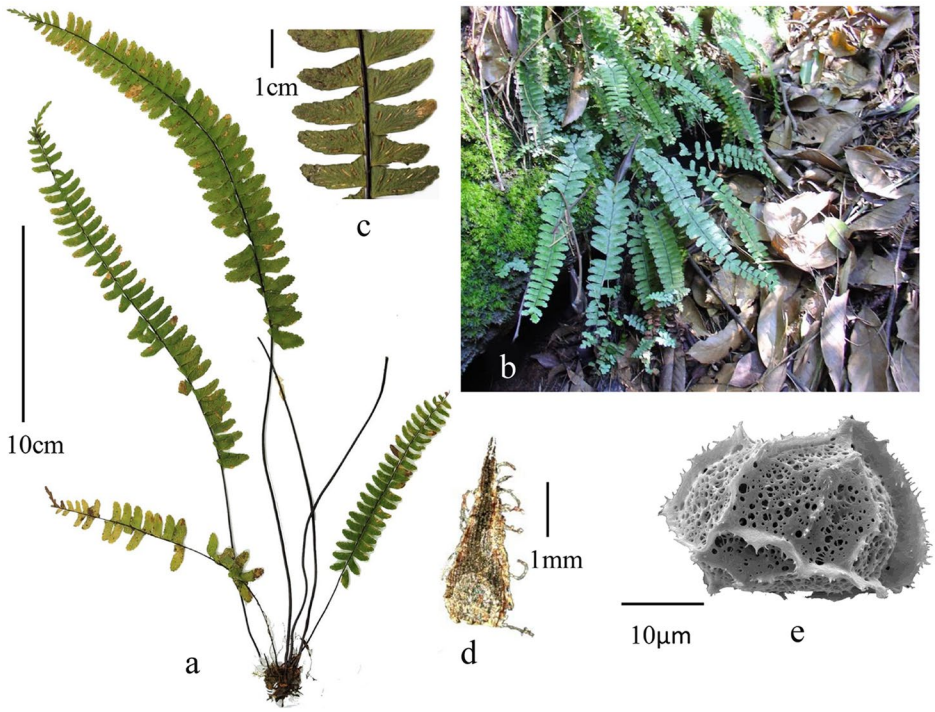

Fig. 6 *Asplenium normaloides*. **a** Plant; **b** habit; **c** middle part of leaf; **d** rhizome scale; and **e** spore

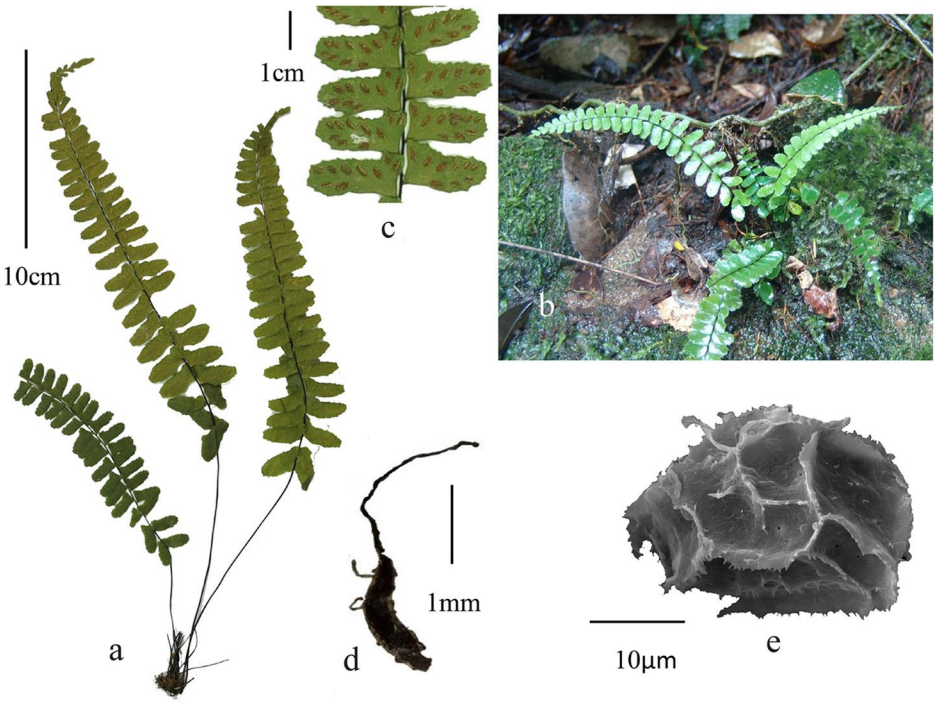

Fig. 7 *Asplenium guangdongense*. **a** Plant; **b** habit; **c** middle part of leaf; **d** rhizome scale; and **e** spore

Asplenium guangdongense Yan Fen Chang & H. Schneid., **sp. nov**.

The new species is similar to *A. normale* D. Don, but differs by having no buds on the rachis, broader pinnae with usually more sori, scales on rhizome dark brown with long fibrous apices, and spores with more crests.

Type: China. Guangdong Province, Ruyuan, Nanling, Nature Reserve: Zhang 390. 2016. (holotype, HITBC; isotype, KUN) (Fig. 7).

Plants 20–40 cm tall. Rhizome erect, 3–4 mm in diameter, densely covered with scale; scale lanceolate, blackish brown, with long fibrous apices, ca 4.1 mm × 0.6 mm,

apex filiform, margin fimbriate. Fronds caespitose; stipe castaneous-brown, shiny, 8–15 cm long, 1–2 mm in diameter, terete to tri- or tetragonous. rachis adaxially with a deep furrow. lamina linear-lanceolate, 15–30 cm × 2–4 cm, 1-pinnate; pinnae 20–30 pairs, alternate, sessile, middle pinnae trapeziform 10–18 mm × 4–6 mm, base asymmetric, acroscopic side truncate and subauriculate, basiscopic side narrowly cuneate, margin serrate, apex obtuse; basal pinnae often somewhat reflexed. Venation pinnate, single or forked. Sori 8–16 per pinna, linear, 1.0–2.0 mm long, median on subtending vein; indusium brown, semi-elliptic, membranous, entire, opening toward costa. Spores with crest perispores surface, 64 spores per sporangium. Plants diploid.

Distribution and habitat: *A. guangdongense* is only known in Guangdong Province of southern China. The new species grows on mossy rocks or soil in dense forests at alt. ca. 500 m.

Specimens examined (Paratypes): China. Guangdong Province, Ruyuan, Nanling, Nature Reserve, 2012, Guocheng Zhang, 403 (HITBC). This species is currently only known from the type locality.

Identification key for the *Asplenium normale* complex

1 Pinnae dissected……..***A. oligophlebium*** Baker

1 ' Pinnae subentire……...2

2 Fronds with no buds on the rachis…….…...…...3

3 Rachis adaxially without a deep furrow; spores with cristate perispores……..…… ……..***A. kiangsuense*** Ching & Jin

3 ' Rachis adaxially with a deep furrow; spores with lophate perispores…….……4

4 Sori less than 5 and mostly only on, and parallel to the basiscopic side of pinnae… ……..***A. pifongiae*** Li-Yaung Kuo, Fay-Wei Li & Yi-Han Chang

4 ' Sori normally more than 5 and on both basiscopic side and acroscopic sides…… 5

5 Mean spore size 27-32 μm…..….***A. guangdongense*** Yan Fen Chang & Schneider

5 ' Mean spore size 34-37 μm……...…….***A. boreale*** (Ohwi ex Sa. Kurata) Nakaike

2 ' Fronds with buds on the rachis……6

6 Frond buds at both the distal end and middle part of the rachis……..……7

7 Mean spore size 27-32 μm…….… ……......***A. pseudonormale*** W. M. Chu et X. C. Zhang ex W. M. Chu

7 ' Mean spore size 34-37 μm…….Tetraploids in clade II including ***A. hobdyi*** W. H. Wagner and ***A. shimurae*** (H. Ito) Nakaike

6 ' Frond buds only at the distal end of rachis……...8

8 Spores with highly perforate perispore; Sori 10–14 per pinna, scales with ciliate protrusions from margin; diploids only…….. ……...***A. normaloides*** Yan Fen Chang & Schneider

8 ' Spores with no or less perforate perispore; Sori less than 10 per pinna, scales with no ciliate protrusions from margin; diploids and tetraploids…***A. normale*** D. Don

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