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Abstract

Discovery of cryptic diversity is essential to understanding both the process of speciation and the conservation of species. Determining species boundaries in fern lineages represents a major challenge due to few morphologically diagnostic characters and frequent hybridization. Genomic data has substantially enhanced our understanding of the speciation process, increased the resolution of species delimitation studies, and led to the discovery of cryptic diversity. Here, we employed restriction-site-associated DNA sequencing (RAD-seq) and integrated phylogenomic and population genomic analyses to investigate phylogenetic relationships and evolutionary history of 16 tree ferns with marginate scales (Cyatheaceae) from China and Vietnam. We conducted multiple species delimitation analyses using the multispecies coalescent (MSC) model and novel approaches based on genealogical divergence index (*gdi*) and isolation by distance (IBD). In addition, we inferred species trees using concatenation and several coalescent-based methods, and assessed hybridization patterns and rate of gene flow across the phylogeny. We obtained highly supported and generally congruent phylogenies inferred from concatenated and summary-coalescent methods, and the monophyly of all currently recognized species were strongly supported. Our results revealed substantial evidence of cryptic diversity in three widely distributed *Gymnosphaera* species, each of which was composite of two highly structure lineages that may correspond to cryptic species. We found that hybridization was fairly common between not only closely related species, but also distantly related species between genera. Collectively, it appears that scaly tree ferns may contain cryptic diversity and hybridization has played an important role throughout the evolutionary history of this group.

Keywords: cryptic diversity; hybridization; phylogeny; RAD-seq; species delimitation; tree fern.

1. Introduction

Cryptic species are defined as being genetically distinct but morphologically similar species (Bickford et al., 2007). Mounting evidence indicates that cryptic species are apparently widespread across the tree of life and there have been recent calls for identifying cryptic diversity and for properly integrating them into understanding Earth's biodiversity (Bickford et al., 2007; Struck et al. 2018). However, discerning the status of potential cryptic species is not a trivial task due to several factors, including homoplasious and plastic morphological characters and the limited resolution of molecular markers (Quattrini et al., 2019). Identifying cryptic species is even more challenging in the case of fern species due to their remarkable degree of morphological stasis, where diagnostic morphological characters are often scarce (Paris et al., 1989). In addition, the delimitation of species is also subject to different species concepts and ways of diagnosing them (de Queiroz, 2007). In this study, we adopt the generalized lineage species concept (de Queiroz, 2007), which differentiates species as separately evolving metapopulation lineages that remain largely intact when in contact with close relatives.

Hybridization and introgression can blur species boundaries and impose further challenges for both species delimitation and reconstructions of phylogenetic relationships among taxa. Hybridization is particularly common in ferns and has long been accepted as an important evolutionary mechanism in fern lineages (Barrington et al., 1989; Wood et al., 2009; Sigel, 2016). The frequent hybridization in ferns is thought to result from their reproductive strategy that comprise both a free-living sporophyte and free-living gametophyte (Knobloch, 1976). Through this reproductive system, high rates of gene flow are believed to occur among ferns, reducing the rate of formation of reproductive barriers and resulting in less species diversity in ferns compared to angiosperms (Ranker and Sundue, 2015).

Recent progress in the establishment of new genomic approaches for high-throughput sequencing like restriction enzyme-based methods (e.g., RAD-seq, Baird et al., 2008) has enabled genomics at the level of populations to be considered with phylogenomics in studies of the pattern and process of speciation, species delimitation and hybridization between closely species. The development of species delimitation methods based on multispecies coalescent (MSC) model (e.g. Knowles and Carstens, 2007; O'Meara, 2010; Yang and Rannala, 2010; Grummer et al., 2014) provides an objective and operational way to jointly infer species boundaries and phylogeny based on large datasets. While these methods have the ability to account for gene tree conflict in species delimitation tests, they generally assume no gene flow among lineages, this assumption may frequently be violated in natural systems (Bacon et al., 2012). Furthermore, coalescent-based approaches do not account for the information conveyed by the spatial distribution of genetic diversity and consequently lead to potential over-splitting of species diversity when dealing with allopatric populations (Gratton et al., 2016; Sukumaran and Knowles, 2017). To prevent these problems and to delimit species with more accuracy, Jackson et al. (2017) proposed a heuristic criterion, the genealogical divergence index (*gdi*). Leaché et al. (2019) further suggesting that *gdi* helps to differentiate between population structure and species-level divergence. More recently, Hausdorf and Hennig (2020) developed a species delimitation approach that integrates spatial information by testing whether the genetic variation between two candidate species can be explained by isolation by distance (IBD). In parallel, a diverse set of sophisticated statistical tests have been developed that utilize genome-wide data for the detection of hybridization and introgression between closely related species (Gronau et al., 2011; Payseur and Rieseberg, 2016; Dalquen et al., 2017; Wen et al., 2018). In the past two decades, genome-wide multi-locus approaches have been used to resolve difficult phylogenies, uncover cryptic diversity and detect hybridization and introgression in many taxonomic groups including flowering

plants (Morales-Briones et al., 2018; Spriggs et al., 2019) and animals (MacGuigan and Near, 2019; Poelstra et al., 2021), but they have not yet been widely used in fern lineages (Wang et al., 2020; Reviewed by Pelosi and Sessa, 2021).

Cyatheaceae are one of the families with the richest species in ferns and represent ca. 90% of the species diversity in the order Cyatheales (PPG I, 2016). This pantropical family of ferns comprises about 643 species distributed throughout the tropical, subtropical and the south-temperate regions of the world, within the greatest species diversity in tropical areas of America and Malesia (Korall and Pryer, 2014). Phylogenetic studies have shown evidence for four major lineages with Cyatheaceae corresponding to the four recognized genera: *Alsophila* R. Br., *Cyathea* Smith, *Gymnosphaera* Blume, and *Sphaeropteris* Bernh (Korall et al., 2006; Janssen et al., 2008; Dong and Zuo, 2018). Cyatheaceae are featured by the strong development of local endemism (Tryon and Gastony, 1975), with a large number of endemic species restricted to very small ranges in tropical and subtropical montane forests (Janssen et al., 2008; Ramírez-Barahona et al., 2011). Morphological evidence suggests frequent natural hybridization in this group (Conant, 1975, 1990; Conant and Cooper-Driver, 1980; Caluff, 2002; Janssen and Rakotondrainibe, 2007). However, the extent of gene flow within and between different scaly tree fern species is largely unexplored.

Here we focused on 16 scaly tree fern species from China and Vietnam, including 13 *Gymnosphaera* species and three *Alsophila* species (Table S1). A previous phylogenetic analysis based on single-copy nuclear loci indicated multiple instances of paraphyly and revealed the presence of cryptic diversity in the genus *Gymnosphaera* (Dong et al., 2019). One cryptic lineage identified within *G. podophylla* was confirmed to represent a different species, *G. bonii*, which was considered as conspecific to *G. podophylla* by Tardieu-Blot and Christensen (1939), while another one (then labelled as *Gymnosphaera* sp. 1) was described as a new species *G. bachmaensis* (Dong et al., 2022). A divergent lineage identified by Dong

et al. (2019) as *Gymnosphaera* sp. 2 was hypothesized also to be an undescribed species, pending further morphological studies. Dong et al. (2022) also described a new species, *G. saxicola,* from southern Yunnan, China. Phylogenetic relationships among species have not been fully resolved, as previous molecular studies were based on limited loci and taxa (Dong and Zuo, 2018; Dong et al., 2019, 2022). It is likely that additional species-level diversity awaits discovery. Previous attempts to revolve relationships using sequence data were complicated with tree discordance between nuclear genes and between the nuclear and plastid markers (Dong et al., 2019), consistent with hybridization and/or strong incomplete lineage sorting (ILS).

In this study, we use thousands of nuclear loci to determine phylogenetic relationships, population structure, species limits and extent of hybridization in scaly tree ferns from China and Vietnam. To this end, we first reconstruct the phylogenomic and phylogeographical relationships among taxa via population-level sampling across their distribution range to infer evolutionary relationships and determine whether lineage divergences and observed population structure corresponds with statistically-defensible hypothesized species boundaries. Then, we conduct species delimitation analyses using the MSC model and novel approaches based on *gdi* and IBD test to evaluate the current species boundaries and identify possible yet unrecognized cryptic diversity. Finally, we integrate a series of population genetic and phylogenetic approaches to assess hybridization patterns and rate of gene flow between taxa.

2. Materials and Methods

2.1. Sample collection and RAD sequencing

We collected 353 individuals of 13 *Gymnosphaera* species, three *Alsophila* species and an outgroup species (*Sphaeropteris brunoniana*) from 129 populations (Fig. 1, Table S1).

Each ingroup species was represented by 1–23 populations (1–3 individuals per population) which cover their most major distribution. We extracted total genomic DNA using a modified CTAB method (Doyle, 1987) for restriction-site-associated DNA sequencing (RAD-seq; Baird et al., 2008). The RAD libraries were constructed and sequenced by Novogene Bioinformatics Institute (Beijing, China). Paired-end (150bp) sequencing was conducted on an Illumina Hiseq 2000 platform (San Diego, CA).

2.2. SNP calling and data filtering

Raw reads were filtered by removing adapters and low quality sequences using Software FASTX_Toolkit (http://hannonlab.cshl.edu/fastx_toolkit) and a perl script developed by C. Feng (http://github.com/scbgfengchao/). Reads containing more than 5% sites with Phred scores <20 were discarded. The retained clean reads were assembled using the *de novo* assembly pipeline of STACKS v2.1 (Rochette et al., 2019). At least five identical reads were required to create a stack using *ustacks* (option –m 5) for each individual, and a catalog of all loci across populations was constructed by *cstacks*. After matching each sample against the catalog using *sstacks*, *tsv2bam* and *gstacks* were executed to incorporate paired-end reads to identify and phase the SNPs. Variant call format (vcf) and haplotype format of called variants were exported using *populations*. We created different datasets by different filtering and sub-sampling for population genomic analysis, species delimitation and phylogeny reconstruction. For all analyses, only two alleles were allowed at each locus, SNPs with observed heterozygosity above 0.5 and extremely high coverage (greater than E+2SD) were discarded. For population genomic analysis, we excluded the outgroup and selected the first SNP per locus (--write-single-snp) to ensure unlinked SNPs were retained. Loci with minor allele frequencies <0.02 were removed. In addition, we created five more data sets using the same filtering options for the further analyses of genetic structure based on the following subset of

176 samples: (1) *Alsophila*, (2) *Gymnosphaera*, (3) *G. podophylla*, (4), *G. henryi*, and (5) *G.*
177 *denticulata*. For phylogenomic analyses, we created different data sets with changed missing
178 rates by controlling p (minimum number of populations a locus must be present in to process
179 a locus: 9, 10, and 11) and r (minimum percentage of individuals across populations required
180 to process a locus: 50% and 70%). As hybrid individuals could affect the inference of
181 phylogenic trees, we excluded all admixed samples (admixture proportions > 5%) identified
182 by Structure for SVDQuartets analyses, and the same filtering strategy was applied as in the
183 population genetic analyses. For BFD*(Bayes factor delimitation with genomic data) and
184 SNAPP species tree analysis, dataset was down-sampled for computational feasibility by
185 selecting three individuals per *Gymnosphaera* species with the least missing data and
186 admixture proportion. We kept unlinked SNPs shared by all these selected individuals for
187 each clade of *Gymnosphaera*. For BPP analyses, we randomly selected 500 RAD loci longer
188 than 400bp from the same data set used in SNAPP analyses. Besides, variants of the
189 chloroplast genome were also called for phylogenetic analyses. Clean reads were mapped to
190 the published chloroplast genome of *G. podophylla* (Liu et al., 2017), and SNPs were
191 identified using SAMtools (Li et al., 2009) and VARSCAN (Koboldt et al., 2012).
192 VCFTOOLS (Danecek et al., 2011) was used to remove sites with missing rates > 25%. All
193 sample information can be found in Table S1.
194
195 *2.3. Concatenated phylogenetic inference*
196 To evaluate the relationships between both individuals and populations, phylogenetic
197 trees based on concatenation analysis using maximum likelihood (ML) were constructed
198 based on nuclear and chloroplast SNP data sets separately. ModelFinder (Kalyaanamoorthy et
199 al., 2017) implemented in IQTREE v2 (Nguyen et al., 2015) was used to choose the best-fit
200 nucleotide substitution models based on Bayesian information criterion (BIC). For each

dataset, IQTREE was run with ascertainment bias correction (+ASC) model (Lewis, 2001) and 1000 ultrafast bootstrap replicates.

2.4. Clustering of species and populations

Population structure was examined using a Bayesian clustering algorithm implemented in STRUCTURE v2.3.4 (Pritchard et al., 2000). The initial analysis was performed with all 352 individuals of *Gymnosphaera* and *Alsophila*. Further runs were executed for each genus (240 individuals for *Gymnosphaera* and 112 individuals for *Alsophila*) in order to detect genetic structure within them. As each of the three broadly distributed species (*G. henryi*; *G. podophylla*; *G. denticulata*) was resolved into two distinct lineages based on phylogenetic analyses, we also conducted three more runs of STRUCTURE for these three species. For each dataset, we ran eight independent replicates for 150,000 generations with the first 50,000 generations discarded as burn-in. The range of K for each dataset were determined based on the species/populations number and size of datasets (*Gymnosphaera* + *Alsophila*: K=1–16; *Gymnosphaera*: K=1–10; *Alsophila*: K=1–5; *G. henryi*: K=1–9; *G. podophylla*: 1–10; *G. denticulata*: K=1–10). The optimal K value was determined according to the method of Evanno et al. (2005). The output of the analysis that produced the best K was aligned by CLUMPP (Jakobsson and Rosenberg, 2007) and then visualized by Distruct (Rosenberg, 2004).

2.5. Species delimitation

We developed species hypotheses according to our ML phylogeny and STRUCTURE results, and different approaches were used to test whether samples clustered into monophyletic lineages should be regarded as distinct species. Due to computational constraints, we performed species delimitation analyses separately for each groups of the

genus *Gymnosphaera*.

First, we conducted Bayes factor delimitation (BFD*; Leaché, 2014) and Bayesian Phylogenetics and Phylogeography (BPP) analyses to compare several candidate species models (Table S2). BFD* analysis was performed using SNAPP (Bryant et al., 2012) implemented in BEAST V2.6 with unlinked SNP shared by all selected individuals. For each candidate species model, marginal likelihood estimate (MLE) was calculated through path sampling with 48 steps. Path sampling parameters were set as follows: ChainLength = 1000,000, PreBurnin=50,000, BurnInPercentage=10% and alpha=0.3. We ranked candidate species models by MLE and calculated Bayes factors (BF) to compare different models. As BPP analysis expect full-length alignments, we randomly selected 500 RAD-loci with minimum length longer than 400bp from the same dataset used in SNAPP analysis. Analysis A10 (species delimitation based on user-specified tree; Yang and Rannala, 2010; Rannala and Yang, 2013) were performed based on reversible-jump MCMC algorithm 0 (ε=2) and algorithm 1(α =2, m = 1). We sampled every 50 generations for 10,000 samples for each run, and the first 2000 samples were discarded as burn-in. Estimated sample size (ESS) was used as an indication of convergence when the value was greater than 200. In addition, we also calculated *gdi* (Jackson et al., 2017; Leaché et al., 2019) for each putative sister species pairs based on the equation: $gdi = 1\text{-}e^{-2\tau/\theta A}$, where τ is the divergence time of species pairs and θ is effective population size. A00 (estimate population parameters with fixed species tree; Rannala and Yang, 2013; Flouri et al., 2020) analysis was conducted to get these two parameters using BPP. According to Jackson (2017), high *gdi* values (> 0.7) indicate that populations should be classified as two distinct species and low *gdi* values (< 0.2) suggest a single species, while *gdi* values fail in the range of 0.2 to 0.7 indicate ambiguous status.

Finally, we performed isolation by distance (IBD) tests for putative sister species pairs for the three broadly distributed species by taking geographic information into consideration. IBD

tests were conducted using R package prabclus (Hausdorf and Hennig, 2020). The rejection of the null hypothesis indicated that the genetic distance between two populations could not be explained by their geographic distance alone, suggesting that candidate species should be regarded as distinct species.

2.6. Coalescent phylogenetic inference

We used three different approaches to reconstruct the coalescent-based phylogeny of all putative species: 1) ASTRAL-III (Zhang et al., 2018), a summary-based method to infer species trees from partially resolved gene trees, 2) SVDQuartets (Chifman and Kubatko, 2014) implemented in PAUP*, a site-based coalescent method to estimate species tree from SNPs data using quartet algorithm, and 3) SNAPP implemented in Beast2 to infer species trees from bi-allelic unlinked SNPs directly based on Bayesian MCMC algorithm.

For both ASTRAL and SVDQuartets analysis, a total of 331 samples were used after excluding all admixed individuals. As previous studies found that alignments < 400bp might result in erroneous species trees (Zhang at al. 2018; Linan et al. 2021), we only kept RAD-loci longer than 500bp and contained at least one PIS in our ASTRAL analysis. After filtering, a total of 7326 RAD-loci with mean length of 569bp in the p10r0.7 dataset were used to infer gene trees. To test the robustness of our estimates, we kept 25%, 50%, 75% and 100% of the loci with the highest PIS number for different species tree inferences. Finally, 1831, 3663, 5494 and 7326 gene trees were used as input in ASTRAL to summarize the species tree. We contracted low support branches (BS <10) from gene trees followed Zhang et al. (2018) to improve the accuracy. For SVDQuartets analyses, a total of 11,429 unlinked SNPs were retained after filtering. Analyses were run with 500,000 quartets randomly selected and node support was assessed with 1000 bootstrap replicates.

SNAPP analysis was performed on a subset of 56 individuals with 1,022 unlinked bi-

allelic SNPs shared by all species due to computationally limitation. Four independent replicate Markov-chain Monte Carlo (MCMC) runs were conducted with chain length of two million generations. Convergence was confirmed when estimated sample sizes (ESS) score was greater than 200. After excluding the first 10% as burn-in, results of each run were combined using Logcombiner (http://beast.community/logcombiner.html) and the Maximum clade credibility trees were generated by TreeAnnotator (https://beast.community/treeannotator) implemented in Beast 2.6.

2.7. Divergence time estimation

We estimated the divergence times among species using the method developed by Stange et al. (2018), which extended the Bayesian species-tree inference SNAPP to calibrate time with a molecular clock model. We used the same dataset with the inference of SNAPP species tree. The previously estimated origin time of the crown group of Cyatheaceae based on fossil evidence (Sosa et al., 2016) was used as the root age of our phylogeny. We specified a lognormal distribution calibration (mean=96.9 Mya, standard deviation=0.021) on the root and generated the input file using the Ruby script "snapp_preb.rb" (Stange et al., 2018). Convergence was checked using the same method used in SNAPP species tree analysis.

2.8. Estimation of phylogenetic discordance

We used Quartets Sampling (QS) to assess the level of conflict and support of our phylogeny estimation (Pease et al., 2018). This method can also distinguish branches with low information from those with multiple highly supported but mutually exclusive phylogenetic relationships. For each internal node, QS provides three scores: (i) quartet concordance (QC; the more concordant the closer to 1, discordance increases as the score approaches −1), (ii) quartet differential (QD; the more equal the frequencies of discordant topologies the closer to

1, 0 indicates that only one other discordant topology was found), and (iii) quartet informativeness (QI; 1 for all replicates informative, 0 for no replicates informative; Pease et al., 2018). All RAD-loci shared by at least 14 (70%) species with length longer than 500bp were selected to prepare the concatenated alignment. We performed Quartets Sampling analysis with species phylogeny constructed by ASTRAL-III with all 20 putative species, and 100 replicates were performed for per branch with other options in default.

2.9. Testing for gene flow

To test for historical introgression between species in the nuclear genome, we used Patterson's *D*-statistic (ABBA-BABA statistics; Durand et al., 2011). This *D*-statistic is based on the comparison of relative abundance of two classes of topology patterns in polymorphic sites known as "ABBAs" and "BABAs" (Durand et al., 2011). Briefly, for a given relationship of (((P1, P2) P3) P4), alleles carried by P4 (outgroup) was defined as ancestral state (A), and the derived state was denoted as B. When there is no asymmetric gene flow between P1 and P3 or P2 and P3, the two sister populations/species (P1 and P2) should carry the same proportion of derived alleles (B) with P3. All trios consistent with our inferred species tree with *Sphaeropteris brunoniana* set as the outgroup (P4) were tested using Dsuite software (Malinsky et al. 2021), except for sister taxa, which cannot be accommodated in the *D*-statistic framework. In order to control the false discovery rate (FDR), Benjamini-Hochberg (BH) correction were applied to P values. The *D* values significantly deviated from zero (adjusted P-values < 0.05) indicated the signal of introgression.

In addition, we applied Dfoil-statistics, an extension of *D*-statistics which applies a five-taxon symmetric phylogeny donated as (((P1, P2), (P3, P4)) O), to infer direction of introgression between non-sister species as well as the ancient introgression between the ancestor of a species pairs (P1P2) and extant species (P3/P4) (Pease and Hahn, 2015). To

investigate the ancient introgression and direction of post-speciation introgression, we performed Dfoil analysis with ExDfoil (Lambert et al., 2019), which is more suited for RAD-seq data. As Dfoil analysis requires that the divergence of P1 and P2 should not be earlier than the divergence of P3 and P4, we conducted Dfoil tests for all five-taxon phylogeny consistent with our time-calibrated species tree where *S. brunoniana* was set as the outgroup (O). Individuals used for SNAPP (three individuals per species) as well as admixed individuals were subject to Dfoil analysis. As a result, a total of 947 unique five-taxon phylogenies with 209,070 individual combinations were tested.

3. Results

3.1 Characteristics of the datasets

We generated RAD-seq data for 353 individuals representing 16 scaly tree ferns from China and Vietnam (Fig. 1; Table S1). After quality filtering, a total of 11,281,731,473 reads were obtained across all samples, ranging from 20,664,129 to 72,127,249 (mean=31,959,579) reads per individual (Table S1). The number of RAD loci and SNPs obtained from each data set were summarized in Table S3. The total number of obtained loci used for concatenated maximum likelihood (ML) tree calculation ranged between 8,393(p11r0.7) and 42,647(p9r0.5). For SNAPP analyses, the 56-sample alignment with no missing data contained 1,022 unlinked SNPs. The final cpDNA matrix has 2,520 SNPs, of which 1,806 (72%) SNPs are parsimony informative sites (PIS, Supplementary Table S3).

3.2. Phylogenetic relationships and clustering

The ML phylogenies for each of the six concatenated data sets were highly resolved and were overall congruent despite variation in the proportion of missing data (Fig. S1). Therefore, we hereafter only refer to the phylogeny obtained from the data set with p=10,

r=0.7 for a total of 179,539 SNP with 140,983 PIS. Our ML phylogeny revealed that all individuals were firstly resolved into two highly supported clades, the *Alsophila* clade and the *Gymnosphaera* clade (Fig. 2a). In the *Alsophila* clade, two samples of *A. latebrosa* were nested with those of *A. costularis*, and two samples, each from *A. latebrosa* and *A. costularis* respectively, were nested with those of *A. spinulosa*. The *Gymnosphaera* clade resolved into four main groups: (1) the *G. henryi* group including *G. henryi*, *G. andersonii*, *G. glabra* and *G. khasyana*, (2) the *G. denticulata* group containing *G. denticulata* and *G. metteniana*, (3) the *G. podophylla* group including *G. bachmaensis*, *G. bonii*, *G. podophylla* and a putative new species *Gymnosphaera* sp. 2, and (4) the *G. salletii* group containing *G. salletii, G. saxicola* and *G. austroyunnanensis* (Fig. 2a). All *Gymnosphaera* species were monophyletic with strong support, except *G. metteniana*, which formed a ladder-like cluster between *G. denticulate* and the *G. henryi* group. Interestingly, each of the three broadly distributed species, i.e. *G. henryi, G. denticulata* and *G. podophylla*, was separated into two well-supported, reciprocally monophyletic lineages (Fig. 2a), which generally grouped according to their geographical distribution (Fig. 1c–e). Relationships among individuals in the cpDNA tree show several differences with the RAD tree, but supports for these conflicting topologies were general low (Supplementary Figs. S2–3).

The first run of STRUCTURE at K=2 split the samples of two genera into distinct clusters with almost no admixture (Fig. 2b; Fig. S4a.). Genetic structure analyses within the genus *Alsophila* largely grouped each species into its own genetic cluster (K=3; Fig. 2c; Fig. S4b) with several admixed individuals; this included one *A. costularis* individual which shared ancestry (54%) with *A. spinulosa* and nine *A. latebrosa* individuals with 7% ~ 46% genetic admixed. These results are corroborated by our result of phylogenetic analysis and indicative of interspecific hybridization. Further analysis within the genus *Gymnosphaera* identified K=4 as the best-fit number of clusters, which are concordant with the four lineages

recovered in the ML phylogeny (Fig. 2a, c; Fig. S4c). We observed substantial genetic admixture between lineages. For example, as expected for its allopolyploid hybridization origin (Wang et al., 2020), nearly half of *G. metteniana*'s genetic ancestry is from the *G. henryi* (formerly misapplied with the name *G. gigantea*, cf. Dong et al., 2020) group. Similar a pattern of genetic admixture was also found for *G. khasyana* and the putative new species *Gymnospheara* sp. 2. STRUCTURE analyses also identified several individuals of *G. denticulate* as genetically admixed (Fig. 2c). To explore cryptic diversity within nominal species, we performed separate STRUCTURE analyses for the three broadly distributed species, in each of which we identified an optimal value of K=2 (Fig. S4d–f). There was little admixture between the two lineages for *G. henryi* and *G. podophylla*, whereas one population of *G. denticulata* was observed to have substantial admixture between lineages (Fig. 1c–e). Together with the result of the phylogenetic analysis, this suggests that these three species each represents two cryptic species.

3.3. Species delimitation

Both BPP and BFD analyses support the splitting each of three species, i.e. *G. henryi*, *G. denticulata* and *G. podophylla*, into two separate species (Tables S4–5). These analyses also support species status of the recently described species *G. bachmaensis* and *G. saxicola*, and the undescribed species *Gymnosphaera* sp. 2 (Tables S4–5).

We calculated *gdi* scores for five hypothetical species comparisons (Fig. 3a; Table S6). In two comparisons, the *gdi* values for at least one of the bi-directional comparisons are below 0.2 and indicate a lack of firm species status as defined by Jackson et al. (2017). In contrast, the *gdi* values estimated for two comparisons (*G. austroyunnanensis*-*G. saxicola* and *Gymnosphaera* sp. 2 -*G. podophylla*-1+ *G. podophylla*-2+*G. bonii*+ *G. bachmaensis*) surpassed the 'distinct species' zone (Fig. 3a), while one lineage of *G. denticulata* fell into the

401 ‘ambiguous delimitation’ zone of species delimitation.

402 IBD test showed that the genetic distances between individuals as well as populations
403 belonging to the two sub-clusters of *G. henryi* could not be explained by their geographical
404 distances (Fig. 3b; Fig. S5a; Table S7), indicating that these two sub-clusters should be
405 classified as two distinct species. Similar significant signatures were also detected between
406 the two sub-clusters of *G. podophylla* (Fig. 3c; Fig. S5b; Table S7). Due to limited population
407 sampling for *G. denticulata*, *G. saxicola, and G. austroyunnanensis*, IBD test was only
408 performed at an individual level, and results demonstrated the validity of *G. saxicola* and
409 supported splitting *G. denticulata* into two distinct species (Fig. 3d–e; Table S7). The results
410 of different species delimitation analyses are summarized in Figure 4.

411

412 *3.4. Species tree and divergence time analyses*

413 To examine relationships among species or lineages, we used the species delimitation
414 results from BPP/BFD* for subsequent species tree analysis. The species tree inferred from
415 ASTRAL-III analyses based on different proportion of PIS exhibited identical topologies with
416 only slight changes in branch support (Fig. S6), suggesting that the topology was insensitive
417 to the data filtering with different phylogenetic information per loci. Therefore, the ASTRAL
418 species tree with the highest posterior probabilities is presented here (Fig. 5). The normalized
419 quartet score for this tree is relatively high (0.796), indicating a low level of gene tree
420 discordance. The resulting ASTRAL tree is well supported and is largely consistent with the
421 inferred ML tree (Fig. 5). They differed only in the placements of *G. khasyana* and *A.*
422 *costularis*: (i) *G. khasyana* was sister to the rest of the species in the *G. henryi* group in the
423 ML tree, but sister to all other species of the *G. henryi* group and the *G. denticulata* group in
424 the ASTRAL species tree; (ii) in the ML tree, *A. costularis* was sister to *A. latebrosa*, whereas
425 in the ASTRAL species tree, *A. costularis* was sister to *A. spinulosa*. The species trees

426 inferred from both SVDquartet and SNAPP analyses had much lower bootstrap support
427 values across many nodes. For example, there is one node with less than 55% bootstrap
428 support in SVDquartet tree and three nodes with less than 80% bootstrap support in SNAPP
429 tree (Fig. 5). The topological conflicts between the SVDquartet tree and the ML tree involved
430 *G. metteniana*, which is sister to the *G. henryi* group in the SVDquartet tree, whereas it is
431 sister to *G. denticulata* in the ML tree. The uncertain position of *G. metteniana* might be due
432 to a hybrid origin via *G. henryi* and *G. denticulata* (Wang et al., 2020).
433 The divergence time for *Alsophila* and *Gymnosphaera* was estimated at around 95.67
434 Mya (95% HPD 91.23–100.86 Mya; Fig. S7). Within *Gymnosphaera*, the *G. henryi* + *G.*
435 *denticulata* groups diverged from *G. podophylla* + *G. salletii* groups at around 28.76 Mya
436 (95% HPD 25.41–32.3 Mya), which is later than previous estimates of the *Gymnosphaera*
437 'Asian-clade' (46.51 Mya, Loiseau et al., 2020). The *G. salletii* group subsequently diverged
438 from *G. podophylla* group at 28.22 Mya (95% HPD 24.98–31.85 Mya), and the *G. henryi*
439 group diverged from the *G. denticulate* group at 10.34 Mya (95% HPD 8.64–12.02 Mya).
440 Divergences amongst species within the main groups of *Gymnosphaera* and *Alsophila* ranged
441 from 1.61–23.23 Mya. Our result is congruent with a previous study which found that most
442 species divergence of *Gymnosphaera* and *Alsophila* occurred in the late Miocene to the
443 Pliocene (Dong, 2019).
444
445 *3.5. Quartet Sampling Analyses*
446 To estimate the robustness of resolved relationships, we analyzed species-tree discord
447 using the Quartet Sampling method, which aims to measure branch support in large sparse
448 alignments (Pease et al., 2018). The informativeness of the replicates was generally high with
449 QI values ranging from 0.99 to 1.0 (Fig. S7). Three nodes in the backbone of the groups of *G.*
450 *henryi* and *G. denticulata* as well as the most recent common ancestor of the *G. podophylla*

group show a low QC (-0.19–0.21) in combination with a QD value of 0, indicating strong discordance and a single alternative topology. In contrast, the ancestor to the *G. podophylla* group and the *G. salletii* group is characterized by a medium QD (0.56) and the divergence of *A. spinulosa* and *A. costularis* is characterized by a low QD (0.24). Such low-to-medium QD values indicate a single discordant topology is inferred.

3.6. Hybridization and gene flow

ABBA-BABA tests revealed that about 28% (274/969) of tested four-taxon phylogenies had a significant signal of introgression ($P < 0.05$, non-zero *D*-statistics) (Fig. 6a; Table S8). *D*-statistics ranged from 0.09–0.94 for all the significant tests. Weak introgression was found between *A. costularis* and *G. saxicola/ Gymnosphaera* sp. 2 as well as *A. latebrosa* and *G. austroyunnanensis/ G.podophylla*-1*/G.saxicola* with *D*-statistics varied from 0.10 to 0.23 (Table S8). When only considering triplets involving species of *Gymnosphaera,* 48% (267/560) of *D*-statistics showed significant gene flow. Significant excesses of shared derived alleles were detected between all species of the *G. saxicola* group and all other species of *Gymnosphaera* except for *Gymnosphaera* sp. 2 and *G. bachmaensis*. Similar signals of gene flow were also found in all species pairs between the *G. denticulate* group and *G. podophylla* group.

To further explore the direction of gene flow and determine whether gene flow occurred between ancestral lineages we performed complementary five-taxon Dfoil analysis (Pease and Hahn, 2015). In all 947 unique five-taxon phylogenies, significant evidence of gene flow was detected in 39.2 % (371/947) of quintets (Table S9). When only considering quintets of *Gymnosphera*, 43.2 % (357/827) species combinations showed significant gene flow. Amongst all these significant tests, up to 68.7 % (Fig. 6b; Table S9) of quintets revealed ancestral gene flow without clear direction (P1+P2 and P3/P4) and 31.3% showed recent gene

476 flow (P1 to P3/P4; P2 to P3/P4; P3 to P1/P2; P4 to P1/P2; Fig. 6b; Table S9) among extant
477 species, suggesting that frequently gene flow was likely to have occurred between the
478 ancestors of extant species. Specifically, our analyses detected signals of widespread ancient
479 genomic exchange between all four mainly groups of *Gymnosphera* (Fig. 6c; Table S9),
480 ancient gene flow was also found to have occurred between the ancestors of species of
481 *Alsophilav* and species of the *G. denticulate*-clade (*G. denticulate*-1*; G.denticulata*-2) and *G.*
482 *podophylla*-clade *(G.podophylla*-1+ *G.podophylla*-2+*Gymnosphera* sp. 2*).* Post-speciation
483 introgression was detected in 30 species pairs, and 13 pairs of which were bidirectional (Fig.
484 6c; Table S9). In all these species pairs, gene flow was detected in about 0.2 % ~51.4% of
485 individual combinations, the minimum and maximum ratios were found in gene flow events
486 between *A. costularis* and *G. podophylla*-1, and *G. saxicola* to *G. denticulata*-2, respectively.
487 All these results suggest a high frequency of gene flow amongst both closely related species
488 and distantly related scaly tree fern species.
489

490 **4. Discussion**

491 *4.1 Phylogenetic relationships among species*

492 A robust phylogenetic framework is a prerequisite for understanding evolutionary history
493 and affinities among taxa in any evolutionary radiation. Previous phylogenies inferred using
494 DNA sequences provided poor support for the species relationships of mainland Asian tree
495 ferns (Dong and Zuo, 2018; Dong et al., 2019, 2022). Our ML phylogeny inferred from
496 thousands of RAD loci fully resolved the evolutionary relationships among *Gymnosphaera*
497 taxa and recovered four well supported clades (Fig. 2). The ASTRAL species tree was highly
498 congruent to the ML tree with only two topological differences involved *G. khasyana* and *A.*
499 *costularis*, respectively (Fig. 5). Our other species tree approaches (SVDQuartets and
500 SNAPP) revealed several phylogenetic discords, but these inferences were weakly supported

501 (Fig. 5). Because of the computational time it took to run SNAPP analysis, only a subset of
502 samples with limited SNPs (~1000) were used, which may reduce the resolution. Chou et al.
503 (2015) found that coalescent-based summary methods (i.e., ASTRAL-II) were generally more
504 accurate than SVDquartets, likely due to greater phylogenetic signal across individual loci.
505 We view the ASTRAL topology as more likely to be accurate since ASTRAL is designed to
506 work with multilocus data and the ASTRAL species tree was more similar to concatenation
507 estimates with higher support values overall. Our findings are consistent with other recent
508 studies that found lower node support values for SVDQuartets tree than trees inferred through
509 ML and ASTRAL (Chou et al., 2015; Leaché et al., 2015; Hosner et al., 2016; Molloy and
510 Warnow, 2018).
511 The differences between species-tree inference methods is a familiar problem
512 encountered for phylogenomic datasets (Huang and Knowles, 2009; Lanier et al., 2014;
513 Gatesy and Springer, 2014). This might reflect underlying evolutionary processes such as
514 gene flow and ILS (Maddison, 1997; Knowles and Carstens, 2007; Degnan and Rosenberg,
515 2009). It has been hypothesized that in cases of high levels of ILS or introgression,
516 coalescent-based methods should produce more congruent topologies than concatenation (Liu
517 et al., 2009; Leaché and Rannala, 2011; Mirarab et al., 2016). On the other hand,
518 concatenation methods can be more accurate than coalescent approaches (Mirarab et al.,
519 2016; Springer and Gatesy, 2016) as weak phylogenetic signal in RAD locus might result in
520 poorly resolved gene trees. In our case, concatenated and summary-based coalescent
521 approaches produced largely congruent topologies, suggesting both coalescence and
522 concatenation approaches should be effective in reconstructing relationships of tree ferns. The
523 phylogenetic discordance involving the *G. khasyana* and *A. costularis*, is likely caused by
524 hybridization and introgression. Our quartet sampling analyses revealed that most cases of
525 discordance were skewed toward a single alternative topology (i.e. QD=0; Fig. S7) as is

consistent with hybridization. Indeed, *G. khasyana* displayed substantial genomic admixture between the *G. henryi* group and *G. salletii* group (Fig. 2b–c). Given the prevalent gene flow and hybridization detected among species (discussed below), strictly bifurcating trees may not adequately depict evolutionary history in tree ferns. Nonetheless, our well-supported and generally congruent phylogenies inferred concatenated and summary-based coalescent methods suggest that RAD-seq data could be used to infer the phylogeny for such "living fossil" groups with large genome size (Mean 1C=7.62 pg; Pellicer and Leitch, 2020).

4.2. Cryptic diversity in tree ferns

Despite the increasing availability of genomic data and sophisticated approaches for delimiting species (Carstens et al., 2013; Yang and Rannala, 2014; Jackson et al., 2017; Hausdorf and Hennig, 2020), species delineation remains a difficult task, even for specialists. Specifically, identifying and defining cryptic species is even more challenging in the case of fern taxa due to the lack of morphological divergence accompanying speciation events (Paris et al., 1989). Hence, we adopted an integrative approach to establish putative species limits by considering different sources of information (genetic and geographic factors), as suggested by Carstens et al. (2013).

Our species delimitation analysis based on RAD-seq data confirmed the validity of the recently described species *G. saxicola* and *G. bachmaensis* (Dong et al., 2022). Evidence for the species status of *G. saxicola* was strong and consistent across all applied species delimitation approaches (Fig. 4). In addition to being genetically divergent, it is morphologically distinctive in several morphological characteristics such as frond dimorphism, plant habit, and stipe appendages (Dong et al., 2022). Furthermore, no evidence of gene flow was found between this species and the other species of the *G. salletii* group (Fig. 6a). Similarly, the species status for *G. bachmaensis* was also supported by all

551 delimitation approaches with the exception for *gdi* (Fig. 3a), which provide an ambiguous
552 support for *G. bachmaensis* as a distinct species. In addition, we found strong evidence
553 supporting species status of the undescribed species (*Gymnosphaera* sp. 2; Dong et al., 2019).
554 Samples of this species were collected from southern Vietnam. It is deeply divergent from
555 other species of the *G. podophylla* group by long branch lengths and, our different
556 delimitation approaches all support the recognition as a separate species (Fig. 4). However,
557 we acknowledge that the new species descriptions should await further study of phenotypic
558 differences in specimens, as we so far have not obtained even one fertile frond of it.
559 Through analysing a larger number of geographically representative samples and
560 genome-wide molecular markers, we identified two distinct evolutionary lineages for each of
561 the three widespread species, i.e. *G. henryi, G. denticulate, and G. podophylla*. For *G. henryi*,
562 our genetic clustering analyses showed a clear distinction between populations from Hainan
563 Island and mainland of China and Vietnam. Two discrete and allopatric lineages were also
564 recovered for *G. denticulate* and *G. podophylla* with population genetic and phylogenetic
565 analyses. Our species delimitation with BPP and BFD* approaches provide decisive support
566 for recognition of these lineages as distinct species (Fig. 4). However, a number of recent
567 studies have demonstrated that MSC models have a tendency to oversplitting in species
568 delimitation (Sukumaran and Knowles, 2017; Leaché et al., 2019; Chambers and Hillis,
569 2020). This is particularly true in naturally fragmented systems (Dong et al., 2019), as the
570 case of tree ferns. Jackson et al. (2017) proposed the incorporation of the *gdi* as an attempt to
571 reduce the oversplitting populations into multiple species. In our case, all of the six lineages
572 but the *G. denticulate*-1 have not passed the heuristic criterion of *gdi* as distinct species (Fig.
573 3a). A recent study showed, however, that the *gdi* method is sensitive to gene flow and in the
574 presence of asymmetrical gene flow the *gdi* will lump genuinely distinct species into the same
575 species (Jiao and Yang, 2021). Given the widespread gene flow detected among species, it

576 seems that the model assumption underlying the *gdi* method might have been violated when
577 applied to our system. This led us to further incorporate geographic information. Consistent
578 with the results of MSC-based species delimitation, our IBD analyses support the two
579 independent entities in each of the three widespread species (Fig. 3b–d; Table S7). Taking
580 these results together, we speculate that each of the three species is composited of two cryptic
581 species that exhibiting a clear geographical structure. Currently, we found little or no
582 morphological divergence between the two lineages within each of the three species.
583 Accordingly, these can mostly be regarded as truly cryptic species (i.e. genetically divergent
584 but morphologically indistinguishable species).

585 Ferns are well known to harbor cryptic diversity (Paris et al., 1989; Bauret et al., 2017;
586 Zhang et al., 2019; Kinosian et al., 2020); however, the underlying mechanisms for origin of
587 cryptic species have largely unexplored. Recently, Fišer et al. (2018) reviewed three possible
588 hypotheses have been proposed to explain the occurrence of cryptic diversity: recent
589 divergence, morphological convergence, and niche conservatism. In the case of
590 *Gymnosphaera*, the morphological convergence hypothesis seems unlikely, as this hypothesis
591 infers that morphological similarity evolved among distantly, rather than closely related
592 species, whereas each of the three pairs of identified cryptic lineages are in sister
593 relationships. The three pairs of sister cryptic lineages are young, with divergence time
594 between 1.61Mya and 2.44 Mya (Fig. S7), which seems to fit the recent divergence
595 hypothesis. However, distinct morphological difference were observed between the more
596 recently divergent species of *G. bachmaensis* and *G. bonii,* suggesting that the origin of
597 cryptic species in the genus *Gymnosphaera* cannot been explained by the recent divergence
598 hypothesis alone. In spite of its high species-richness, the family Cyatheaceae were found to
599 be highly conserved in morphological and niche evolution (Bystriakova et al., 2011; Sosa et
600 al., 2016; Loiseau et al., 2020). The fact that tree ferns are restricted to warm and humid

601 montane habitats suggests that morphological stasis might have acted as the most important
602 mechanisms for the occurrence of cryptic species in this group.
603
604 *4.3. Widespread ancient and contemporary hybridization*
605 Hybridization has long been proposed as a major evolutionary mechanism in vascular
606 plants (Abbott et al., 2016), and especially in ferns (Barrington et al., 1989; Sigel, 2016). In
607 the family Cyatheaceae, natural hybridization events have been reported in numerous
608 instances based on morphological and cytological studies (Holttum and Sen, 1961; Conant,
609 1975; Conant and Cooper-Driver, 1980; Large and Braggins, 2004; Edwards, 2005; Janssen
610 and Rakotondrainibe, 2007). We used an integrative approach of phylogeny and population
611 genetics to test for hybridization among closely and distantly related species and found that
612 hybridization was fairly common within and between the two genera of *Gymnosphaera* and
613 *Alsophila*. STRUCTURE analyses indicated that three species, i.e. *G. metteniana*, *G.*
614 *khasyana* and *Gymnosphaera* sp. 2, showed substantial genomic admixture (Fig. 2),
615 suggesting ongoing or recent hybridization between these species. Of them, *G. metteniana*
616 was proved to be an allotetraploid origin via hybridization between *G. henryi* and *G.*
617 *denticulate* accompanied by gene flow (Wang et al. 2020). STRUCTURE analyses also
618 revealed shared ancestry between *A. latebrosa* individuals and *A. costularis* as well as *A.*
619 *spinulosa*, indicating that gene flow has been occurred within *Alsophila*. Our analyses using
620 the ABBA/BABA test detected significant historical introgression for most species pairs (Fig.
621 6). Given that ABBA/BABA test only identifies gene flow between non-sister taxa, the actual
622 degree of introgression could be higher. Furthermore, the results of Dfoil analysis suggested a
623 decline in the proportion of the genome introgression from ancient lineages to more recent
624 splits. This indicates that descendant lineages are on diverging trajectories and genetic
625 incompatibilities may be accumulated, albeit in a slow rate. Collectively, our results provide

clear evidence that hybridization and introgression are likely to be a common phenomenon during diversification of tree ferns such as *Gymnosphaera* and *Alsophila*.

The widespread introgression detected here could be explained by several aspects of fern biology and geography. In comparison with angiosperms, ferns are vulnerable to hybridization owing to their relative ease of dispersal via wind-mediated spores, the lack of physical barriers restricting fertilization and perceived simple reproductive strategy (Knobloch, 1976; Barrington et al., 1989). Our observation of frequent hybridization between distantly related species is consistent with the prevalence of hybrids and reticulation evolution within and even between some fern genera (e.g. Barrington et al., 1989; Paris et al., 1989; Wagner et al., 1992; Grusz et al., 2009; Yatabe et al., 2009; Sessa et al., 2012; Rothfels et al., 2015; Kinosian et al., 2020), and supports the view that reproductive boundaries may generally be weak in ferns (Barrington et al., 1989).

5. Conclusions

In this study we demonstrated the utility of RAD-seq to generate robust phylogenetic relationships at the family level in Cyatheaceae and to objectively infer species boundaries by testing alternative delimitation hypotheses. By using genome-wide SNP data, we were able to reconstruct a high-supported phylogeny of scaly tree ferns from China and Vietnam, which facilitated our understanding the evolutionary history of this living fossil plant group. Our integrative species delimitation approaches provide strong evidence supporting the existence of cryptic diversity in the three broadly distributed members of the genus *Gymnosphaera*. In addition, we found genome-wide evidence for prevalent hybridization between not only closely related species, but also distantly related species between genera. Overall, our results suggest that scaly tree ferns may contain cryptic diversity and hybridization might have played an important role throughout the evolutionary history of this group.

Data availability

Data supporting the findings of this work is available within the paper and Supplementary Information files. All of the raw sequence reads and assemblies described in this manuscript have been submitted to the National Center for Biotechnology Information (NCBI) under accession codes PRJNA849405.

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Supplementary materials

Figure S1 Concatenated maximum likelihood (ML) tree topologies derived from nuclear SNPs with different assemble parameters of Stacks (*populations*). Different color correspond to different species. Bootstrap support values of 100% are indicated by black points on each node.

Figure S2 Concatenated maximum likelihood (ML) tree topologies derived from chloroplast SNPs. Different color correspond to different species. Bootstrap support values of 100% are indicated by black points on each node.

Figure S3 The discordance among the nuclear (left) and chloroplast (right) SNPs-based maximum likelihood tree. All branch lengths are fixed to simplify viewing of the tree topology. Different color correspond to different species.

Figure S4 The calculation of ΔK of STRUCTURE analyses for **(a)** all 352 individuals of 13 *Gymnosphaera* species and three *Alsophila* species, **(b)** 240 individuals of 13 *Gymnosphaera* species, **(c)** 112 individuals of three *Alsophila* species, **(d)** 64 individuals from 23 populations of *G. henryi*, **(e)** 54 individuals from 20 populations of *G. podophylla*, and **(f)** 23 individuals from 9 populations of *G. denticulata*.

Figure S5 Relationship between genetic and geographical distances in pairs of populations of candidate species. Black and red circles represent the distance between populations belonging to the first or second candidate species, green circles represent the distance between populations belong to different candidate species. See more annotation for lines in Figure 3.

Figure S6 ASTRAL-III summary coalescent species tree. Numbers at nodes suggest the local posterior probabilities for different proportion (25%/50%/75%/100%) of parsimony informative sites (PIS) are used.

Figure S7 Time-calibrated phylogeny and Quartet Sampling (QS) analyses. The number on nodes indicate the median age estimates and the horizontal bars indicate the 95% highest

posterior density intervals. Pie charts below nodes indicate the Quartet Concordance (QC)/quartet Differential (QD)/Quartet Informativeness (QI) score for the internal branches of the phylogeny. Red represents QS score of 1/NA/1, meaning full support to the focal branch, pink represents a QS score of 0.66/0/1, meaning strong support, orange represents a QS score of 0.21/0.56/1 or 0.18/0/0.99 or 0.14/0/1, meaning weak support; green represents a QS score with QC<0 and QD is low, meaning counter support.

Table S1. Sampling information and details of datasets used for different analyses.

Table S2. Species delimitation hypotheses.

Table S3. Parameter combinations used in data assembly and data characteristics for all analysis.

Table S4. Results of Bayes factor delimitation with genomic data (BFD*).

Table S5. Results of guided delimitation with BPP.

Table S6. Genealogical divergence index (*gdi*) for putative sister species pairs.

Table S7. *P* values obtained for the isolation-by-distance (IBD) tests. The first test, H_{01}, hypothesis that the relationship between the genetic and geographical distance within each candidate species can be modeled by the same regression. If H_{01} is not rejected, H_{02} (the regression fitted on all within-group distances also fits the distances between the two candidate species) is further tested. If H01 is rejected, H_{03} (the regression fitted on between-group distances is at least consistent with the regression fitted on one of the candidate species) is further tested. The significantly rejection to the H_{02} (or the both tests of H_{03}) indicate that the two candidate species might represent two distinct species.

Table S8. Details of ABBA-BABA statistics tested using *D*-suites. *Sphaeropteris brunoniana* is fixed as the outgroup for all tests.

Table S9. Summary of Dfoil analyses using ExDfoil. *Sphaeropteris brunoniana* is fixed as the outgroup for all tests. The total number of individual combinations, and the number of significant tests for corresponding introgression type for each species combination are reported.

Figure Legends

Figure 1 Sampling locations for all species investigated. Separated maps were generated for **(a)** *Gymnosphaera*, **(b)** *Alsophila*, and the three broadly distributed species of *Gymnosphaera*: **(c)** *Gymnosphaera henryi*, **(d)** *G. podophylla*, and **(e)** *G. denticulate*. In **(c)** to **(e)**, bar charts show the results of clustering analyses using STRUCTURE at the best K=2, each vertical bar represents an individual.

Figure 2 Phylogenetic relationships and ancestry proportions. **(a)** Concatenation analysis of Maximum-likelihood (ML) phylogeny based on analysis of 179,539 SNPs. Bootstrap support values of 100% are indicated by black points on each node. **(b)** Clustering results analyzed using STRUCTURE at the best K=2 for all *Gymnosphaera* and *Alsophila* species. **(c)** Clustering results at the best K=4 and K=3 for *Gymnosphaera* and *Alsophila,* respectively. In **(b)** and **(c)**, each horizontal bar represents an individual.

Figure 3 Genealogical divergence index (*gdi*) and isolation by distance (IBD) tests at an individual level for putative sister species. **(a)** *gdi* for putative sister species pairs. Black line within boxes indicate the median values, the black points indicate the mean values, and the error bars indicate the maximums and minimums. High *gdi* values (> 0.7) suggest two distinct species; low *gdi* values (< 0.2) suggest a single species; *gdi* values fail in the range of 0.2 to 0.7 indicate ambiguous status. **(b)** to **(**e**)** Relationship between genetic and geographical distance in pairs of individuals or populations of two candidate species. All geographical distance are log-transformed. Black and red circles represent the distance between individuals

belonging to the first or second candidate species, green circles represent the distance between individuals belong to different candidate species. Blank and red dashed lines represent the regression lines fitted within the two candidate species, respectively. Black and red solid lines represent the regression lines fitted on the distance of first or second species as well as the between-group distances, respectively. Blue lines represent the mean of geographical distance of the between-groups. Green dashed lines represent the regression lines fitted on the within-group distances, which taking black and red circles into consideration together. Green solid lines represent the regression lines fitted on the all distances, which taking all black, red, and green circles into consideration.

Figure 4 Summary of species delimitation. Box with solid edges indicate that species within the box should be delimited as the same species. Box with dashed edges indicate the ambiguous status of species within the box.

Figure 5 Comparison of topological differences between the concatenated maximum likelihood tree (ML) and species trees (Astral-III, SVDQuartets and SNAPP). All nodes are supported with a bootstrap support values ≥ 95% or a Bayesian posterior ≥ 0.95 unless labeled. Dashed lines indicates the difference between the ML tree and the species tree.

Figure 6 The extent of introgression between species. **(a)** Heatmap indicating pairwise *D*-statistics estimated using Dsuite. Grey squares correspond to the sister species pairs which fails to be tested. **(b)** Summary of gene flow signals detected by ExDfoil analysis. P1 and P2 are the younger sister species pair and the P3 and P4 are the older species pair on a five-taxon phylogeny. The number above/under the horizontal arrows indicates the proportion of species combinations which detected significant signals for the corresponding pattern of introgression. **(c)** All possible introgression signals detected by ExDfoil. The grey squares correspond to species pairs which fail to meet the requirement of the five-taxon phylogeny. The blank squares indicate no introgression. The green squares indicate ancient introgression

and the orange indicate post-speciation introgression. Numbers in squares show the proportions (%) of individual combinations which detected significant signal of introgression. The dashed line represents the ancestor of the nearest nested species pair.