SYSTEMATICS AND PHYLOGENY

Species diversity and reticulate evolution in the *Asplenium normale* complex (Aspleniaceae) in China and adjacent areas

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Abstract The *Asplenium normale* complex contains the widespread *A. normale* and several geographically restricted species: *A. boreale*, *A. hobdyi*, *A. kiangsuense*, *A. oligophlebium* and *A. shimurae*. The taxonomy of this group is unclear with some entities treated infraspecifically or as synonyms. Furthermore, the existence of diploids and tetraploids in this species complex is suggestive of reticulate evolution. In order to formulate a natural classification and to investigate the relationships in this complex, phylogenetic analyses of plastid and nuclear sequence data and inference of ploidy level were used to assess the distinctiveness of putative taxonomic units and their relationships. The *Asplenium normale* complex was recovered as a monophyletic group comprising six principal chloroplast lineages. The results support the hypothesis that *A. normale* s.l. contains several species. Based on our inferences, we outline an improved species classification recognizing three diploid and four tetraploid entities. Incongruence of chloroplast and nuclear phylogenies was interpreted to be a result of recurrent reticulation events in the *A. normale* complex.

Keywords Aspleniaceae; *Asplenium*; chloroplast DNA; genome size; *pgiC*; polyploidy; reticulation; taxonomy

Supplementary Material The alignment files were deposited in TreeBase (http://purl.org/phylo/treebase/phylows/study/TB2:S14444).

Received: 18 Jan. 2013; revision received: 22 Mar. 2013; accepted: 3 May 2013. DOI: http://dx.doi.org/10.12705/624.6

INTRODUCTION

Asplenium L. is one of the most species-rich fern genera with about 700 species (Schneider & al., 2004). It is also one of the most widespread fern genera and occurs in temperate and tropical regions of all continents, except Antarctica. Polyploidy is common in *Asplenium*, and both autopolyploidy and allopolyploidy may be driving forces of evolution in this fern group (Wagner, 1954; Lovis, 1978; Reichstein, 1981). The complex history of *Asplenium* makes it a model group for the study of fern evolution (Wagner, 1954; Lovis, 1978; Reichstein, 1981). However, as a consequence of hybridization and auto- and allopolyploidy, there are a large number of recognized species complexes with ambiguous boundaries between species (Herrero & al., 2001; Yatabe & al., 2001, 2009; Van den Heede & al., 2003; Yatabe & Murakami, 2003; Perrie & Brownsey, 2005; Dyer & al., 2012). Limited morphological diversity further complicates species delimitation in *Asplenium*. In the last decade, DNA sequencing studies, particularly comparisons of phylogenetic analyses of nuclear and chloroplast sequences among polyploids and their proposed progenitors, have revealed reticulate evolutionary patterns and resolved taxonomic challenges in some *Asplenium* species complexes (Van den Heede & al., 2003; Yatabe & al., 2001, 2009; Yatabe & Murakami, 2003; Dyer & al., 2012).

The *Asplenium normale* complex is one of the newly identified fern species complexes among the "black-stemmed" spleenwort group, which also includes the *A. monanthes* complex, the *A. trichomanes* complex, the *Diellia* complex and *A. viride* (Schneider & al., 2004, 2005; Dyer & al., 2012). The distribution of the *A. normale* complex ranges from East Africa, Madagascar and throughout the Indian Ocean, Southeast Asia, Malaysia, northern Australia, Pacific islands to Hawai'i in the east, and central China and Japan in the north. The taxonomy of the *A. normale* complex remains poorly understood and controversial. Most authors accept one broadly defined species, *A. normale* D. Don, but several local treatments accept segregates. For example, *A. normale* in Japan was treated as one species with three varieties based on slightly different morphologies: *A. normale* var. *normale*, *A. normale* var. *boreale* Ohwi ex Sa. Kurata, and *A. normale* var. *shimurae* H. Ito (Kurata, 1963; Ito, 1972; Nakaike, 1992; Iwatsuki, 1995). Subsequent studies

of Japanese *A. normale* recovered strong evidence for four species in the *A. normale* complex, i.e., *A. boreale* (Ohwi ex Sa. Kurata) Nakaike, *A. normale*, *A. shimurae* (H. Ito) Nakaike, and *A. oligophlebium* Baker (Matsumoto, 1975; Matsumoto & al., 2003). *Asplenium oligophlebium* is distinct in the dissection of its pinnae but otherwise very similar to other Japanese members of the complex. These four taxa—*A. oligophlebium* and the three taxa treated previously under *A. normale*—show distinct patterns in flavonoid composition (Iwashina & Matsumoto, 1994; Iwashina, 2000; Matsumoto & al., 2003). Further evidence for the separation of these four taxa was recovered by sequencing the chloroplast gene *rbcL* (Murakami & al., 1999; Ebihara & al., 2010). However, the latest edition of *Flora of China* (Lin & Viane, 2012) recognizes only *A. normale* and rejects suggestions that *A. boreale* and *A. shimurae* are distinct.

Phylogenetic studies have confirmed that the tetraploid Hawaiian endemic *A. hobdyi* W.H. Wagner (Wagner, 1993), which is morphologically similar to *A. normale*, is also a member of this complex (Schneider & al., 2005). This taxon was recovered as more closely related to an African sample than to a Hawaiian sample of *A. normale* (Schneider & al., 2005). Studies of this complex should also include *A. gulingense* Ching & S.H. Wu (Lin, 1999) and *A. kiangsuense* Ching & Jin (Lin & Viane, 2012) because of morphological similarities to *A. normale*. *Asplenium gulingense* was reduced to a synonym of *A. kiangsuense* in the English edition of *Flora of China* (Lin & Viane, 2012). There are currently no obvious morphological differences between taxa in the *A. normale* complex. Therefore, a phylogenetic study of this fern group is urgently needed to achieve a natural classification of the complex.

Diploid and tetraploid chromosome counts have been recorded for *A. normale* and its relatives (Ghatak, 1977; Matsumoto & Nakaike, 1988; Wang, 1988; Weng & Qiu, 1988; Wagner, 1993), and sterile hybrids have been reported among these entities (Matsumoto, 1975; Nakaike, 1992). This indicates the possibility of reticulate evolution in the *A. normale* complex, similar to that in temperate *Asplenium* species such as the Appalachian spleenworts (Wagner, 1954; Werth & al., 1985), the *Ceterach* complex (Pinter & al., 2002), New Zealand spleenworts (Perrie & Brownsey, 2005; Perrie & al., 2010), and the *A. trichomanes* complex (Lovis, 1978) which is closely related to *A. normale* (Murakami & al., 1999; Schneider & al., 2004, 2005).

In this study, we aim to identify the number of lineages that form the *A. normale* complex and clarify the relationships among these lineages. Sampling covered the distribution range of the whole complex, including China, East Africa (Tanzania), Madagascar, the Pacific Islands (Hawai'i), Japan, Indochina (Vietnam), and Malesia (Malay Peninsula). Chinese populations of the *A. normale* complex are particularly suited for an initial study of this group because they cover a continuous climatic gradient from tropical climates in the extreme south to temperate climates in the north. The genetic diversity and phylogeny of the complex were studied using four regions of the maternally inherited chloroplast genome (*trnL-trnF*, *rps4-trnS*, *trnG-trnR*, *rbcL*) that have been used in previous studies on intra-/interspecies differentiation in ferns (Gastony & Yatskievych, 1992; Vogel & al., 1998). In order to detect reticulation in the *A. normale* complex, we employed a biparentally inherited marker, the single-copy nuclear gene *pgiC*. The *pgiC* gene has recently been successfully used in several phylogenetic studies of hybridization in ferns as well as in flowering plants (Russel & al., 2010; Juslen & al., 2011; Kamiya & al., 2011; Dyer & al., 2012; Sessa & al., 2012; Wang & al., 2012). Additionally, ploidy levels of the samples were determined by measuring spore size (Barrington & Paris, 1986) and determining DNA C-values by flow-cytometric analysis of selected specimens (Dolezel & al., 2007).

■ MATERIALS AND METHODS

Plant materials. — Our samples comprehensively represent the geographical distribution, known morphological diversity and previous taxonomic treatments of the *Asplenium normale* complex. Species with small distribution ranges, such as *A. hobdyi* endemic to Hawai'i, *A. kiangsuense* endemic to central China, and *A. oligophlebium* endemic to Japan, were included in this study. The *A. kiangsuense* sample was obtained from Guling, China, where it had been reported as *A. gulingense*. Samples representing proposed segregates such as *A. boreale* and *A. shimurae* were also included. Unfortunately, we were unable to obtain fresh samples from the type locality of *A. pseudonormale* W.M. Chu & X.C. Zhang ex W.M. Chu which was treated as a species by Zhu (Zhu, 1992) but reduced to a synonym of *A. normale* in *Flora of China* (Lin, 1999).

Type specimens of each species in the *A. normale* complex were studied and distinguishing characters were defined (Table 1). Samples were identified using the limited morphological characteristics available (Table 1). However, because the lack of reliable morphological differences made it difficult to distinguish between the three varieties of *A. normale*, they were all included in *A. normale* s.l. Specimens of *A. kiangsuense* and *A. oligophlebium* were identified by their bud position and pinna shape (Table 1). *Asplenium hobdyi* was distinguished from Hawaiian *A. normale* based on the presence of buds at the apex and other parts of the rachis. However, this character state was also found in *A. normale* s.l. from Southeast Asia.

In total, 156 individuals of the *A. normale* complex from 25 localities were collected, mainly representing the diversity in China and adjacent regions such as Japan and Vietnam, but with specimens also from Hawai'i, Tanzania, Madagascar, and the Malay Peninsula (see Fig. 1 and Appendix 1 for further details). At least five specimens from each location were included in analyses where possible.

Ploidy analyses. — In ferns, spore diameter is considered a good indicator of ploidy level when comparisons are made among close relatives (Barrington & Paris, 1986). Spores of the *A. normale* complex from nearly all locations (Table 2) were examined with an Olympus BX-51 light microscope to look for aborted spores and to measure spore size. Mature sporangia from each specimen were removed and ruptured with a needle tip. The length and width of 25 randomly selected spores with exine were measured for each specimen (Table 2).

Table 1. Taxon name, common synonym, holotype (HT), followed by herbarium acronym, publication of species name, and three diagnostic characters of the *Asplenium normale* complex. *Asplenium boreale* and *A. shimurae* are usually considered varieties of *A. normale*. *Asplenium gulingense* is considered a synonym of *A. kiangsuense*.

Taxon	Synonym	Type	Published in	Buds	Pinnae	Lamina width
A. normale D. Don		*F.B. Hamilton s.n.*, Nepal. HT: BM	Prodr. Fl. Nepal.: 7. 1825	Present, one or two at the frond apex	Entire	>2 cm
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	New Fl. Jap. Pterid.: 839. 1992	Absent	Entire	>2cm
A. shimurae (H. Ito) Nakaike	*A. normale* var. *shimurae* H. Ito	*Y. Shimura s.n.*, Japan, Shizuoka, Sakuma-machi, Kazuma. HT: TI	New Fl. Jap. Pterid.: 839. 1992	Present, several at the frond apex and other parts of the rachis	Entire	>2 cm
A. oligophlebium Baker		*Mabies s.n.*, Japan. HT: K	Gard. Chron. 14: 494. 1880	Present, one or two at the frond apex	Dissected	>2 cm
A. gulingense Ching & S.H. Wu		*C.E. Devol s.n.*, China, Jiangxi, Guling, Mt. Lu Shan. HT: PE	Bull. Bot. Res. Harbin 9: 84. 1989	Absent	Entire	<2 cm
A. kiangsuense Ching & Y.X. Jin	*A. gulingense* Ching & S.H. Wu	*C.Q. Yuan & al. 75014*, China, Jiangsu, Yixing. HT: JSBI	Fl. Jiangsuensis 1: 465, f. 63. 1977	Absent	Entire	<2 cm
A. hobdyi W.H. Wagner		*W.H. Wagner 87164*, Hawai'i, East Maui. HT: MICH	Contr. Univ. Michigan Herb. 19: 63–82. 1993	Present, several at the frond apex and other parts of the rachis	Entire	>2 cm

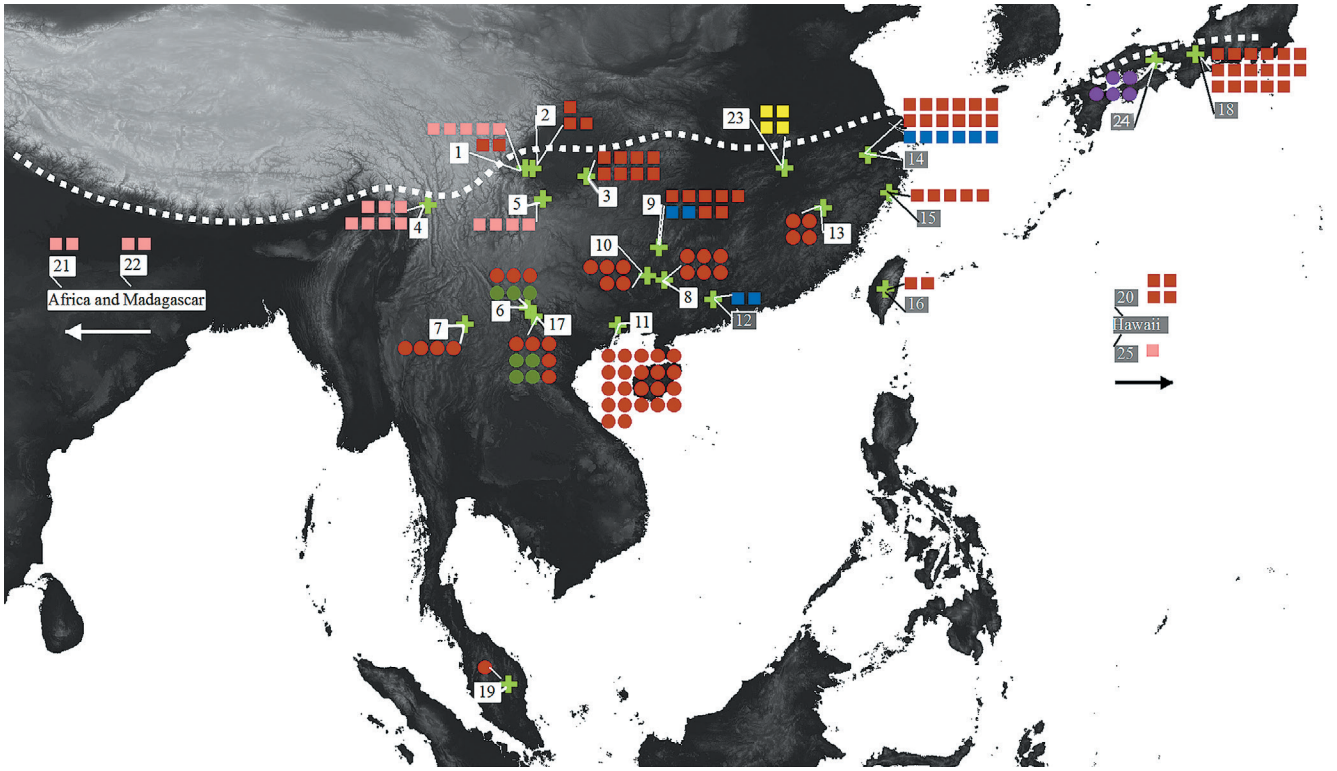

Fig. 1. Distribution of cytotypes and genotypes in the *Asplenium normale* complex in China and adjacent areas. White dotted line indicates the approximate northern distribution of the complex. Green crosses indicate sample locations. Each location is connected with a box showing location number and symbols corresponding to ploidy level and position in the chloroplast phylogeny. Symbol shape: circle = inferred diploid; square = inferred tetraploid. Symbol colour: red = clade I; pink = clade II; green = clade III; blue = clade IV; yellow = *A. kiangsuense*; purple = *A. oligophlebium*.

Table 2. Cytological examination of the *Asplenium normale* complex. Ploidy levels were inferred using spore and genome size.

Location no.	Sequence name	Mean spore diameter [µm]	Genome size [pg/C]	Inferred ploidy level
1	Mt Emei 1 China	35 (33–39)	18.2	4*x*
1	Mt Emei 2 China	35 (33–39)		4*x*
2	Leshan China	36 (35–40)		4*x*
3	Mt Jinyun 1 China	37 (33–41)		4*x*
3	Mt Jinyun 2 China	37 (33–41)		4*x*
3	Mt Jinyun 3 China	37 (33–41)		4*x*
3	Mt Jinyun 4 China	37 (33–41)		4*x*
4	Gongshan China	37 (33–43)		4*x*
5	Zhaotong China	37 (34–39)		4*x*
6	Jinping 1 China	31 (28–33)	9.8	2*x*
6	Jinping 2 China	31 (28–33)		2*x*
7	Xishuangbanna China	27 (24–31)		2*x*
8	Mt Dayao 1 China	31 (27–34)		2*x*
8	Mt Dayao 2 China	31 (27–34)		2*x*
9	Huaping 1 China	38 (34–44)		4*x*
9	Huaping 2 China	39 (34–45)		4*x*
9	Huaping 3 China	39 (35–44)		4*x*
10	Mt Shengtang 1 China	30 (27–32)		2*x*
10	Mt Shengtang 2 China	30 (27–32)		2*x*
10	Mt Shengtang 3 China	30 (27–32)		2*x*
11	Mt Shiwandashan 1 China	29 (26–35)		2*x*
11	Mt Shiwandashan 2 China	30 (29–35)		2*x*
11	Mt Shiwandashan 3 China	30 (29–35)		2*x*
11	Mt Shiwandashan 4 China	29 (26–35)		2*x*
11	Mt Shiwandashan 5 China	30 (29–35)		2*x*
12	Mt Dinghushan China	35 (31–42)		4*x*
13	Mt Wuyi China	32 (29–34)		2*x*
14	Hangzhou 1 China	38 (34–44)		4*x*
14	Hangzhou 2 China	38 (34–44)		4*x*
14	Hangzhou 3 China	38 (34–41)		4*x*
15	Mt Yandang China	34 (30–38)	18.4	4*x*
16	Is Taiwan	34 (31–37)	18.1	4*x*
17	HL 1 Vietnam	30 (27–34)		2*x*
17	HL 2 Vietnam	No spores		–
17	HL 3 Vietnam	30 (28–34)		2*x*
18	Honshu Japan	38 (35–40)		4*x*
19	Malaysia	29 (26–34)		2*x*
20	Hawai‘i 1	34 (33–40)		4*x*
20	Hawai‘i 2	35 (33–40)		4*x*
21	Mt Kilimanjaro Tanzania	No spores		–
22	Madagascar	35 (32–38)		4*x*
23	*A. kiangsuense*	37 (33–39)		4*x*
24	*A. oligophlebium*	30 (26–33)	9.1	2*x*
25	*A. hobdyi*	Not checked		–

In addition to spore measurements, DNA ploidy levels (Suda & al., 2006) were determined by measuring DNA C-values of specimens with accessible living leaf material (Table 2). DNA C-values were measured using standard protocols (Dolezel & al., 2007) with an Accuri C6 Flow Cytometer (Accuri Cytometers, Inc., Ann Arbor, Michigan, U.S.A.), and *Glycine max* (L.) Merr. as the internal standard with a DNA C-value of ca. 1.13 pg/C (http://data.kew.org/cvalues/). Freshly collected leaves were chopped using razor blades and transferred into a mixture containing calibration standard and 0.5 ml of tris-HCl buffer. The suspended nuclei were filtered through a 30 µm nylon mesh (Partec, Münster, Nordrhein-Westfalen, Germany). Finally, 100 µl of propidium iodide (PI) solution with 1.5 µl 0.1 mg/ml RNase A was added. Samples were kept on ice for 30 minutes. Approximately 5000 nuclei were measured in each run. The average 2C DNA content value was compared with data from the Plant DNA C-values database (http://data.kew.org/cvalues/), in particular with the measurements of tetraploid *Asplenium trichomanes* subsp. *quadrivalens* (Redondo & al., 1999; Bainard & al., 2011).

Chloroplast DNA sequencing. — For each sampled individual, genomic DNA was extracted from silica gel-dried leaves using a modified CTAB method (Doyle & Doyle, 1987). Four regions of the chloroplast genome were amplified using established primers and protocols. The *trnL-trnF* region including the *trnL* intron and the *trnL-trnF* intergenic spacer was amplified using the primer combination of Fern-1 (Trewick & al., 2002) and f (Taberlet & al., 1991). The *rps4-trnS* region including part of the *rps4* gene and the *rps4-trnS* intergenic spacer was amplified using the primers reported in Schneider & al. (2005). The *trnG-trnR* region including the *trnG* intron and the *trnG-trnR* intergenic spacer was amplified using primers reported in Grusz & al. (2009). The *rbcL* gene was amplified using the primers reported in Schneider & al. (2004). Extracted DNA products were amplified and sequenced following the protocols described in Schneider & al. (2004). Using diluted total DNA in 10 ng/µl as reaction templates, PCR amplification of each region was run for 35 cycles under the following conditions: 94°C for 45 s; 48°C (*trnL-trnF*), 52°C (*rps4-trnS*), 53°C (*trnG-trnR*), or 59°C (*rbcL*) for 45 s; and 72°C for 75 s. Primers of each region were used for cycle sequencing reactions with BigDye Terminator version 3.1 (Applied Biosystems, Foster City, California, U.S.A.). Each sample was sequenced using an ABI 310 genetic analyzer (Applied Biosystems). For each of the four chloroplast regions, identical sequences of specimens from the same location were reduced to a single exemplar sequence and deposited in GenBank (see Appendix 1 for accession numbers).

Nuclear DNA sequencing. — Initial sequences for the *pgiC* nuclear region were obtained via PCR amplification (35 cycles: 94°C for 45 s, 59°C for 45 s, 72°C for 75 s) using primers 14F and 16R (Ishikawa & al., 2002). PCR amplification conditions for *pgiC* were the same as for the chloroplast regions. The amplified region of *pgiC*

corresponds to part of exon 14, intron 14, exon 15, and intron 15 (Ishikawa & al., 2002). A single copy (~600 bp) of *pgiC* was recovered in this group of *Asplenium* ferns. PCR products were cleaned using Montage PCR Plates (Millipore Corporation, Billerica, Massachusetts, U.S.A.). The purified PCR products were ligated into a pGEM-T Vector with a Promega Kit (Promega Corporation, Madison, Wisconsin, U.S.A.). At least five positive clones from each putative diploid and ten from each putative tetraploid individual were randomly selected for sequencing. The plasmid was extracted using 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). The sequenced products were run on an ABI 310 genetic analyzer (Applied Biosystems).

In some cases considerably more PCR products of an individual were cloned for sequencing in an attempt to detect all homologous sequences. To account for false sequence variation attributable to PCR error 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 subsequent analyses of the nuclear dataset and deposited in GenBank (see Appendix 1 for accession numbers).

Sequence alignment and phylogenetic analyses. — Sequences of the four chloroplast regions and nuclear *pgiC* were edited and assembled using the Staden Package (Staden & al., 2000), aligned with Clustal X (Thompson & al., 1997) and manually corrected with BioEdit v.7.0.1. Ambiguous indels were excluded and unambiguous indels were coded and scored using Gapcoder (Young & Healy, 2003). Five datasets comprising the sequences from the four chloroplast regions and one nuclear gene were analyzed independently using maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI). Maximum parsimony analyses were carried out in PAUP* v.4.0b10 (Swofford, 2002) using the heuristic search mode, 1000 random starting replicates, and TBR branch swapping, with MULTREES and Collapse on. Bootstrap values were estimated using 1000 bootstrap replicates under the heuristic search mode, each with 100 random starting replicates. Maximum likelihood analyses were carried out in PhyML v.3.0 (Guindon & al., 2010) using default settings, and the best-fit models for the parameter-based analyses were selected using jModelTest (Posada, 2008) with the Akaike information criterion (Akaike, 1974). Parameter values were estimated simultaneously with the analyses. Bayesian inference was carried out in MrBayes v.3.1.2 (Huelsenbeck & Ronquist, 2001) with four chains and the model selected by jModelTest with the Akaike information criterion. Chains were run for two million generations, and trees were sampled every 100 generations. Convergence was evaluated by examining the standard deviation of split frequencies among runs and by plotting the log-likelihood values from each run using Tracer v.1.5 (http://beast.bio.ed.ac.uk/). These diagnostics indicated that runs reached convergence within the first 400,000 generations, and trees sampled during this period were excluded before obtaining clade posterior probabilities. TreeAnnotator (BEAST v.1.6.2., http://beast.bio.ed.ac.uk/) was used to compute the consensus majority tree of all trees recovered in the stationary phase.

Because a comparison of phylogenies of the four chloroplast datasets revealed no substantial incongruence across methods (MP, ML, BI) or datasets (*trnL-trnF*, *rps4-trnS*, *trnG-trnR*, *rbcL*), the four chloroplast partitions were combined into a single dataset, and the three phylogenetic analyses (MP, ML, BI) were repeated. The combined chloroplast dataset contained 50 distinct sequences. In addition to our newly generated sequences, we included *trnL-trnF* and *rps4-trnS* sequences from the endemic Hawaiian *A. hobdyi* (Schneider & al., 2005) and *rbcL* from Japanese *A. normale* var. *normale*, *A. normale* var. *shimurae* and *A. normale* var. *boreale* (Ebihara & al., 2010) from GenBank (http://www.ncbi.nlm.nih.gov). The inclusion of these additional taxa allowed us to compare samples in our phylogeny with the taxonomic units widely recognized in Japan (Kurata, 1963; Ito, 1972; Nakaike, 1992; Iwatsuki, 1995). The plastid phylogenetic tree was rooted using outgroup taxa based on the results reported in Schneider & al. (2005). The outgroup included representative specimens of *A. viride* Huds, *A. trichomanes* complex (*A. azoricum* Lovis & al., *A. trichomanes* subsp. *inexpectans* Lovis, *A. trichomanes* subsp. *quadrivalens* D.E. Mey., *A. trichomanes* subsp. *trichomanes*), *A. monanthes* complex (*A. formosum* Willd., *A. heteroresiliens* W.H. Wagner, *A. monanthes* L., *A. resiliens* Kunze) and *Diellia* complex (*A. dielerectum* Viane, *A. dielfalcatum* Viane, *A. dielmannii* Viane, *A. dielpallida* Viane, *A. ×lauii* Viane, *A. unisora* Viane) using *trnL-trnF* and *rps4* regions available from GenBank. New sequences were generated for one specimen of *A. trichomanes* to root the *trnG-trnR* tree. To address uncertainty in the phylogenetic results reported in previous studies (Schneider & al., 2004, 2005), we carried out several analyses using different outgroup compositions.

Average uncorrected *p*-distances between the principal cpDNA lineages were calculated using MEGA4 (Tamura & al., 2007). The species delimitation plugin (Masters & al., 2011) of Geneious v.5.3.6 (http://www.geneious.com), which implements the methods of Rosenberg (2007) and Rodrigo & al. (2008), was used to calculate the probability of reciprocal monophyly of the chloroplast phylogeny under the assumption of random coalescence (Rodrigo & al., 2008; Ross & al., 2008). Rosenberg's reciprocal monophyly P(AB) and Rodrigo's randomly distinct P(RD) were estimated for taxa that included more than one haplotype. A P(RD) value smaller than 0.05 defines a distinct species (see Rodrigo & al., 2008) and a P(AB) value smaller than 10^{-5} is considered to be significant. These values were found to be reliable in a recent sequence-based species delimitation study (Boykin & al., 2012). We also employed the independent network approach (Pons & al., 2006) to reconstruct networks of chloroplast haplotypes using statistical parsimony as implemented in TCS v.1.3 (Clement & al., 2000). Chloroplast haplotypes were identified using DnaSP (Rozas & al., 2003) and using the haplotype collapse command in ALTER (Glez-Pena & al., 2010). The TCS calculations were carried out with gaps treated as missing in order to avoid ambiguity introduced by gaps comprising more than one base pair.

We also explored the impact of the 90%–99% cut-off of the maximum number of mutational connections.

For the nuclear gene (*pgiC*) analyses, *A. viride* was used as the outgroup as it is clearly distinguishable from the *A. normale* complex and the relevant sequence was available from GenBank. The resulting consensus tree of nuclear *pgiC* sequences was a multi-labelled tree, in which some terminals represent different homologous sequences of the same accession. The multi-labelled tree was transformed into a network, using the algorithm described in Huber & al. (2006) and the open-source PADRE software for analyzing and displaying reticulate evolution (Lott & al., 2009). Based on the results from PADRE, the network was redrawn and edited using Adobe Illustrator CS3.

■ RESULTS

Ploidy analyses. — The spores of all studied specimens were well-formed and evidence for aborted spores was not found. Specimens were sorted into two groups according to mean spore sizes: 27–32 µm and 34–39 µm (Table 2; Fig. 2).

DNA C-values were determined for five specimens, of which two had spores with a mean size of 30 or 31 µm and three had spores with a mean size of 34 or 35 µm (Table 2). The genome sizes of two specimens with small spores were 8.05 (9.1 pg/C) and 8.67 (9.8 pg/C) times that of the internal standard (*Glycine max* ca. 1.13 pg/C), and those of three specimens with large spores were 16.02 (18.1 pg/C), 16.11 (18.2 pg/C) and 16.28 (18.4 pg/C) times that of the internal standard (Table 2; Fig. 3). These results indicate different ploidy levels exist in the *Asplenium normale* complex.

Chloroplast DNA phylogeny. — The total length of the chloroplast sequence alignment was 4155 bp. Sequences of the *rbcL* region were 1188 bp long, *trnL-trnF* 862–896 bp,

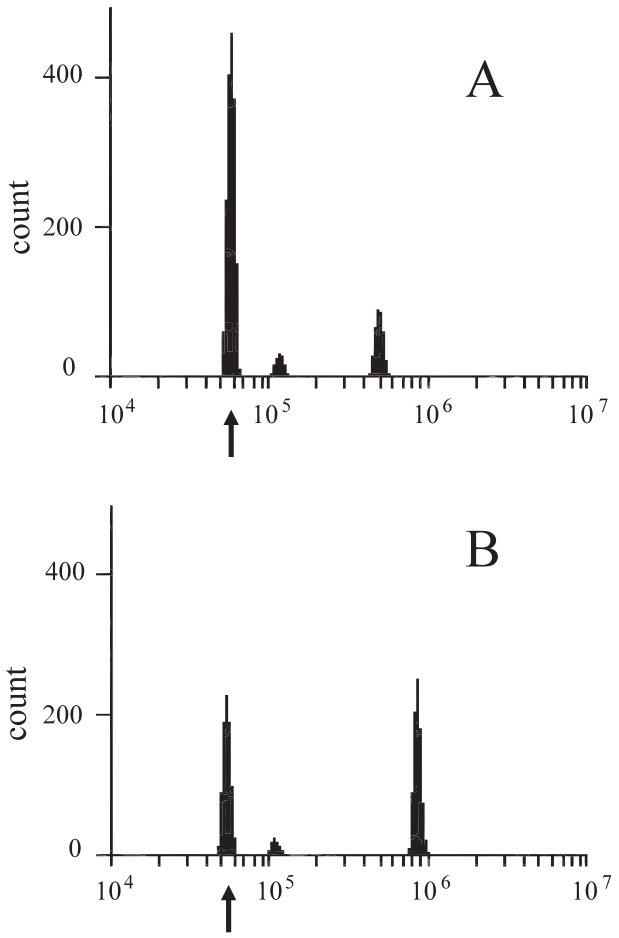

Fig. 3. Flow cytometric determination of ploidy level. DNA content calibrated with the internal standard *Glycine max* (arrows); count = number of nuclei measured. **A,** measurements interpreted as 2*x* (Jinping 1 China); **B,** measurements interpreted as 4*x* (Mt Emei 2 China).

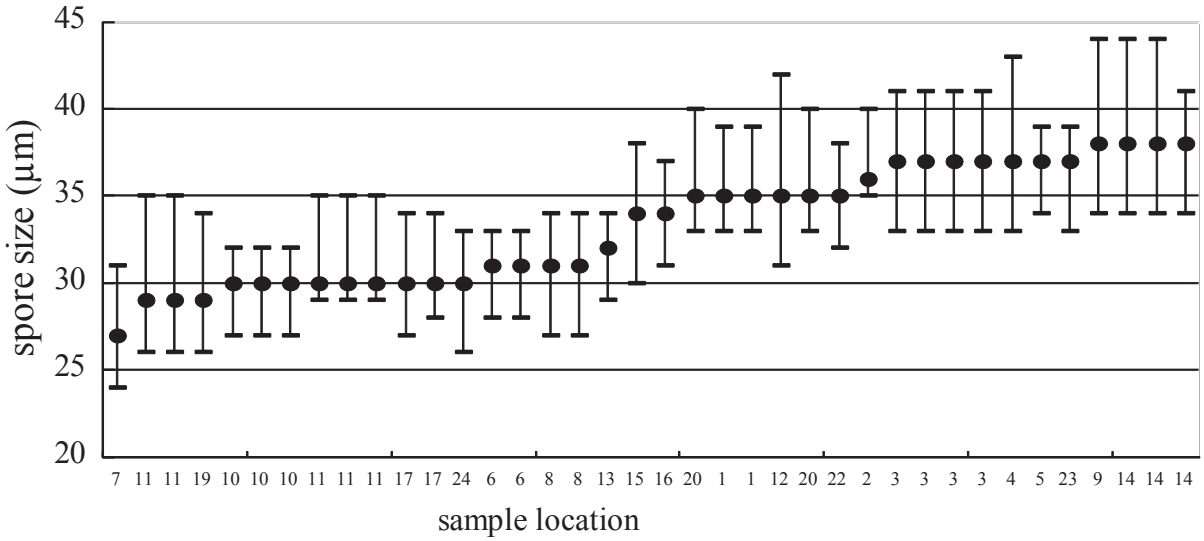

Fig. 2. Spore sizes for each sample location arranged by increasing size. Dot = mean value, interval = maximum and minimum values measured. See also Table 2.

rps4 982–992 bp, and *trnG-trnR* 1050–1079 bp. No substantial conflict was found among the separate phylogenetic analyses (MP, ML, BI) of each region. The three phylogenetic analyses of the combined chloroplast dataset recovered the same topology. Varying the composition of outgroup taxa did not affect the high support (i.e., bootstrap value = 100%; posterior confidence values = 1.0) for the monophyly of the *A. normale* complex and several intraclade relationships.

Phylogenetic analyses recovered four principal clades comprising more than one haplotype plus two distinct haplotypes unique to two morphologically distinct species, *A. kiangsuense* and *A. oligophlebium* (Fig. 4). These two species formed a clade

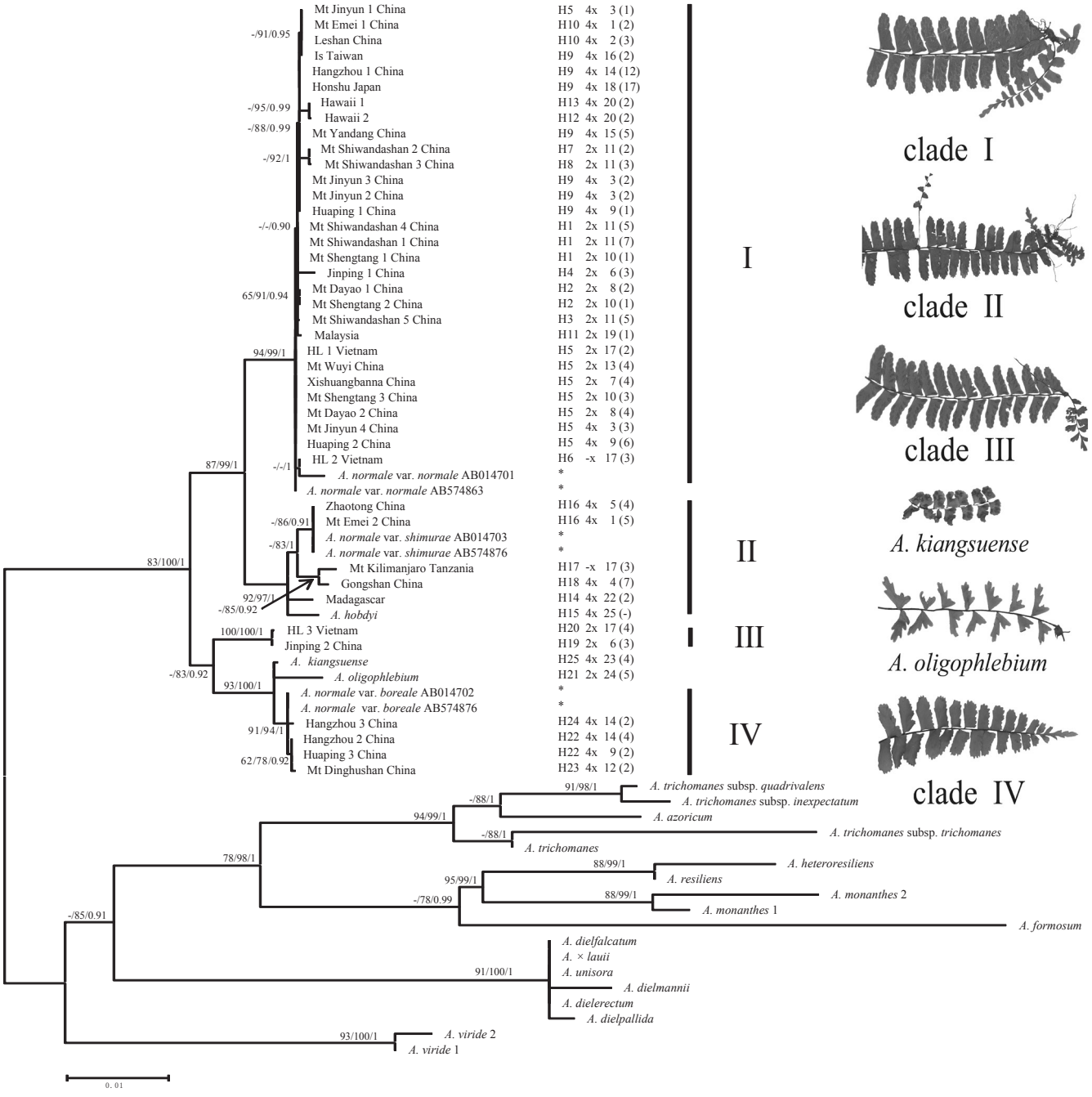

Fig. 4. Maximum likelihood phylogeny of the chloroplast dataset. Branch lengths correspond to estimated probability of substitution events. Maximum parsimony and Bayesian inference recovered identical topologies with respect to relationships among the main lineages of the *A. normale* complex. Node support is indicated for all three analyses: maximum parsimony/maximum likelihood/Bayesian inference as bootstrap percentages and posterior probability, respectively. Japanese samples downloaded from GenBank are indicated by stars. Columns on the right refer to haplotypes (H1 to H25), inferred ploidy levels (2*x*, 4*x*), sample locations (1–25, see Appendix 1 for details), numbers of individuals with identical sequences at each location in parentheses, and clade abbreviations (I–IV). Frond sketches of sample specimens from each lineage are given.

together with clade IV. The latter was supported by high bootstrap (≥90%) and posterior probability values (PP= 1; Fig. 4). Clade III was recovered as sister to the clade including clade IV, *A. kiangsuense* and *A. oligophlebium*, while clades I and II were sister to one another; these clades were recovered with high support (Fig. 4). Genetic distances (uncorrected *p*-distance) between groups ranged from 0.004 to 0.030 (Table 3). We separated *A. hobdyi* as a putatively distinct taxon in order to determine its genetic distance from other members of clade II as well as from other lineages; the distance between *A. hobdyi* and clade II was the smallest of all those calculated. Genetic distances between clade IV, *A. kiangsuense*, and *A. oligophlebium*, and between clade I and clade II were smaller (0.09–0.011) than those of other comparisons (0.21–0.30; Table 3).

Clade I comprised samples from various parts of southern and central China (Chongqing, Fujian, Guangxi, Sichuan, Yunnan, Zhejiang), and also Taiwan Island, Japan, northern Vietnam, Malay Peninsula, and Hawai'i (Fig. 4), with a total of 13 haplotypes (H1–H13). Its sister clade, clade II, included specimens collected in southwestern China (Sichuan, Yunnan), East Africa (Tanzania) and Madagascar as well as the Hawaiian *A. hobdyi*, with five haplotypes (H14–H18). Clade III included specimens collected in northern Vietnam and southwestern China, with two very similar haplotypes (H19, H20). Clade IV included specimens collected in southern and central China (Guangdong, Guangxi, Zhejiang) and comprised three haplotypes (H22–H24). Haplotype 21 and haplotype 25 corresponded to two species, *A. oligophlebium* endemic to Japan and *A. kiangsuense* endemic to China. Sequences of Japanese taxa downloaded from GenBank were nested within three clades: *A. normale* var. *normale* in clade I, *A. normale* var. *shimurae* in clade II and *A. normale* var. *boreale* in clade IV.

Based on the topology obtained from analyses of the chloroplast data and the morphological distinctiveness of *A. kiangsuense* and *A. oligophlebium*, we considered each of the six cpDNA lineages as possible diploid species for the purposes of the species delimitation analyses (Table 4). The results indicated that clade I was distinct with P(RD) < 0.05, and significant with P(AB) < 10^{-5} (= 2.3×10^{-8}; Table 4). Clade II was distinct under P(AB) (= 2.3×10^{-8}) but not significantly so under P(RD) (= 0.06; Table 4). Clades III and IV did not pass either criteria. *Asplenium kiangsuense* and *A. oligophlebium* were not fit for the species delimitation analyses because each had only one haplotype. TCS analysis failed to connect all lineages but recovered three independent networks with H1, H19 and H22 as ancestral haplotypes (Fig. 5). Clade III was resolved as an independent network and comprised two similar haplotypes (H19, H20). Clade II (H14–H18) connected with clade I (H1–H13) with a long branch of 10 steps. Haplotype 21 and haplotype 25 connected with clade IV (H22–24), also with long branches (Fig. 5).

Table 3. Number of specimens included and genetic distances (uncorrected *p*-distance) between the six cpDNA lineages and *Asplenium hobdyi*.

	Number of specimens	Uncorrected p-distance of cpDNA lineages					
		Clade II	Clade III	*A. kiangsuense*	*A. oligophlebium*	Clade IV	*A. hobdyi*
Clade I	110	0.011±0.003	0.027±0.005	0.029±0.005	0.030±0.005	0.029±0.005	0.013±0.004
Clade II	20		0.021±0.004	0.022±0.005	0.023±0.005	0.023±0.005	0.004±0.002
Clade III	7			0.020±0.004	0.021±0.005	0.021±0.005	0.023±0.005
A. kiangsuense	4				0.009±0.002	0.011±0.002	0.025±0.005
A. oligophlebium	5					0.011±0.002	0.025±0.005
Clade IV	10						0.024±0.005
A. hobdyi	0						

Table 4. Comparison of the six cpDNA lineages based on cpDNA variation and ploidy.

	Clade I	Clade II	Clade III	*A. kiangsuense*	*A. oligophlebium*	Clade IV
N Loc	16	6	2	1	1	3
N Seq	110	20	7	4	5	10
N Hap	13	5	2	1	1	3
N 2*x*	42	0	7	0	5	0
N 4*x*	65	18	0	4	0	10
P(RD)*	<0.05	0.06	0.86	NA	NA	0.17
P(AB)*	2.3×10^{-8}	2.3×10^{-8}	0.01	0.05	0.07	0.07

Row headers are as follows: N Loc, Number of locations studied; N Seq, number of sequences obtained; N Hap, Number of haplotypes recovered; N 2*x*, number of diploid specimens; N 4*x*, number of tetraploid specimens; P(RD), probability that the focal clade forms a distinct species, a value <0.05 is expected for distinct species; P(AB), probability for reciprocal monophyly, $P < 10^{-5}$ is considered to be significant.
NA, insufficient sample size for analysis.
*Analyses were carried out using the "Species Delimitation" plugin in Geneious v.5.3.6.

Nuclear DNA phylogeny. — A total of 140 cloned *pgiC* sequences were grouped into 21 consensus sequences, each of which represented two to ten cloned sequences. With *A. viride* as the outgroup, the aligned matrix was 616 bp long. Six indels were scored as present/absent (1/0) and added to the matrix, resulting in a total of 88 parsimony informative characters. The three phylogenetic analyses (MP, ML, BI), employed to analyze the nuclear *pgiC* DNA dataset, recovered the same topology with high support for five clades (Fig. 6, clades A–E). The nuclear phylogeny (Fig. 6) was discordant with the chloroplast phylogeny (Fig. 4). In the nuclear phylogeny, clades A and B comprised accessions of clades I and II of the plastid phylogeny whereas clade C included only accessions of clade I; clade D comprised plastid phylogeny accessions of tetraploids from clade I, clade IV, and *A. kiangsuense*, and diploid *A. oligophlebium*; clade E comprised cpDNA diploid clade III and one copy from a tetraploid Hawaiian sample of chloroplast clade I.

In the network (Fig. 7), which was constructed from the strict consensus parsimony tree, reticulate relationships were summarized from the distribution of nuclear copies of each accession. Both accessions of clade II shared copies with clade I. Accessions of clade I had copies both in clade III and clade IV. However, *A. oligophlebium* and *A. kiangsuense* showed no evidence of reticulation. These specimens had one or two copies but all were recovered in the same clade.

■ DISCUSSION

Our combined chloroplast dataset supports the monophyly of the *Asplenium normale* complex, which includes the broadly distributed *A. normale* and less widely distributed species, i.e., *A. kiangsuense* from central China, *A. hobdyi* from Hawai'i and *A. oligophlebium* from Japan. Four well-supported monophyletic clades (clades I, II, III, IV) were recovered with high bootstrap and posterior support values, along with distinct haplotypes for *A. kiangsuense* and *A. oligophlebium* (Figs. 4, 5). The results are consistent with studies on Japanese members of this complex (Matsumoto, 1975; Nakaike, 1992; Matsumoto & al., 2003; Ebihara & al., 2010) which indicated the presence of four independent taxonomic units in Japan: *A. normale* (= *A. normale* var. *normale*), *A. boreale* (= *A. normale* var. *boreale*), *A. shimurae* (= *A. normale* var. *shimurae*) and *A. oligophlebium*.

The incongruence between the chloroplast and nuclear phylogenies (Figs. 4, . 6) and the co-existence of diploids and tetraploids (Table 2; Figs. 2, 3) in the *A. normale* complex suggest that reticulate evolution through allopolyploidy has occurred, possibly alongside autopolyploidy. This result resembles reticulate evolution in two closely related *Asplenium* species complexes: the *A. trichomanes* complex (Lovis, 1978) and the *A. monanthes* complex (Dyer & al., 2012).

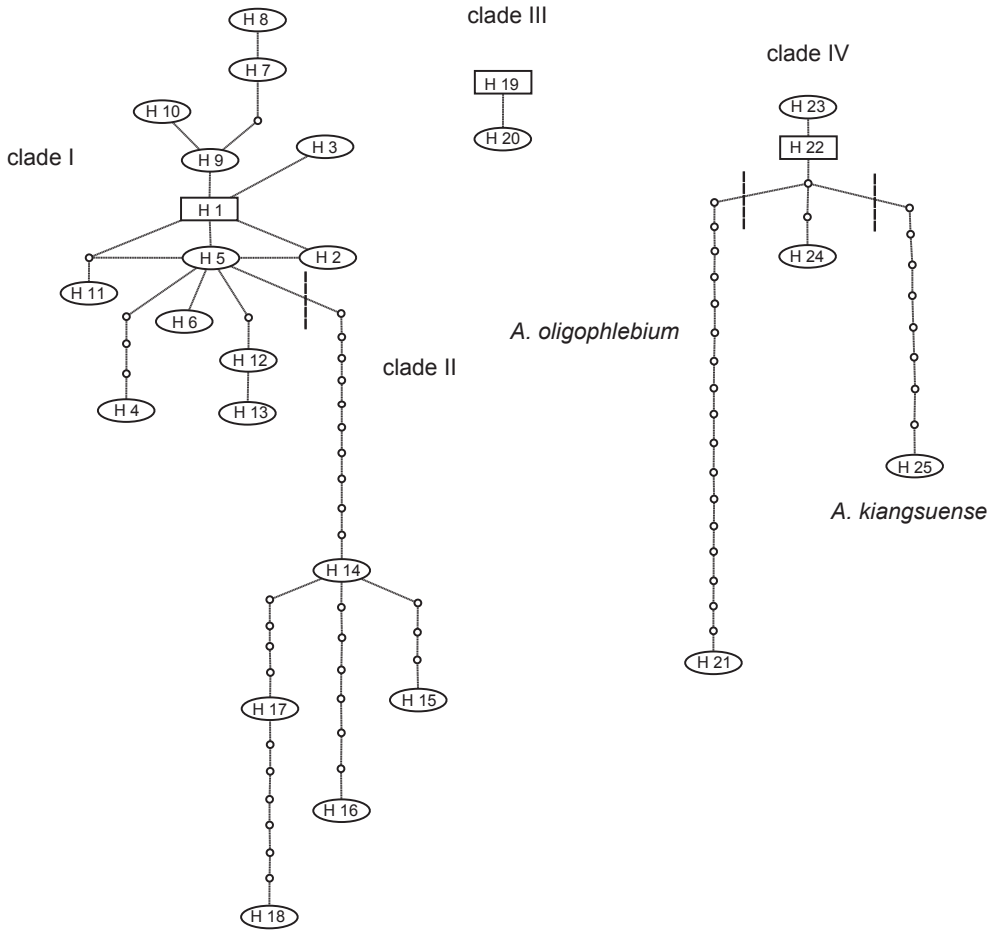

Fig. 5. Three independent minimum spanning networks of 25 chloroplast DNA haplotypes detected in the *Asplenium normale* complex. Dashed lines separate connected lineages in each network. Clade and haplotype numbers are identical with those in Fig. 4. Each line between haplotypes represents one mutational step and small circles indicate hypothetical haplotypes. A square indicates the inferred potential ancestral haplotype of each network.

Chloroplast lineages. — We identified four distinct clades (I–IV) including specimens identified as *A. normale* s.l. using morphological features alone (Figs. 4, 5; Table 3). Clade I is distributed throughout most of Southeast Asia and extends to Hawai'i and Malaysia (Fig. 1). It includes both diploid and tetraploid plants with buds occurring at the frond apex. Clade II also has a wide geographical distribution, from East Africa and Madagascar to southeastern China and also occurs in Hawai'i. This lineage includes only tetraploid specimens with buds not restricted to the frond apex. Clade III is restricted to areas in southern Yunnan (China) and northern Vietnam and includes only diploids with buds at the frond apex. Clade IV is found in southern and central China and includes only tetraploid specimens without frond buds. *Asplenium oligophlebium* is a diploid with dissected pinnae restricted to Japan. *Asplenium kiangsuense* is a tetraploid without frond buds restricted to central China.

Evidence from phylogenetic and TCS analyses distinguish lineages consistent with morphologically diagnostic taxa. Further analyses were carried out to explore the distinctiveness of these six lineages using Rodrigo's P(RD) and Rosenberg's P(AB) (see Table 4). These coalescence-based species delimitation analyses are not appropriate for taxa with a single haplotype, such as *A. kiangsuense* and *A. oligophlebium*, because they assume accumulation of genetic diversity at tip nodes. However, these two lineages are recognizable by their distinct morphologies. Of the four lineages that comprised several haplotypes (clades I–IV), the clades found to be distinct by the coalescent species delimitation analyses (clades I and II) comprise more haplotypes compared to the unsupported clades (clades III and IV). These results may indicate a putative bias through sampling density, which is expected given the foundation of these measures in coalescence theory. Thus, these analyses are fit for well-sampled clades like clades I and II but not clades III and IV which have small sample sizes. However, the lack of support for the monophyly of clade IV may not only be due to insufficient sampling but also the inclusion of polyploids because polyploidy can imply a high probability of relatively rapid speciation processes resulting in further deviation of the coalescence analyses and leading to lack of support in P(AB) and P(RD) (Rosenberg, 2007; Rodrigo & al., 2008; Ross & al., 2008; Master & al., 2011; Boykin & al., 2012).

Overall, our results suggest that the *A. normale* complex consists of six distinct chloroplast lineages of which two are significantly supported by the species delimitation analyses using Rodrigo's P(RD) and Rosenberg's P(AB). The lineage diversity

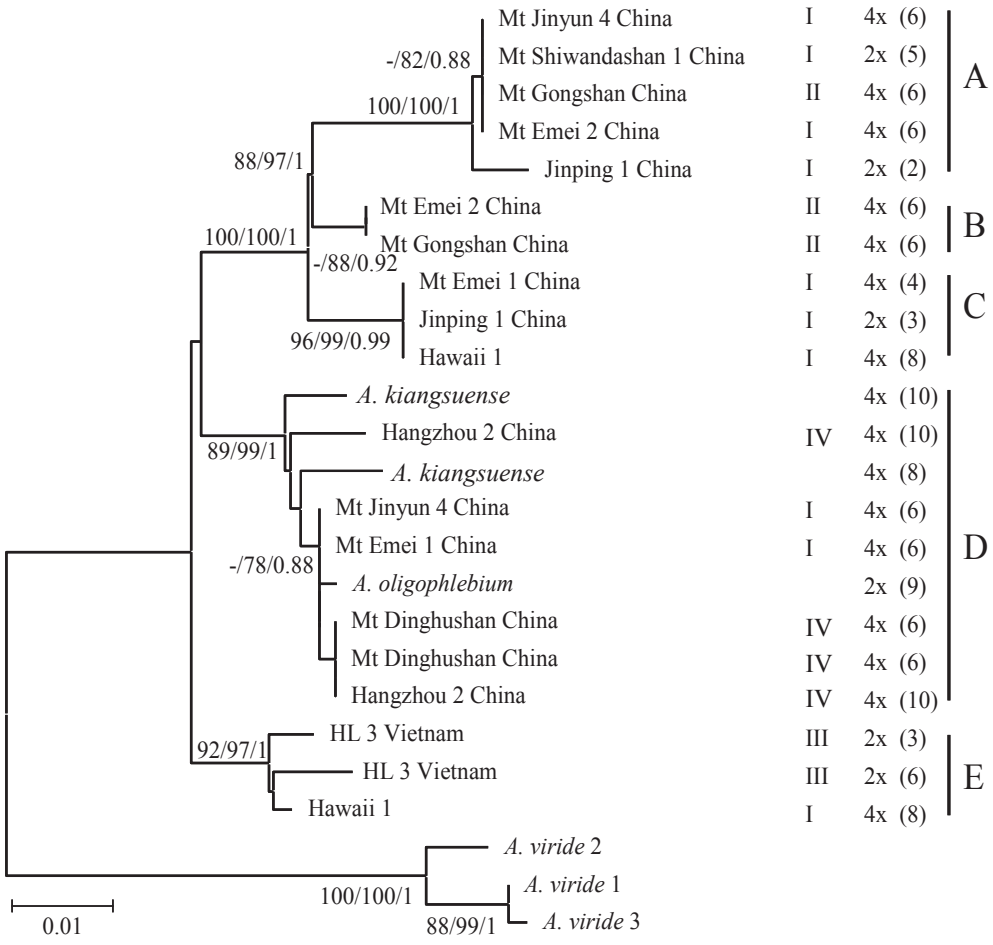

Fig. 6. Maximum likelihood phylogeny of the nuclear gene *pgiC* dataset. Maximum parsimony and Bayesian inference recovered identical topologies. Node support is indicated for all three analyses: maximum parsimony/maximum likelihood/Bayesian inference as bootstrap percentages and posterior probability, respectively. Columns on the right indicate clade abbreviations (I–IV) obtained from the chloroplast phylogeny (Fig. 4), inferred ploidy levels (2*x*, 4*x*), numbers of clones of each consensus sequence, and nuclear phylogeny clade labels (A–E).

in this complex supports the hypothesis that the *A. normale* complex comprises several distinct species (Nakaike, 1992; Iwatsuki, 1995; Iwashina, 2000; Matsumoto & al., 2003).

Molecular evidence for recurrent reticulation. — The occurrence of reticulate evolution in the *A. normale* complex is strongly supported by the incongruence of the plastid and nuclear phylogenetic results, and also by the observation of tetraploid individuals containing two divergent nuclear *pgiC* copies each characteristic of diploids in different cpDNA lineages (Figs. 4, 6, 7). This finding is consistent with studies of Japanese members of the *A. normale* complex that suggested the existence of natural hybrids (Matsumoto, 1975; Nakaike, 1992; Matsumoto & al., 2003). In addition to hybridization, conflicting gene trees can be the result of other biological events such as lineage sorting or paralogy. The best approach to solve this problem is to combine many unlinked gene trees into a phylogenetic network (Rieseberg, 1995; Sang & Zhong, 2000; Sang, 2002; Doyle & al., 2003; Smedmark & al., 2005; Brysting & al., 2007, 2011). Particularly powerful in *Asplenium* is the combination of organelle genome (plastid DNA) and nuclear genome markers because organelle DNA is usually uniparentally inherited in asplenioid ferns (Vogel & al., 1998). Our network based on sequences of two unlinked genomes suggests that hybridization and polyploidization are key evolutionary processes in this complex. Additionally, inferences of ploidy levels of specimens in each lineage enable us to detect the origin of tetraploids and the existence of incomplete lineage sorting in diploids.

Three diploid and four tetraploid entities were recognized in the *A. normale* complex in this study. The three diploid entities are *A. oligophlebium*, chloroplast clade I and clade III. The independence of these diploids and the absence of incomplete lineage sorting are supported by the nuclear data (Fig. 6). The four tetraploid entities correspond to chloroplast clades I, II, IV and *A. kiangsuense*. The absence of diploids in some chloroplast clades (clades II, IV and *A. kiangsuense*) may be the result of small sample size but it is also possible that the diploid progenitors have gone extinct. Tetraploids of chloroplast clade I are probably allopolyploid since their accessions contain nuclear copies from two lineages, i.e., clades III and IV. Tetraploids of chloroplast clade II, which contain specimens identified as *A. hobdyi* and *A. shimurae*, share nuclear copies with clade I (Fig. 7), implying that they also have an allopolyploid origin with diploid clade I as one parent. Tetraploids of chloroplast clade IV have nuclear copies found only in individuals of chloroplast clade IV implying that they may be autopolyploid. *Asplenium kiangsuense* appears to be autopolyploid as it has two *pgiC* copies that belong to the same group. Further evidence is needed to confirm this hypothesis because the PCR-based cloning of the nuclear gene may overlook copies as a result of PCR bias.

Taxonomic implications. — For a formal taxonomic designation of the six putative cpDNA-taxa obtained in this study, further studies including geographical distribution of diploid taxa and detailed morphological investigations are necessary. Here we suggest taxonomic assignments based on our chloroplast and nuclear phylogenies, morphological observations such as pinna shape and presence and location of frond buds (Table 1), and the strong associations of the three currently accepted Japanese varieties or species of *A. normale* (i.e., *A. normale* var. *normale*, *A. normale* var. *shimurae*, *A. normale* var. *boreale*) with chloroplast clades I, II and IV, respectively. (Fig. 4).

Diploid chloroplast clade I corresponds to *A. normale* s.str., and is found throughout most of Southeast Asia (Fig. 1). Plants in this group often have a single bud near the frond apex. Tetraploid clade I has a wider distribution and reaches Japan in the north and Hawai'i in the east with buds occurring at the tip of fronds like in diploid clade I. It is likely to be an allotetraploid with diploid clade I and undetected diploid clade IV as its progenitors. However, morphologically, tetraploid clade I shares features with diploid clade I and differs from tetraploid clade IV. Thus, we treat tetraploid clade I as part of *A. normale* s.str..

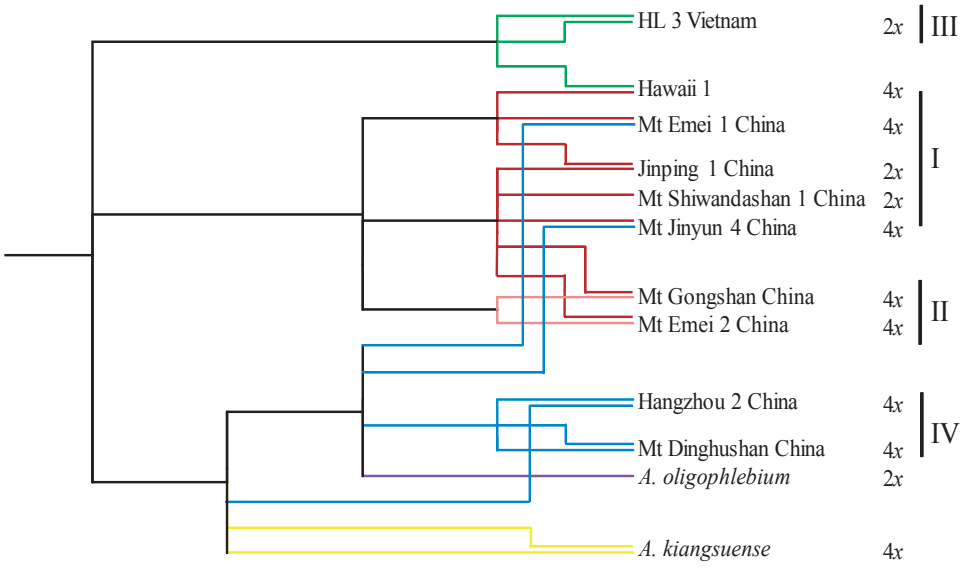

Fig. 7. The reticulate evolutionary history of the *A. normale* complex as revealed by the *pgiC* network. The network was constructed from the *pgiC* maximum parsimony strict consensus tree. Plastid lineages are indicated by different colours: red = clade I; pink = clade II; green = clade III; blue = clade IV; yellow = *A. kiangsuense*; purple = *A. oligophlebium*. Columns on the right indicate inferred ploidy levels (2*x*, 4*x*) and clade abbreviations (I–IV) obtained from the chloroplast phylogeny (Fig. 4).

Tetraploid clade II corresponds to *A. shimurae*. It has a wide geographical distribution from East Africa to East Asia and also occurs in Hawai'i (Fig. 1). This lineage differs from others in having buds near the frond apex as well as on other parts of the rachis. It is an allopolyploid with diploid clade I and undetected diploid clade II as its progenitors (Fig. 7). Based on its morphological and molecular distinctness, we define this tetraploid lineage as *A. shimurae*. *Asplenium hobdyi* is morphologically similar to tetraploid clade II and is part of the same clade. The low *p*-distance value between *A. hobdyi* and other clade II members also fails to support *A. hobdyi* as an independent species (Table 3). In the context of species name priority, the species names relevant for clade II were published in 1992 (*A. shimurae*) and 1993 (*A. hobdyi*). *Asplenium normale* was used in the description of a Malagasy specimen (Tardieu-Blot, 1958). However, our single Malagasy sample is nested within clade II and is morphologically similar to *A. shimurae*. More samples from Madagascar are needed for further study since other clades may also be present there. Here, we advocate the use of *A. shimurae* for clade II until further results clarify the taxonomy of this clade.

Diploid clade III has only been found in southern Yunnan (China) and northern Vietnam. Further fieldwork is necessary to confirm the range of this diploid taxon in the eastern parts of southern China. This lineage has no morphological features distinguishing it from *A. normale* s.str. and appears to be a cryptic species. It supports the notion that cryptic species may be much more common among ferns than previously assumed, particularly within widespread species (e.g., Paris & al., 1989; Hauk & Haufler, 1999; Masuyama & al., 2002; Yatabe & al., 2009). Current assessments of fern diversity may substantially underestimate real species diversity.

Tetraploid clade IV corresponds to *A. boreale*. It is distributed from southern China to Japan. This lineage is probably an autopolyploid since both chloroplast and nuclear DNA sequences fall into the same clade (Fig. 7). It has no frond buds. After examining the *A. normale* var. *boreale* type specimen, we suggest that this lineage corresponds to *A. boreale*.

Asplenium kiangsuense and *A. oligophlebium* are accepted as species since they are morphologically and genetically distinct (Tables 1, 3; Figs. 4–6). Diploid *A. oligophlebium* is restricted to Japan and has distinctly shaped pinnae. Tetraploid *A. kiangsuense* is restricted to central China and is probably an autopolyploid. The distinctiveness of this lineage is mainly based on its small size and lack of frond buds.

As a general conclusion, our results suggest that the *A. normale* complex consists of six distinct species. Traditional morphology-based taxonomy poorly represents the diversity in this complex. This is consistent with studies on other species complexes of *Asplenium* as well as other derived ferns (Yatabe & al., 2001, 2009; Yatabe & Murakami, 2003; Sprunt & al., 2011; Wang & al., 2011). In light of our phylogenetic results, further analyses of morphology may recover currently unrecognized species characters (Sprunt & al., 2011). This study revealed that the presence and location of frond buds is a potentially informative character in the *A. normale* complex.

■ ACKNOWLEDGEMENTS

We thank Dr. Alain Vanderpoorten, Dr. Joachim W. Kadereit and anonymous reviewers for their helpful reviews of the manuscript. We extend our appreciation to Dr. Pelin Kayaalp for revising the English and several colleagues who assisted with collections, including Liyuan Guo in Taiwan Island, Mitsuyasu Hasebe in Japan, Ngan Lu Thi and Duo Van Truong in Vietnam, and Renxiang Wang, Rihong Jiang and Xianchun Zhang in China. Harald Schneider acknowledges the senior visiting professorship granted by the Chinese Academy of Sciences.

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Appendix 1. Specimens used in the DNA sequence analyses.

GenBank accession numbers are given for sequences deposited in GenBank. Only one sequence was submitted for sets of identical sequences. Data strings are organized as follows: species name, location number, locality; sequence name, collector and number (herbarium), haplotypes, GenBank accession numbers for four plastid loci: *trnL-trnF*/*rps4-trnS*/*trnG-trnR*/*rbcL*; *pgiC*: GenBank accession (number of clones). Species names: *A. normale* accepted here as sensu lato, only *A. kiangsuense*, *A. hobdyi*, and *A. oligophlebium* are accepted as segregated.

***Asplenium normale* 1:** China, Sichuan Province, Mt. Emei; Mt Emei 1 China, *Yanfen Chang 100103* (HITBC), H10, JQ724184/JQ724268/JQ724226/JX152745; *pgiC*: JX237503 (4), JX237504 (6); Mt Emei 2 China, *Yanfen Chang 100105* (HITBC), H16, JQ724185/JQ724269/JQ724227/JX152758; *pgiC*: JX237505 (6), JX237506 (6). **2:** China, Sichuan Province, Leshan; Leshan China, *Yanfen Chang 100204* (HITBC), H10, JQ724186/JQ724270/JQ724228/JX152752. **3:** China, Chongqing; Mt Jinyun 1 China, *Yanfen Chang 100301* (HITBC), H10, JQ724187/JQ724271/Q724229/–; Mt Jinyun 2 China, *Yanfen Chang 100303* (HITBC), H9, JQ724188/JQ724272/JQ724230/JX152747; Mt Jinyun 3 China, *Yanfen Chang 100306* (HITBC), H9, JQ724189/JQ724273/JQ724231/–; Mt Jinyun 4 China,

Appendix 1. Continued.

Yanfen Chang 100311 (HITBC), H5, JQ724190/JQ724274/JQ724232/JX152746; *pgiC*: JX237511 (6), JX237512 (6). **4:** China, Yunnan Province, Gongshan; Gongshan China, *Yanfen Chang 100402* (HITBC), H18, JQ724191/JQ724275/JQ724233/JX152741; *pgiC*: JX237507 (6), JX237508 (6). **5:** China, Yunnan Province, Zhaotong; Zhaotong China, *Shugang Lu 100502* (HITBC), H16, JQ724192/JQ724276/JQ724234/–. **6:** China, Yunnan Province, Jinping; Jinping 1 China, *Shugang Lu 100602* (HITBC), H4, JQ724193/JQ724277/JQ724235/–; *pgiC*: JX237491 (2), JX237492 (3); Jinping 2 China, *Shugang Lu 100604* (HITBC), H19, JQ724194/JQ724278/JQ724236/JX152742. **7:** China, Yunnan Province, Xishuangbanna; Xishuangbanna China, *Yanfen Chang 100701* (HITBC), H5, JQ724195/JQ724279/–/–. **8:** China, Guangxi Province, Mt Dayao; Mt Dayao 1 China, *Shugang Lu 100801* (HITBC), H2, JQ724196/JQ724280/JQ724237/–; Mt Dayao 2 China, *Shugang Lu 100803* (HITBC), H5, JQ724197/JQ724281/JQ724238/JX152750. **9:** China, Guangxi Province, Huaping; Huaping 1 China, *Renxiang Wang 100901* (HITBC), H9, JQ724198/JQ724282/JQ724239/JX152733; Huaping 2 China, *Renxiang Wang 100903* (HITBC), H5, JQ724199/JQ724283/JQ724240/JX152734; Huaping 3 China, *Renxiang Wang 100905* (HITBC), H22, JQ724200/JQ724284/JQ724241/JX152735. **10:** China, Guangxi Province, Jinxiu, Mt Shengtang; Mt Shengtang 1 China, *Xianchun Zhang 101001* (HITBC), H1, JQ724201/JQ724285/JQ724242/–; Mt Shengtang 2 China, *Xianchun Zhang 101003* (HITBC), H2, JQ724202/JQ724286/JQ724243/JX152743; Mt Shengtang 3 China, *Xianchun Zhang 101005* (HITBC), H5, JQ724203/JQ724287/JQ724244/JX152744. **11:** China, Guangxi Province, Shangsi, Mt Shiwandashan; Mt Shiwandashan 1 China, *Yanfen Chang 101108* (HITBC), H1, JQ724204/JQ724288/JQ724245/–; *pgiC*: JX237517 (5); Mt Shiwandashan 2 China, *Yanfen Chang 101112* (HITBC), H7, JQ724205/JQ724289/JQ724246/–; Mt Shiwandashan 3 China, *Yanfen Chang 101115* (HITBC), H8, JQ724206/JQ724290/JQ724247/JX152736; Mt Shiwandashan 4 China, *Yanfen Chang 101119* (HITBC), H1, JQ724207/JQ724291/JQ724248/JX152737; Mt Shiwandashan 5 China, *Yanfen Chang 101121* (HITBC), H3, JQ724208/JQ724292/JQ724249/–. **12:** China, Guangdong Province, Zhaoqing, Mt Dinghushan; Mt Dinghushan China, *Shugang Lu 101203* (HITBC), H23, JQ724209/JQ724293/JQ724250/–; *pgiC*: JX237501 (6), JX237502 (6). **13:** China, Fujian Province, Nanping, Mt Wuyi; Mt Wuyi China, *Yanfen Chang 101302* (HITBC), H5, JQ724210/JQ724294/JQ724251/–. **14:** China, Zhejiang Province, Hangzhou; Hangzhou 1 China, *Yanfen Chang 101403* (HITBC), H9, JQ724211/JQ724295/JQ724252/JX152740; Hangzhou 2 China, *Yanfen Chang 101406* (HITBC), H22, JQ724212/JQ724296/JQ724253/JX152748; *pgiC*: JX237477 (10), JX237478 (10); Hangzhou 3 China, *Yanfen Chang 101411* (HITBC), H24, JQ724213/JQ724297/JQ724254/JX152739. **15:** China, Zhejiang Province, Wenzhou, Mt Yandang; Mt Yandang China, *Yanfen Chang 101507* (HITBC), H9, JQ724214/JQ724298/JQ724255/JX152749. **16:** Taiwan Island; Is Taiwan, *Liyuan Guo 101602* (HITBC), H10, JQ724215/JQ724299/JQ724256/JX152754. **17:** Vietnam, Lao Cai Province, Hoang Lien National Park; HL1 Vietnam, *Ngan Lu Thien & Harald Schneider V7-10* (BM), H5, JQ724216/JQ724300/JQ724257/–; HL 2 Vietnam, *Ngan Lu Thien & Harald Schneider V18-10* (BM), H6, JQ724217/JQ724301/JQ724258/JX152757; HL 3 Vietnam, *Ngan Lu Thien & Harald Schneider V22-10* (BM), H20, JQ724218/JQ724302/JQ724259/JX152756; *pgiC*: JX237526 (3), JX237527 (6). **18:** Japan, Kyoto prefecture, Honshu; Honshu Japan, *Harald Schneider J18-10* (BM), H9, JQ724219/JQ724303/JQ724260/JX152753. **19:** Malay Peninsula, Pahang, Cameron Highlands; Malaysia, *E. Schuettpelz 762A* (DUKE), H11, JQ724220/JQ724304/JQ724261/JX152755. **20:** Hawai'i; Hawai'i 1, *Tom Ranker 102001* (HITBC), H12, JQ724221/JQ724305/JQ724262/–; *pgiC*: JX237480 (8), JX237481 (8); Hawai'i 2, *Tom Ranker 102003* (HITBC), H13, JQ724222/JQ724306/JQ724263/JX152759. **21:** Tanzania, Mt. Kilimanjaro; Mt. Kilimanjaro Tanzania, *Andreas Hemp 104* (BM), H17, JQ724223/JQ724307/JQ724264/JX152760. **22:** Madagascar; Madagascar, *Thomas Janssen 2388/* (P), H14, -/JQ724308/JQ724265/–. ***A. kiangsuense*** **23:** China, Jiangxi Province, Guling, Mt Lushan; *A. kiangsuense*, *Yanfen Chang 102303* (HITBC), H25, JQ724224/JQ724309/JQ724266/JX152738; *pgiC*: JX237473 (10), JX237474 (8). ***A. oligophlebium*** **24:** Japan, Kyoto prefecture, Honshu; *A. oligophlebium*, *Xianchun Zhang 102404* (HITBC), H21, JQ724225/JQ724310/JQ724267/JX152751; *pgiC*: JX237475 (9). ***A. hobdyi*** **25:** Hawai'i; *A. hobdyi*, H15, AY549839/AY549785/–.