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Research Article

Likely allopatric origins of *Adiantum* × *meishanianum* (Pteridaceae) through multiple hybridizations

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Abstract *Adiantum* × *meishanianum* F. S. Hsu ex Y. C. Liu & W. L. Chiou was regarded as an endemic species in Meishan Village, Kaohsiung, Taiwan, China and a hybrid between *A. malesianum* Ghatak (the maternal parent) and a sexually reproducing diploid cryptic species of *A. philippense* L. (the paternal parent), as revealed by chloroplast and nuclear markers. However, morphological research revealed that *A.* × *meishanianum* is also disjunctively distributed in Yunnan and that its paternal parent is possibly *A. menglianense* Y. Y. Qian. Thus, this study aimed to confirm these findings by using two chloroplast regions and a low-copy nuclear marker in DNA barcoding and phylogenetic analyses, spore measurement, and flow cytometry. Our results indicated that *A.* × *meishanianum* in Yunnan is triploid and abortive, the same as *A.* × *meishanianum* in Taiwan, and they both originated from the hybridization between the maternal parent of *A. malesianum* and the paternal parent of *A. menglianense*, but not *A. philippense*. In conclusion, *A.* × *meishanianum* probably originated from multiple hybridizations in Taiwan and Yunnan.

Key words: *Adiantum* × *meishanianum*, *A. menglianense*, cryptic species, disjunctive distribution, multiple hybrids.

Hybridization is an important, dominant force that shapes the evolutionary history of plant species (Mallet, 2007). Due to the absence of many speciation barriers described for seed plants, hybridization occurs more often in ferns (Knobloch, 1976; Barrington et al., 1989). Hybrids are thus expected in most cases when fern species co-occur and they are usually sterile (Haufler, 2008). However, at least in some cases, the hybrids are fertile, which can promote allohomoploid speciation (Conant & Cooper-Driver, 1980; Mullenniex, 1998) or can result in allopolyploid species by chromosome doubling (Ranker et al., 1989; Barrington, 1990). Therefore, although sterile hybrids in ferns can be identified by the presence of sterile spores (Wagner et al., 1986), it is not adequate for explaining all events of origin and evolution of hybrid species. Moreover, compared to traditional hybrid identification methods, such as morphological differences, chromosome number, and pairing behavior (Manton, 1950; Walker, 1961; Mayer & Mesler, 1993), molecular markers (allozyme analysis or DNA analyses of the nuclear and plastid genomes) have proven more convenient for identifying fern hybrids (Werth, 1991; Chang et al., 2009; Zhang et al., 2013, 2014; Wang et al., 2015b) or inferring reticulate evolution in fern, for example, the *Asplenium normale* complex (Chang et al., 2013) and *Dryopteris* Adans in North America (Sessa et al., 2012) and Japan (Hori et al., 2014). Furthermore, the high diversity of genetic variation found in some of these studies indicates that multiple hybridizations could occur if the parent lineages co-occur independently in different sites (Chang et al. 2009; Hunt et al., 2011; Chao et al., 2012).

Adiantum × *meishanianum* F. S. Hsu ex Y. C. Liu & W. L. Chiou, which is an endangered species that can only produce aborted spores (Zhang et al., 2014), is considered endemic in Meishan Village in Taiwan, China (Liu et al., 2009; Wang et al., 2012). Using chloroplast regions, a low-copy nuclear gene marker, and flow cytometry (FCM), Zhang et al. (2014) confirmed that *A.* × *meishanianum* is a triploid hybrid between *A. malesianum* Ghatak as the maternal parent and a sexual diploid cryptic species of *A. philippense* L. as the paternal parent (hereafter denoted as "the paternal species in Taiwan"). However, a series of specimens collected from Yunnan were also identified as *A.* × *meishanianum* based on morphological characteristics and the chloroplast *rbcL* gene (Wang et al., 2014), although nuclear genetic evidence is lacking. This finding indicates that multiple hybridization

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events of *A.* × *meishanianum* may occur in Yunnan and Taiwan. A hybrid zone, which is an area where the parental species overlap, is a primary condition that enables the formation of multiple hybrids. The maternal parent *A. malesianum* is widely distributed in tropical Asia, including Yunnan and Taiwan. However, limited information is available about "the paternal species in Taiwan". Further studies should be carried out to gather novel information regarding *A.* × *meishanianum*, especially whether "the paternal species in Taiwan" is also distributed in Yunnan.

The morphological characteristics of "the paternal species in Taiwan" described by Zhang et al. (2014) are consistent with *A. menglianense* Y. Y. Qian in Yunnan (Qian, 1992). However, *A. menglianense* has rarely been investigated since it was first published; this species has only been briefly described in the *Flora Yunnanica* (Zhang, 2006) and has yet to be recorded in the *Flora of China* (Lin et al., 2013). In most herbaria in China, *A. menglianense* is identified as *A. philippense* because of the similarities between them. Despite their similarities, *A. menglianense* and *A. philippense* differ in terms of pinna, which is lobed by more than one-third, segments with an obtuse or emarginate apex, and sori, with 6–10 found in each pinna of *A. menglianense*. Moreover, 64 spores are formed in each sporangium of *A. menglianense*, whereas 32 spores are formed in *A. philippense*; this characteristic suggests that sexual reproduction occurs in *A. menglianense* according to the hypothesis acknowledged by Knobloch (1966), who stated that individuals bearing 64 spores per sporangium are sexual, whereas individuals with 32 or 16 spores per sporangium are apogamous (Walker, 1979). A phylogenetic tree based on the *rbcL* gene also indicates that *A. menglianense* is distinct from *A. philippense* monophyly as a sister group (Wang et al., 2014).

This study aimed to resolve the hybrid origins of *A.* × *meishanianum* obtained from Yunnan by using a combination of maternally inherited plastid markers, a bi-parental inherited nuclear DNA marker, and ploidy analyses. The two hypotheses that we aimed to test were: (i) whether *A.* × *meishanianum* from the new distribution site shares allopatric hybrid origins with that from Taiwan; and (ii) whether *A. menglianense* is just the paternal species of *A.* × *meishanianum*.

Material and Methods

Taxon sampling

Five *Adiantum menglianense*, one *A. malesianum*, and one *A.* × *meishanianum* individuals were collected from Yunnan (Table S1). The sequences of the chloroplast DNA (cpDNA) and nuclear regions of *A.* × *meishanianum* from Taiwan and other *Adiantum* species reported by Zhang et al. (2014) were downloaded from GenBank (Table S1).

DNA extraction, PCR amplification, and sequencing

Total genomic DNA was extracted from silica-dried leaves using a plant total genomic DNA kit (Tiangen, Beijing, China). The sequences of *matK* and *rps16-matK* intergenic spacer (IGS) regions for the chloroplast dataset were obtained using primers and protocols designed by Zhang et al. (2014). For the nuclear dataset, we used the first intron of the low-copy nuclear marker *CRY2* developed by Zhang et al. (2014). The nuclear PCR products were gel purified and cloned when direct sequencing failed because of superimposed peaks at multiple sites. The purified nuclear DNA was then subcloned into the TA cloning vector pMD18-T (TaKaRa, Dalian, China) and then sequenced using the M13 universal and reverse primers. Ten positive colonies of *A. malesianum* and *A.* × *meishanianum* were selected for sequencing (Table S1).

DNA barcoding analyses

For species delimitation among *A. menglianense*, *A. philippense*, and the "paternal species in Taiwan", the DNA barcoding gap method based on the Kimura two parameter (K2P) distance was used with chloroplast sequences. Intra- and inter-taxa genetic distances were evaluated using MEGA 5.0 (Tamura et al., 2011).

Sequence alignment and phylogenetic analyses

Phylogenetic analysis was used to compare the hybrid origin of *A.* × *meishanianum* obtained from Taiwan and Yunnan, with both chloroplast and nuclear markers used as powerful methods of species delimitation. DNA sequences were aligned using CLUSTALW and manually edited using BioEdit (Hall, 1999). The best-fit nucleotide substitution model of cpDNA (TPM3uf+G) and nuclear ribosomal DNA (HKY) was selected based on the Bayesian information criterion using jModelTest 3.7 (Posada, 2008) for subsequent phylogenetic analyses. Maximum likelihood (ML) trees were reconstructed by a rapid bootstrap analysis on the RAxML web server (Stamatakis, 2006). Tree-search parameters were calculated using our selected settings. Maximum likelihood bootstrap values (MLBS) were obtained by running 1000 replicates with the same criteria. Moreover, Bayesian inference analysis was carried out in MrBayes version 3.1.2 (Huelsenbeck & Ronquist, 2001; Ronquist et al., 2012) by using the Markov chain Monte Carlo algorithm. To establish the Bayesian inference settings, we used four chains with random initial trees. Trees were constructed for 1 000 000 generations, and sampling was carried out every 100 generations. The first 25% samples were discarded as burn-in before stationarity, and the remaining trees were used to construct majority-rule consensus trees by using PAUP (Swofford, 2003).

Ploidy analyses

Spore size was compared between *A. menglianense* and *A. malesianum* to confirm the ploidy level because spore diameter is considered a good indicator of ploidy level (Barrington et al., 1986). We observed the spores and took photographs using an Axio Scope.A1 microscope (Zeiss, Gottingen, Germany). Equatorial axis (length) and polar axis (width) of 30 spores per species were measured using ImageJ (http://rsb.info.nih.gov/ij/).

We also used 4′,6′-diamidino-2-phenylindole dihydrochloride (DAPI) staining methods to more accurately measure the relative DNA C-values and deduce the ploidy level (Suda et al., 2006; Chang et al., 2013; Wang et al., 2015a) of *A. menglianense*, *A. malesianum*, and *A.* × *meishanianum*. *Adiantum malesianum* was used as an internal standard because many studies (Roy & Holttum, 1965; Sinha & Manton, 1970; Lin, 1990; Zhang et al., 2014) have reported that *A. malesianum* is tetraploid. Furthermore, different ploidy levels have yet to be obtained. We carried out FCM analyses

on young circinate leaves of four sporophytic materials from different populations of all taxa to confirm the ploidy level of *A. menglianense* and *A. meishanianum*. The plant materials used for FCM were prepared in accordance with the standard protocols described in commercial kits. Equal amounts of fresh young leaves from both the targeted samples (*A. menglianense* and *A. meishanianum*) and an internal standard (*A. malesianum*) were chopped using a double-edged razor blade in a plastic Petri dish containing ice and 0.4 mL DAPI nucleus extraction buffer (CyStain UV Precise P05-5002; Partec, Münster, Germany). The samples were subsequently incubated on ice for 3 min. The suspension containing debris was filtered through a 40-μm mesh filter, and placed in a 5-mL cytometry tube with 1.6 mL DAPI staining solution (CyStain UV Precise P). The mixed solution was incubated in the dark for 5 h and then analyzed using an FCM (CyFlow Space; Partec) equipped with a high-pressure mercury arc lamp for UV excitation. For each sample, the fluorescence intensity of 5000–10 000 nuclei was recorded. Fluorescence peaks and relative fluorescence intensity were analyzed using FloMax version 2.3 (Partec).

Specimen examination

Because *A. menglianense* has generally been misidentified as *A. philippense*, we examined the specimens under each name in the HAST, HGAS, HITBC, IBK, KUN, NAS, PE, PYU, QTPMB, TAI, and TAIF herbaria. The distribution was mapped using the obtained results, the records of *A. malesianum* from the Global Biodiversity Information Facility and Chinese Virtual Herbarium databases, and reports of *A.* × *meishanianum* from Zhang et al. (2014) and Wang et al. (2014).

Results

A total of seven new sequences among the total of 27 taxa were generated in the cpDNA matrix of *rps16-matK* IGS + *matK* containing 1605 bp characters with 632 variable sites and 269 parsimony-informative sites. The optimal ML tree showed a negative log-likelihood score (–lnL) of 5928.7397. *Adiantum menglianense* and "the paternal species in Taiwan" formed a highly supported monophyletic group with an MLBS of 100 as sister clades of *A. philippense*. Moreover, all *rps16-matK* IGS + *matK* sequences of "the paternal species in Taiwan" were identical to those of *A. menglianense*. The sequences of *A.* × *meishanianum* from Yunnan and those from Taiwan were also clustered in the *A. malesianum* clade, which had an MLBS of 100 (Fig. 1).

The nuclear DNA matrix of *CRY2*'s first intron contained 510 bp characters, 127 of which were variable and 94 were parsimony-informative. The log-likelihood score was −1747.2164 for the most probable ML tree. A total of 13 haplotypes were generated. *Adiantum menglianense* and "the paternal species in Taiwan" shared the same haplotypes, which were distinct from that of *A. philippense*. Moreover, *A.* × *meishanianum* from Yunnan and Taiwan shared the same three haplotypes. Two of these haplotypes were identical to those of *A. malesianum*, whereas the remaining haplotype was clustered with *A. menglianense* (Fig. 2).

No difference was observed in the *rps16-matK* IGS + *matK* barcoding sequence between *A. menglianense* and "the paternal species in Taiwan", as indicated by their genetic distance of zero. Their inter-taxon distances were significantly larger than their intra-taxon distances compared with *A. philippense* (Fig. 3).

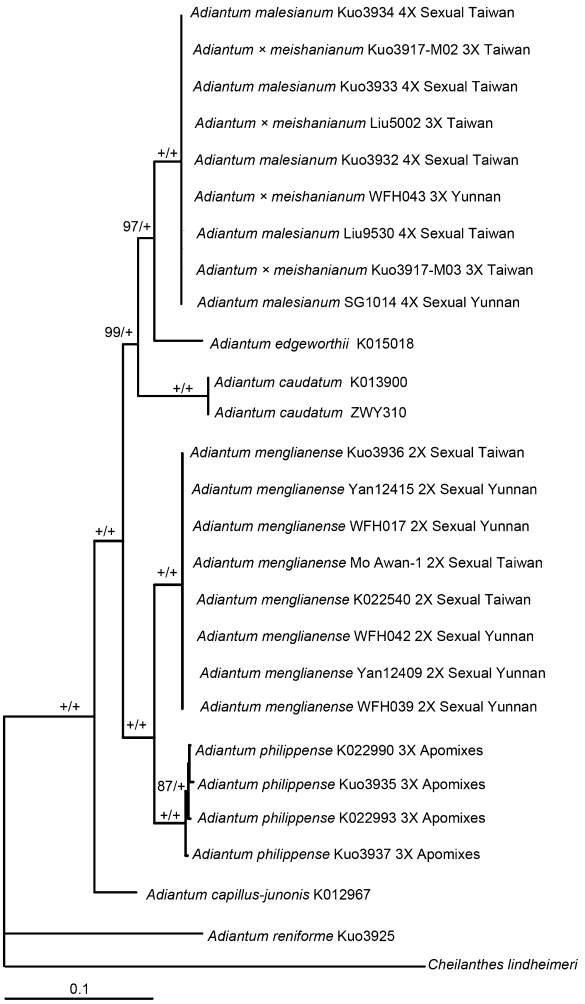

Fig. 1. Phylogeny of 26 *Adiantum* samples and *Cheilanthes lindheimeri* Hook. based on *rps16-matK* IGS + *matK*. Maximum likelihood bootstrap support (MLBS) values and posterior probabilities of Bayesian phylogenetic inference (PP) are indicated on each branch as MLBS/PP. The plus (+) sign represents MLBS = 100 or PP = 1.00.

Because the spores of *A.* × *meishanianum* were aborted and irregularly shaped, its spore size was not compared for ploidy level (Fig. 4A). *Adiantum malesianum* is tetraploid; thus, its spores should be diploid. The spores of *A. menglianense* were smaller than the diploid spores of *A. malesianum* (Fig. 4B). This result indicated that the former must contain only one chromosome complement. Thus, the sporophyte of *A. menglianense* should be diploid, similar to "the paternal species in Taiwan". However, this evidence was rather weak, so we measured the relative DNA C-values of *A. menglianense* and compared the results with the known *A. malesianum* tetraploid. We found that the relative fluorescence intensities were 36.70 ± 2.71 for *A. menglianense*, 58.20 ± 4.53 for *A.* × *meishanianum*, and 80.95 ± 4.53 for the internal

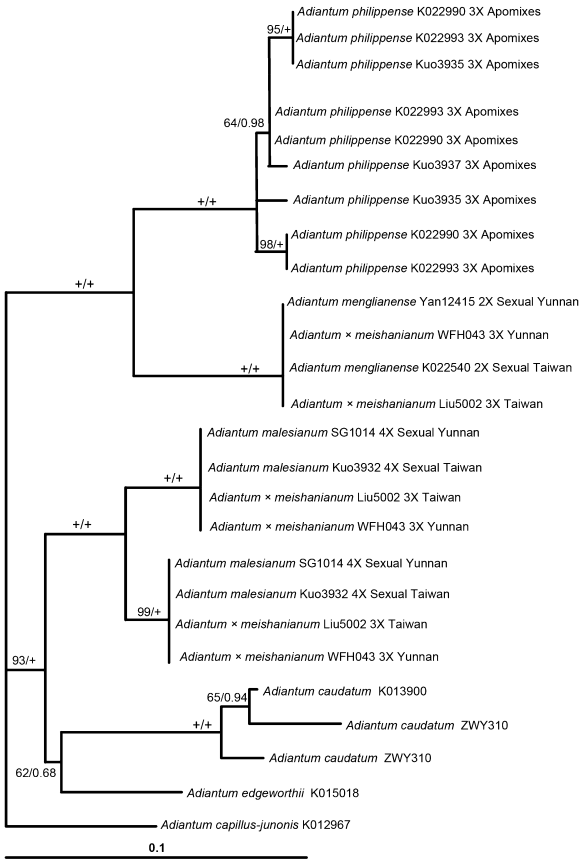

Fig. 2. Phylogeny of *Adiantum* species based on *CRY2*'s first intron. Maximum bootstrap support (MLBS) values and posterior probabilities of Bayesian phylogenetic inference (PP) are indicated on each branch as MLBS/PP. The plus (+) sign represents MLBS = 100 or PP = 1.00.

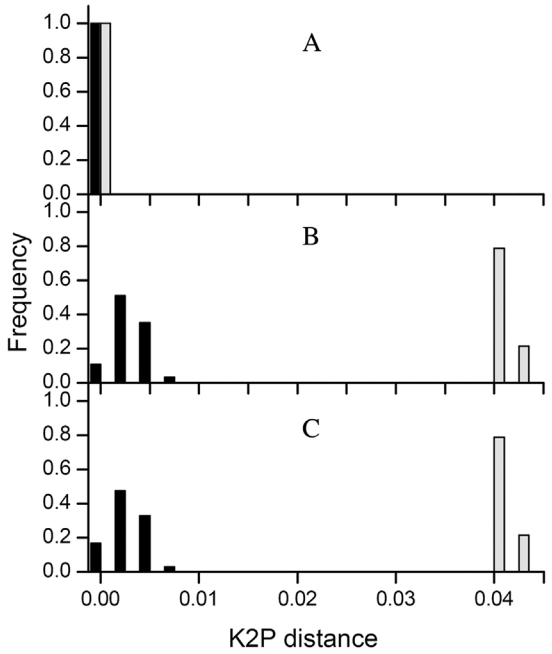

Fig. 3. Distribution of intra-taxa (black) and inter-taxa (gray) Kimura two parameter (K2P) distances based on *rps16-matK* IGS + *matK* sequence as barcode. **A**, *Adiantum menglianense* versus "the paternal species in Taiwan". **B**, *A. philippense* versus "the paternal species in Taiwan". **C**, *A. menglianense* versus *A. philippense*.

standard *A. malesianum*. Therefore, *A. menglianense* was diploid and *A.* × *meishanianum* was triploid because the relative fluorescence intensity of *A. malesianum* accessions was approximately twofold higher than that of *A. menglianense* accessions and 1.5-fold higher than that of *A.* × *meishanianum*. Figure 5 illustrates the flow cytometry histograms of the three plants.

Nearly 60 specimens of *A. menglianense* (Table S2) under the name of *A. philippense* were re-identified. Based on these records, *A. menglianense* had a disjunctive distribution in the region of Pan-Himalaya (especially Yunnan) and Taiwan, whereas *A. malesianum* was widely distributed in tropical Asia, including southern China and some Pacific islands. *Adiantum* × *meishanianum* was recorded only in three

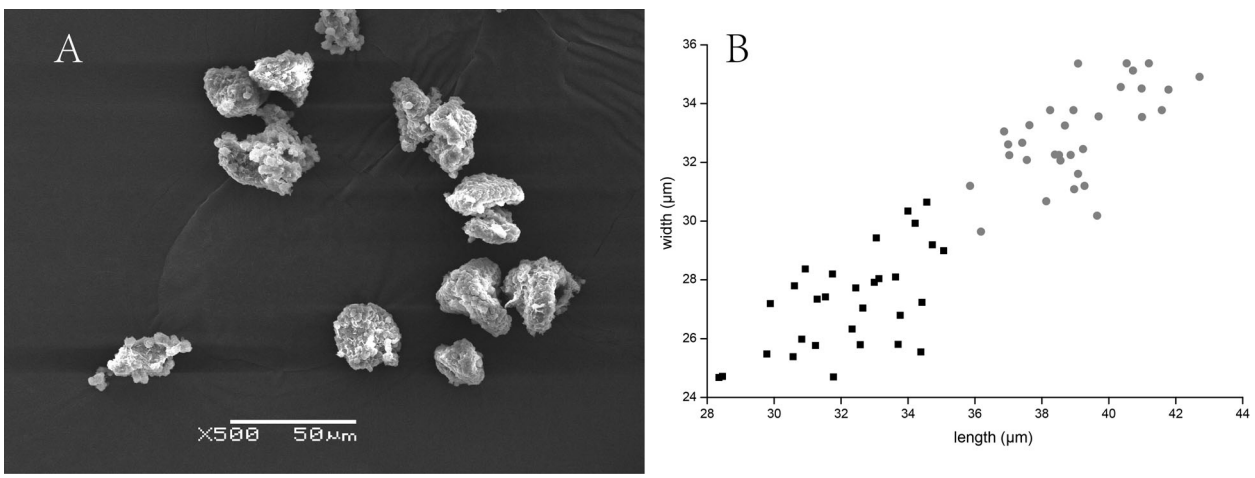

Fig. 4. A, Aborted and irregularly shaped spores of *Adiantum* × *meishanianum*. **B**, Spore sizes of *A. malesianum* (gray cycles) and *A. menglianense* (black squares).

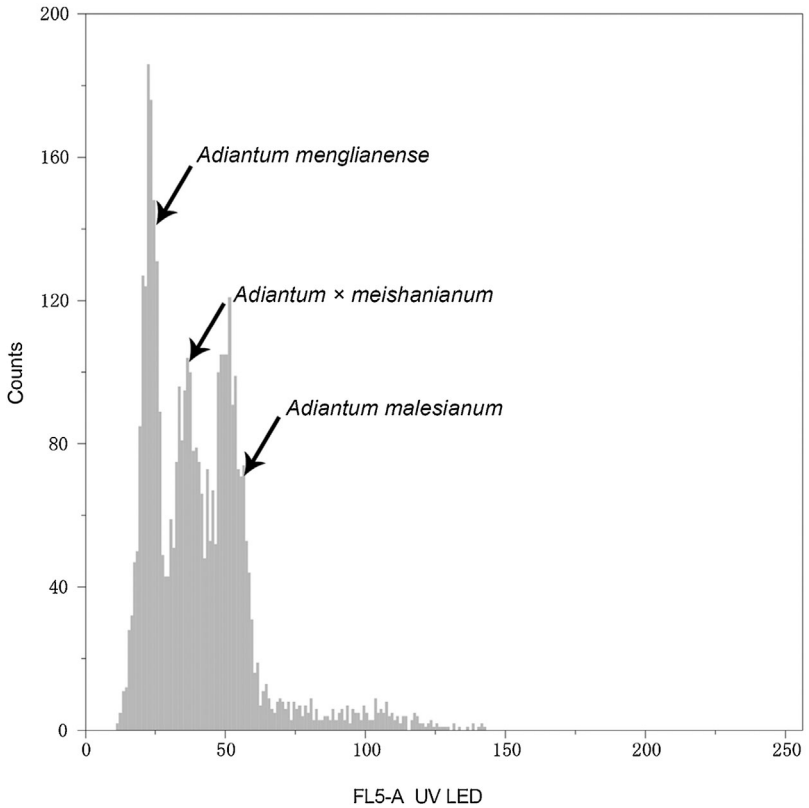

Fig. 5. Flow cytometry histogram of *Adiantum menglianense*, *A.* × *meishanianum*, and *A. malesianum*. FL5-A UV LED, 4′,6′-diamidino-2-phenylindole dihydrochloride fluorescence intensity excited by UV light.

localities overlapping with *A. menglianense* and *A. malesianum*, in Yunnan, and in Taiwan (Fig. 6).

Discussion

Adiantum menglianense as the paternal species

Zhang et al. (2014) identified the paternal parent of *A.* × *meishanianum* as a cryptic species of *A. philippense* because the former is morphologically indistinguishable but genetically divergent. The two species also differ in ploidy and reproductive mode. Our barcoding analysis based on the K2P model revealed a significant gap between the inter- and intra-taxon genetic distances; this result suggested that "the paternal species in Taiwan" should be a distinctive species from *A. philippense*. The barcoding analysis between *A. menglianense* and *A. philippense* showed a similar pattern. Moreover, the K2P distance based on cpDNA was zero between *A. menglianense* and "the paternal species in Taiwan", and they contained the same nuclear haplotype. Ploidy analyses also indicated that *A. menglianense* was diploid. The presence of 64 spores in a sporangium indicated that *A. menglianense* was sexually reproducing, as confirmed by a spore propagation experiment (Zhao et al., 2015, unpublished data). Based on these findings, we can conclude that the paternal parent of *A.* × *meishanianum* should be *A. menglianense*.

Disjunctive distribution and multiple hybridization

Based on the *rbcL* phylogeny, Wang et al. (2014) hypothesized that *A.* × *meishanianum* from Yunnan shared a maternal origin with *A.* × *meishanianum* from Taiwan; this finding was confirmed by our result using the *matK* region with the primers designed by Zhang et al. (2014). The phylogeny reconstructed using a nuclear region revealed that, similar to *A.* × *meishanianum* from Taiwan, *A.* × *meishanianum* from Yunnan contained haplotypes from *A. malesianum* and *A. menglianense*. Thus, *A.* × *meishanianum* from Yunnan and Taiwan were the same hybrid from the same parent species. *Adiantum* × *meishanianum* likely originated from multiple hybridizations in Yunnan and Taiwan, as evidenced by the pattern of disjunctive distribution in the southwestern region and Taiwan in China.

The independent origin of multiple hybridizations can occur in allopatric locations when both parents exist. High mute diversity markers can reveal an origin from different lineages and can thus be used to prove an independent hybrid origin (e.g., Hunt et al., 2011). Unfortunately, the chloroplast and nuclear regions in our study can only distinguish species but cannot identify their origin. The allopatric distribution patterns of hybrids can originate in only two ways: from long-distance dispersal or from multiple hybridizations.

To prove the former, we needed to find evidence that *A.* × *meishanianum* had a high dispersal capacity. Our results showed that spores of *A. meishanianum* were abortive, so long-distance dispersal seems unlikely. Although *A. meishanianum* can reproduce by asexual propagation, propagule dispersion across long distances, especially across the Taiwan Strait, is unimaginable.

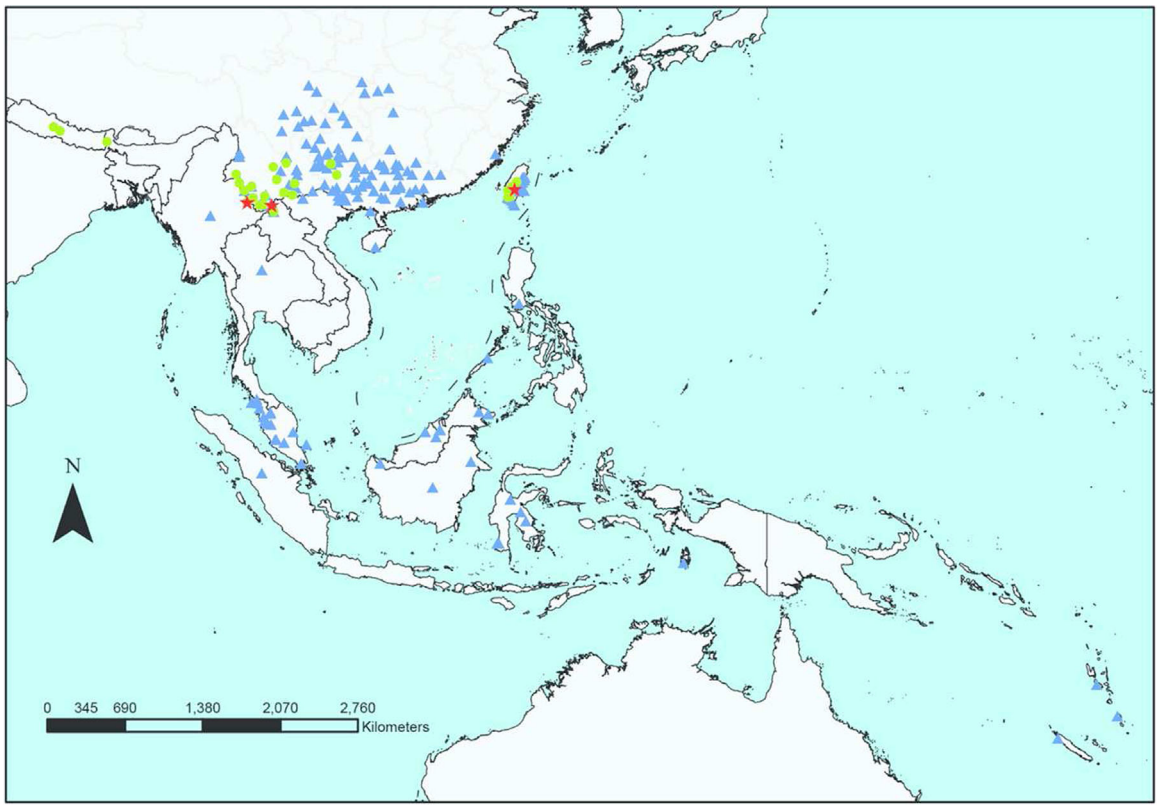

Fig. 6. Distribution of *Adiantum menglianense* (green circles), *A.* × *meishanianum* (red stars), and *A. malesianum* (blue triangles), using a map available from http://219.238.166.215/mcp/index.asp.

Proving the second origin through DNA sequences without variation was difficult. However, some hints were available from the overlapping distribution regions of the disjunctive parents. *Adiantum malesianum*, which is the maternal parent of *A.* × *meishanianum*, was widely distributed in tropical Asia, especially in southern China, whereas *A. menglianense*, as its male parent, was disjunctively distributed in the Pan-Himalaya region and Taiwan according to specimen records. Consequently, the hybrid zone was also disjunctive. This distribution pattern was a precondition for the establishment of an allopatric hybrid.

As mentioned above, both parents of *A.* × *meishanianum* were distributed in Yunnan and Taiwan, and their hybrid offspring cannot disperse over a long distances. Thus, the most probable reason for the disjunctive distribution was multiple hybridizations.

Conclusion

Adiantum × *meishanianum* probably originated from multiple hybridizations in Taiwan and Yunnan, independently. The paternal and maternal parents of this species should be *A. menglianense* and *A. malesianum*, respectively.

Acknowledgements

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Supplementary Material

The following supplementary material is available online for this article at http://onlinelibrary.wiley.com/doi/10.111/jse.12205/suppinfo:

Table S1. List of GenBank accessions used in the phylogenetic analyses of *Adiantum* species.

Table S2. Specimen records of *Adiantum menglianense*.