RESEARCH ARTICLE

Molecular phylogenetic assessment of three major taxa in the *Asplenium scolopendrium* complex (Aspleniaceae)

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Abstract Taxonomic ambiguities of the *Asplenium scolopendrium* complex arise from multiple synonyms and reclassifications, subtle phenotypic variations, and a lack of information on phylogenetic relationships. This study thus aimed to resolve this taxonomic uncertainty and provide insight into identifying evolutionarily significant units (ESUs). We first utilized genomic approaches to resolve the phylogenetic position of the East Asian taxon, *A. komarovii*, which is currently treated as a separate species. Phylogenetic trees based on whole plastomes suggested that *A. komarovii* is a variant of *A. scolopendrium* most closely related to *A. scolopendrium* var. *americanum*, with *A. scolopendrium* var. *scolopendrium* as the sister to these in the clade. This three-lineage relationship was also validated with the nuclear marker *gapCp* and newly developed infraspecific plastid markers. *Asplenium komarovii* should therefore be subsumed into *A. scolopendrium*, rather than remaining a distinct species. In addition, our phylogenetic analyses further revealed that *A. scolopendrium* var. *americanum* consisted of subclades with potential to be treated as distinct ESUs. Our results also grouped a newly discovered population from New Mexico (U.S.A.) as a member of *A. scolopendrium* var. *americanum* and identified a genetically admixed population in New York (U.S.A.) containing putative hybrids. Well-defined taxonomy and ESUs can greatly improve the implementation of tailored conservation actions by adequately reflecting the underlying evolutionary potentials of the *A. scolopendrium* complex.

Keywords *Asplenium komarovii*; *Asplenium scolopendrium* var. *americanum*; *Asplenium scolopendrium* var. *scolopendrium*; evolutionarily significant unit; taxonomic uncertainty

Supporting Information may be found online in the Supporting Information section at the end of the article.

■ INTRODUCTION

Delineating species is challenging due to the gradual process of speciation and the various epistemological views on the species concept (Stanton & al., 2019). Species exist on a continuum rather than as fixed entities because new species do not appear suddenly but rather become apparent over time (Hey, 2001; Mallet, 2001). Accordingly, species concepts inevitably differ as they are both descriptions of historical and modern existence of the organisms as well as explanations for the processes and consequences of speciation (De Queiroz, 2007). In general, taxonomy focuses on the consequences of speciation and aims to describe biodiversity under the assumption that all species are equivalent. Conservation, in contrast, aims to protect species by focusing on speciation processes and subsequently prioritizing evolutionary potentials (Agapow & al., 2004; Forest & al., 2007).

Given that species are operational units that link taxonomy and conservation, conflicts in delimiting species have emerged between the two fields of study (Isaac & al., 2004; Garnett & Christidis, 2017). The implementation of conservation measures without well-resolved taxonomic delimitations raises concerns because decisions made in the absence of taxonomic certainty may ultimately impede conservation efforts, with practical consequences ranging from under- or over-estimates of diversity to decisions of substantial ecological and economic importance (Paris & al., 1989; Mace, 2004; Garnett & Christidis, 2017). Given these risks, evolutionarily significant units (ESUs), which refer to the set of historically isolated populations that likely have distinct potentials, provide a rational basis for defining conservation priorities, particularly as existing taxonomic systems may not adequately reflect underlying genetic diversity (Moritz, 1994).

Classical taxonomy, rooted in the morphological species concept (MSC), follows the phenetic convention of using overall phenotypic similarity as the criterion for grouping individuals into species and some measure of dissimilarity for separating species (Cronquist, 1978). However, the MSC is often less easily applied to fern species. Compared to seed plants, fewer diagnosable morphological characters are available for fern taxonomy due to their relatively simple reproductive and vegetative structures. In addition, recurrent auto- and allopolyploidy events consistently generate multiple cytotypes that reproductively isolate them from diploid progenitors despite less pronounced morphological variations. Morphological differences between species can be further obscured by

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reticulate evolution when hybridization occurs between closely related taxa due to secondary contacts (Barrington & al., 1989). Subsequently, species complexes, groups of very similar and presumably closely related infraspecies or cytotypes, commonly exhibit a wide range of continuous morphological variations (Haufler, 1995). Several molecular phylogenetic studies have shown certain single species are actually composed of multiple discrete clades, suggesting the existence of cryptic species that are morphologically indistinguishable but genetically different from other lineages (Simpson, 2019). Thus, pteridologists encounter particular difficulties when attempting to resolve problematic species complexes using morphological characters (Christenhusz & Chase, 2014). These difficulties are exacerbated in rapidly diversifying taxa, which includes many lineages of leptosporangiate ferns (Schneider & al., 2004).

The recent integration of genomic data and advances in phylogenetic methodologies have notably contributed to testing morphology-based taxonomy. Molecular variations currently provide the highest resolution data and are necessary to interpret evolutionary histories, particularly at the infraspecific level (Palmer & al., 1988; Soltis & al., 1992; Soltis & Gitzendanner, 1999). However, selecting appropriate genetic markers is critical because the amount of homologous variation differs considerably across DNA sequences. Land plants typically present many unique challenges (Chase & al., 2005; Cowan & al., 2006; Kress & Erickson, 2008). Although several markers such as *rbcL*, *trnL-trnF*, and *matK* have been proposed as universal markers for plant phylogeny, they are often too conserved to resolve taxonomically complex groups of closely related species, polyploids, and hybrids (Fazekas & al., 2009). Many studies have subsequently used additional supplementary or more complex marker combinations to attain adequate species discrimination (Newmaster & al., 2006; Chase & al., 2007; Kress & Erickson, 2007). Moreover, combinations of biparentally inherited nuclear DNA markers have increased phylogenetic resolution and also provided useful information for revealing hybrid and polyploid origins (Schuettpelz & al., 2008; Rothfels & al., 2015).

The delineation of the *Asplenium scolopendrium* L. complex, commonly known as the hart's tongue fern complex, has historically presented challenges due to their subtle morphological characters (Emmott, 1964; Futyma, 1980; Cinquemani & al., 1988). Four distinct taxonomic groups have been widely recognized: two European taxa (*A. scolopendrium* var. *scolopendrium* L. and subsp. *antri-jovis* (Kümmerle) Brownsey & Jermy), and two American taxa (*A. scolopendrium* var. *americanum* (Fernald) Kartesz & Gandhi and var. *lindenii* (Hook.) Viane & al.) (Tropicos, https://www.tropicos.org; GBIF, https://www.gbif.org/; World Plants, https://www.worldplants.de/). However, the taxonomic histories of these taxa are extensive and contentious. More than 25 taxonomic names have been ascribed to the *A. scolopendrium* complex as some botanists treat them as separate lineages based on their locations (Heo, 2021). The criteria and evidence for taxonomic classification therefore remain poorly understood and often controversial, as well as their phylogenetic relationships (Testo & Watkins, 2011). In particular, *A. komarovii* Akasawa, currently treated as a separate species, has long been suggested as an Asian member of the *A. scolopendrium* complex due to its high morphological similarity to and same cytotype as the American variety (Emmott, 1964; Viane & Reichstein, 1991). Debate also continues regarding the infraspecific relationships of the rare taxa *A. scolopendrium* subsp. *antri-jovis* and *A. scolopendrium* var. *lindenii*. Arguments have been made to lump both into their respective distributionally widespread and abundant neighboring taxa of *A. scolopendrium* var. *scolopendrium* and *A. scolopendrium* var. *americanum* (Brownsey & Jermy, 1973; Mickel & Smith, 2004: 120). New *A. scolopendrium* populations have also been discovered in previously undocumented regions including New Mexico, U.S.A. (Testo & al., 2021), and new hybrid lineages may continue to emerge due to artificial introduction (Weber-Townsend, 2017). Even with the current use of both morphology and ploidy level screening, the ambiguity in identification and classification of the *A. scolopendrium* complex persists.

This study thus aims to resolve the taxonomic uncertainties surrounding the *Asplenium scolopendrium* complex by employing phylogenetic approaches. We first examined the phylogenetic relationships between *A. scolopendrium* and *A. komarovii* together with other members of the family Aspleniaceae based on plastid genomic sequences. To this end, we sequenced and constructed the complete plastid genomes of two representative members of the *A. scolopendrium* complex and compared them with that of *A. komarovii*. Second, we resolved the infraspecific relationships among the members of the *A. scolopendrium* complex. Given the limitations of using whole plastid genomes in population-level phylogenetic studies, we developed novel plastid DNA markers that provided sufficient resolution to elucidate the infraspecific relationships and identify ESUs. These were also combined with a nuclear DNA marker to examine reticulate patterns of speciation and origins of polyploid American and East Asian taxa. Moreover, this study investigated the relationship of a recently discovered population in New Mexico (U.S.A.) and the occurrence of a genetically admixed population in New York (U.S.A.). A clearer taxonomic delineation of target species or populations from this study can facilitate conservation of this iconic species complex.

■ MATERIALS AND METHODS

Plant materials. — To investigate interspecific relationships between *Asplenium scolopendrium* and *A. komarovii*, we examined the complete plastid genomes of 27 Aspleniineae taxa, including *A. komarovii* and the two newly sequenced infraspecific taxa of the *A. scolopendrium* complex, i.e., var. *scolopendrium* from Portugal and var. *americanum* from the U.S.A. (Appendix 1). In addition, we sampled 47 individuals from 24 different populations (typically two individuals per population) to examine infraspecific phylogenetic relationships

among the members of the *A. scolopendrium* complex (suppl. Fig. S1). We obtained official permits for legal sample collection in areas of North America from the U.S. Fish and Wildlife Service (USFWS), the New York State Office of Parks, Recreation and Historic Preservation (NYSOPRHP), Hiawatha National Forest (HNF), and the Michigan Nature Association (MNA). The GenBank accession numbers of plastome sequences for taxa used in this study can be found in Appendix 1. The GenBank accession numbers of partial plastid and partial nuclear sequences as well as voucher information for collected samples of each taxon are listed in Appendix 2.

DNA sequencing, genome assembly, and annotation. — We extracted genomic DNA using the DNeasy Plant Mini Kit (Qiagen, Carlsbad, California, U.S.A.) according to the manufacturer's instructions. Extracted genomic DNA was used to prepare uniquely indexed paired-end libraries according to the standard protocol provided by the manufacturer using the TruSeq Nano DNA library preparation kit (Illumina, San Diego, California, U.S.A.). DNA sequencing was performed using the Illumina Novaseq 6000 (Illumina, San Diego, California, U.S.A.) by Psomagen (Rockville, Maryland, U.S.A.). *Asplenium scolopendrium* var. *scolopendrium* and var. *americanum* respectively generated 145,211,622 and 186,127,488 reads. We initially assembled the paired-end reads into contigs of multiple k-mers using Velvet v.1.2.10 (Zerbino & Birney, 2008). Based on the reference genome of *A. komarovii* (GenBank accession no. MZ064529), we constructed plastid genomes of *A. scolopendrium* var. *scolopendrium* and var. *americanum* using Geneious v.10.2.6 (Biomatters, Auckland, New Zealand). In addition, we reconfirmed missing regions between contigs and uncertain sequences that varied from the reference genome by Sanger sequencing. Dual Organellar GenoMe Annotator (DOGMA) (Wyman & al., 2004) and tRNAscan-SE (Lowe & Chan, 2016) were respectively used to confirm the annotation of the protein-coding and transfer RNA (tRNA) genes. The protein-coding sequences were reconfirmed using a conserved domain database (Lu & al., 2020). The annotated plastid genomes were deposited in GenBank with the accession numbers MZ329814 (*A. scolopendrium* var. *americanum*) and MZ329815 (*A. scolopendrium* var. *scolopendrium*).

Development of infraspecific plastid DNA markers. — To identify candidate markers for infraspecific delineation, we calculated nucleotide diversity (*Pi*) and detected highly variable sites among the plastid genomes of *Asplenium komarovii*, and the two varieties of *A. scolopendrium* using DnaSP v.6 (Rozas & al., 2017) with 200 bp step size and 600 bp window length. We ranked the candidate markers based on the parameters by Hebert & al. (2003) and Kress & Erickson (2008), which include: (a) total length of the site should be sufficiently short to accommodate current methods of DNA extraction, amplification, and sequencing, and (b) flanking sites should be conserved for developing universal PCR primers. In addition, the regions containing less informative insertions and deletions (indels) which are likely coded or ignored in phylogenetic analyses were avoided (Rouhan & al., 2004; Korall & al., 2007). The regions with consecutive single nucleotide polymorphisms (SNPs) were ignored because they are likely consequences of a single mutational event (Jiménez-Gómez & Maloof, 2009).

We designed primer pairs for the candidate markers using Primer3 (Rozen & Skaletsky, 2000) (suppl. Table S1). The major parameters for primer design were set as follows: optimal primer length of 20 bp, optimal GC content of 50%, and optimal temperature (Tm) of 60°C. The availability of the synthesized primer pairs was tested by PCR. AccuPower PCR PreMix (Bioneer, Oakland, California, U.S.A.) was used for the PCR, and each 20 µl of PCR sample included 1 µl of genomic template DNA, 0.5 µl of 10 µM forward primer, 0.5 µl of 10 µM reverse primer, 0.5 µl of 5% DMSO, and 17.5 µl of distilled water. PCR conditions are shown in suppl. Table S1. PCR products were visually checked through gel electrophoresis and qualified products were sequenced at Psomagen. The efficacies of candidate markers were evaluated based on discrimination accuracy using 17 individuals (4 for *A. scolopendrium* var. *scolopendrium*, 7 for *A. scolopendrium* var. *americanum*, 6 for *A. komarovii*), and compared with markers based on widely used plastid DNA regions (*rbcL*, *trnL-trnF*, *atpB-rbcL*) sequenced in a previous study (Heo & al., 2022). Discrimination accuracy was estimated as the number of taxa in the generated phylogenetic trees that are accurately assigned to their corresponding regional monophyletic groups. To make this calculation, the numerator consisted of the sum of the sampled individuals that were correctly sorted into their monophyletic group, divided by a denominator equal to the total number of sampled individuals in the phylogenetic tree (Costion & al., 2011) (suppl. Fig. S2). These tests were performed based on both individual markers and ones concatenated in different combinations. Genetic distance was calculated by the Tamura-Nei model (Tamura & Nei, 1993) using MEGA X (Kumar & al., 2018). The data on which this article is based can be accessed from the NCBI and detailed accession numbers are given in Appendix 1.

In addition, we designed primers for the nuclear gene *gapCp* based on previously reported *gapCp* sequences from two *Asplenium scolopendrium* samples (JX475226, JX475227). The *gapCp* is a single-copy nuclear marker developed for ferns (Ebihara & al., 2005; Schuettpelz & al., 2008). Our primers covered the second region of the nuclear *gapCp* gene from the regions of partial intron 8 to partial exon 11. The PCR conditions for *gapCp* are shown in suppl. Table S1, and the products were visually checked and sequenced in the same manner as described above. We successfully amplified 37 samples from 21 populations, which were used for phylogenetic analyses. Polymorphic sites in the *gapCp* sequences were designated when a site had double peaks on the same position on both direct and reverse strands of the electropherogram and the minimum intensity for the weakest peak was greater than 25% compared to the strongest signal (Fuertes Aguilar & Nieto Feliner, 2003). The polymorphic sites were described according to the International Union of Pure and Applied Chemistry (IUPAC) ambiguity codes.

Phylogenetic analyses. — We performed two levels of phylogenetic analyses. One investigated phylogenetic relationship at the species level based on the plastid genomes of

27 taxa, including 3 infraspecies of the *Asplenium scolopendrium* complex, 23 Aspleniineae species, and an outgroup (*Cystopteris chinensis* X.C.Zhang & R.Wei). Alignment of whole plastome sequences was conducted using MAFFT v.7, all gaps and poorly aligned positions were refined by Gblocks v.0.91b (Castresana, 2000), and sequences of infraspecific markers were aligned and concatenated using Geneious v.10.2.6 (Kearse & al., 2012). The other phylogenetic analysis resolved the infraspecific relationships among the representative populations of the *A. scolopendrium* complex using the nuclear *gapCp* marker as well as the plastid marker developed by concatenating the novel regions *psbZ-trnS* and *rpl16*. *Asplenium nidus* L. and *A. adiantum-nigrum* L. were respectively used as outgroups for these analyses. The alignment datasets for phylogenetic analyses are provided in suppl. Appendices S1–S5.

We constructed maximum likelihood (ML) and Bayesian inference (BI) phylogenetic trees using IQ-TREE v.1.4.2 (Nguyen & al., 2015) and MrBayes v.3.1.2 (Ronquist & Huelsenbeck, 2003), respectively. The ML phylogenetic tree was obtained with 1000 bootstrap (BS) replications. In all cases, the best models were predicted using ModelFinder (Kalyaanamoorthy & al., 2017) in IQ-TREE and the best model was selected on the basis of the Bayesian information criterion (BIC). For the BI phylogenetic tree, the analysis of each dataset was performed with 100,000–300,000 generations until the standard deviation of split frequencies was below 0.01. Each chain was sampled every 100 generations. The first 25% of the samples was discarded as burn-in, and the rest was used to construct a consensus tree. Phylogenetic trees were visualized using FigTree v.1.4.4. All of the described processes and conditions were applied to both phylogenetic analyses. Considering bifurcating trees' limitations in investigating reticulate speciation patterns among closely related taxa, we additionally performed phylogenetic network analyses using SplitsTree v.4.14.4 (Huson & Bryant, 2006). We used the Neighbor-Net algorithm with Kimura 2-parameter (K2P) distances and ordinary least-squares inference for branch lengths. The EqualAngle method was chosen to draw phylogenetic networks, and confidence values were generated based on bootstrapping with 1000 replicates.

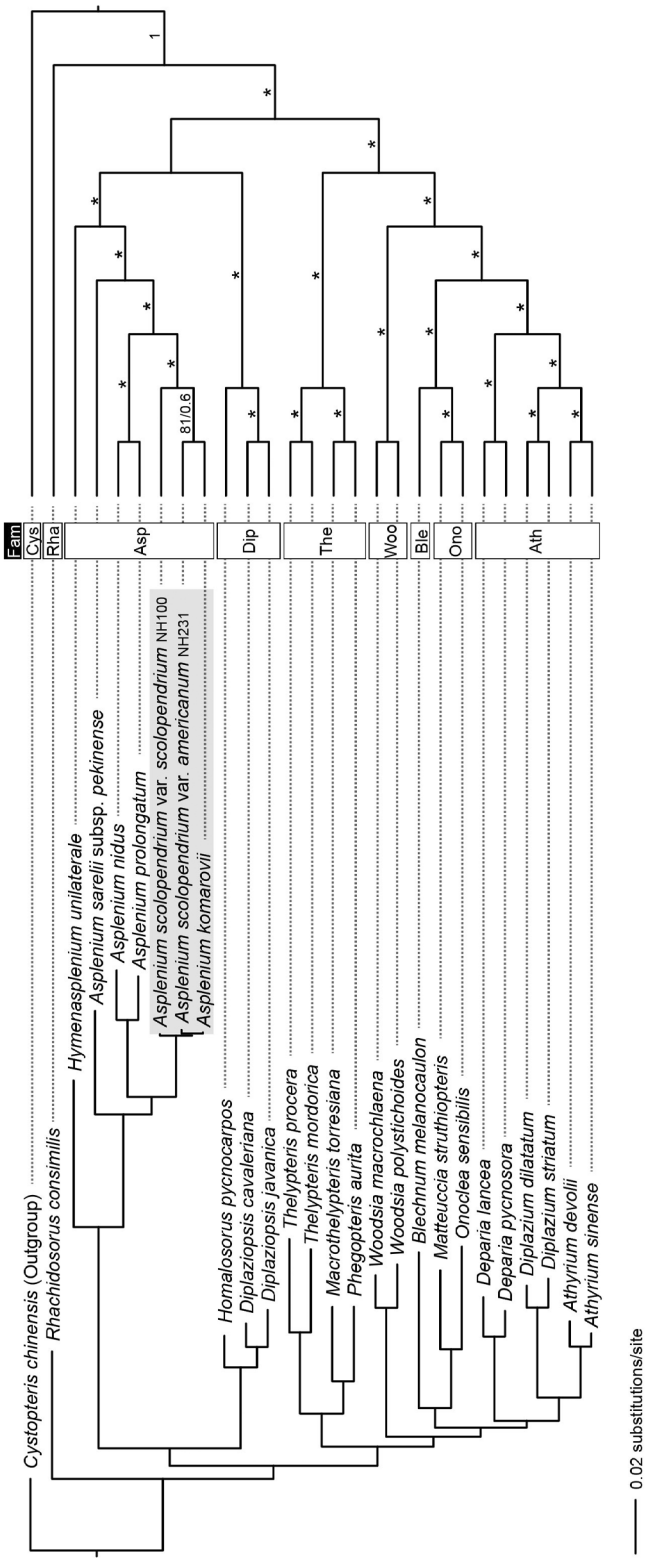
■ RESULTS

Phylogenetic position of *Asplenium komarovii* based on whole plastid genomes. — All major clades of suborder Aspleniineae were congruent with the current taxonomic classification at the family level (Fig. 1). The phylogenetic position of *Asplenium komarovii*, however, was inconsistent with its current taxonomic classification and instead formed a monophyletic group with the two infraspecific taxa of *A. scolopendrium*. This monophyly was strongly supported with 100% bootstrap support (BS) and 1.00 posterior probability (PP). Within the clade, *A. scolopendrium* var. *scolopendrium* was sister to the subclade containing var. *americanum* and *A. komarovii* (BS = 100, PP = 1). *Asplenium komarovii* was positioned as a sister to *A. scolopendrium* var. *americanum* (BS = 81, PP = 0.6).

Development of plastid markers for infraspecific delineation. — Universal DNA markers such as the concatenation of *rbcL*, *trnL-trnF*, and *atpB-rbcL* failed to delineate the *Asplenium scolopendrium* complex and generated several incorrect groupings, requiring the development of high-resolution infraspecific markers (Fig. 2). Out of 16 hypervariable regions detected by comparative genomic analysis ($Pi \geq 0.005$), four candidate markers were selected according to previously described criteria (Table 1, suppl. Table S2, suppl. Figs. S2, S3). Three were primarily located in non-coding regions in the LSC region (i.e., intronic *rpl16*, intergenic *psbZ-trnS*, and *clpP-psbB*), and one in the IR coding region (i.e., *ycf2*). All these markers had a PCR success rate of 100%. *PsbZ-trnS* had the highest number of polymorphisms and informative sites, and nucleotide diversity, followed by *rpl16*, *ycf2*, and *clpP-psbB*. The mean genetic distance generated by each marker was highest in *psbZ-trnS*. However, the intra- and infraspecific resolution of each marker differed. *Rpl16* generally showed a high resolution that distinguished the European from the North American and East Asian lineages, whereas *psbZ-trnS* and *ycf2* generated more genetic distance between the North American and East Asian lineages. *PsbZ-trnS* showed the highest resolution within lineages (Table 1).

Regarding the validation of the infraspecific markers, they generally grouped the taxa into their respective populations and geographic locations but with different topologies and resolutions (suppl. Fig. S2D–G). Both *rpl16* and *ycf2* markers accurately delimited the three major clades of the European, American, and East Asian lineages. While *psbZ-trnS* showed a higher resolution in resolving subclades within the European and East Asian lineages, *clpP-psbB* could not resolve the European and East Asian lineages, leaving unresolved relationships between infraspecies. The accuracy of topology and resolution were higher in certain marker combinations (suppl. Fig. S2H–K). Gene trees constructed with the dataset of concatenated *psbZ-trnS* and *rpl16* markers displayed higher resolution than those using the concatenated dataset of *rbcL*, *trnL-trnF*, or *atpB-rbcL* markers (Fig. 2).

Infraspecific phylogenetic relationships. — The phylogenetic relationships of 24 geographically distinct *Asplenium scolopendrium* populations, including taxonomically uncertain samples from the Sentinel Basin (SB, New York) and New Mexico (NM) populations (U.S.A.), were analyzed using the novel plastid and nuclear *gapCp* markers (Fig. 3). The topologies of the phylogenetic trees based on plastid and nuclear DNA markers were generally congruent, with only the Alabama population forming a subclade in the plastid phylogenetic tree. All three major lineages were monophyletic, excluding the SB population. The monophyly of all three major lineages was highly supported in both phylogenetic trees (BS = 100, PP = 1). However, despite both phylogenetic trees indicating that American and East Asian lineages formed a subclade, the support values in the plastid phylogenetic tree were relatively low (BS = 56, PP = 0.8), compared to that of the nuclear phylogenetic tree (BS = 82, PP = 1.0).



Despite being located in North America (New York), seven out of the eight sampled individuals in SB were positioned within the European clade, while only one individual (SB6) was nested within the American clade. With respect to the population from New Mexico (U.S.A.), both the plastid and nuclear phylogenetic trees indicated that it was mostly related to the American taxon by nesting within the *Asplenium scolopendrium* var. *americanum* clade with high support values.

The topologies of the phylogenetic networks were also generally congruent with those of phylogenetic trees, forming three major clades (BS = 100) (Fig. 4). However, more branching patterns were found in the East Asian and European clades in both plastid and nuclear phylogenetic networks. In addition, three individuals of the SB population (SB2, SB6, SB8) were positioned between the European and central nodes in the phylogenetic network based on the nuclear *gapCp* marker.

Polymorphic sites in the *gapCp* region. — No polymorphic sites in the *gapCp* region were found in any sampled European, East Asian, or American taxa other than SB population in the U.S. Three individuals of the SB population (SB2, SB6, SB8) had 16 polymorphic sites, which all showed additive patterns containing the *gapCp* copies of both European and American taxa (Table 2). All other individuals from the SB population had identical European sequences.

■ DISCUSSION

Phylogenetic position of *Asplenium komarovii*. — The taxonomic position of *Asplenium komarovii* has been questioned by several pteridologists. Initially described by Komarov as *Phyllitis japonica* Kom. in 1932, Akasawa (1962) proposed the alternative name *Asplenium komarovii* primarily due to the replacement of the genus *Phyllitis* and that the potential name *Asplenium japonicum* would have been a homonym to *A. japonicum* Thunb. Emmott (1964) questioned the taxonomic rank of *A. komarovii* based on its cytological consistency and reproductive compatibility with *A. scolopendrium* var. *americanum*, and Viane & Reichstein (1991) also reclassified it as a member of the *A. scolopendrium* complex, naming it *A. scolopendrium* subsp. *japonicum*. However, this classification is not well recognized and is currently treated as a synonym

Fig. 1. Phylogenetic tree of suborder Aspleniineae based on 27 plastid genomes. The left tree is a phylogram with branch lengths proportional to the number of nucleotide substitutions. The right tree is a cladogram emphasizing the relationships of monophyletic groups. The branches with a bootstrap support (BS) value of 100 and a posterior probability (PP) of 1.00 are indicated with an asterisk in the cladogram. The numbers above branches represent the BS and PP, respectively. The scale bar represents the number of nucleotide substitutions per site. Taxonomic classification (i.e., family name) corresponding to each clade is indicated within a box. Fam, Family; Cys, Cystopteridaceae; Rha, Rhachidosoraceae; Asp, Aspleniaceae; Dip, Diplaziopsidaceae; The, Thelypteridaceae; Woo, Woodsiaceae; Ble, Blechnaceae; Ono, Onocleaceae; Ath, Athyriaceae.

(https://www.worldplants.de/). A previous phylogenetic study of the genus *Asplenium* based on six plastid markers suggested that *A. komarovii* is a distinct sister species to *A. scolopendrium* (Xu & al., 2020).

However, all our phylogenetic trees fully supported the monophyly of *Asplenium komarovii* and the two *A. scolopendrium* taxa (BS = 100, PP = 1 in Figs. 1–3), indicating that *A. komarovii* is an East Asian member of the *A. scolopendrium* complex. The trees suggested that *A. komarovii* is more closely related to *A. scolopendrium* var. *americanum*, with *A. scolopendrium* var. *scolopendrium* as the sister to these. This phylogenetic relationship between *A. komarovii* and *A. scolopendrium* var. *americanum* is also supported by their cytological consistency (i.e., tetraploid taxa) and reproductive compatibility (Emmott, 1964). Inconsistencies with the previous phylogenetic study likely arose from the greater sample size and resolution of molecular markers in this study, which are considered vital to robust phylogenetic analyses (Hollingsworth & al., 2011).

All populations of *Asplenium komarovii* formed a monophyletic group in the phylogenetic trees/networks and had no additive polymorphisms in *gapCp* gene sequences, suggesting that gene flow with other *A. scolopendrium* members has been very limited, and its polyploidy likely arose through a single autopolyploidization event in the common ancestor of *A. scolopendrium* var. *americanum* and *A. komarovii*. A high degree of geographic segregation due to historical vicariance events (e.g., disappearance of the Bering land bridge) may have led to geographically/taxonomically independent reproduction (Heo & al., 2022). Consequently, *A. komarovii* should be reclassified as an infraspecies of *A. scolopendrium*. Considering the spatial resolution of other *A. scolopendrium* taxa at the continental level, we propose the designation *A. scolopendrium*

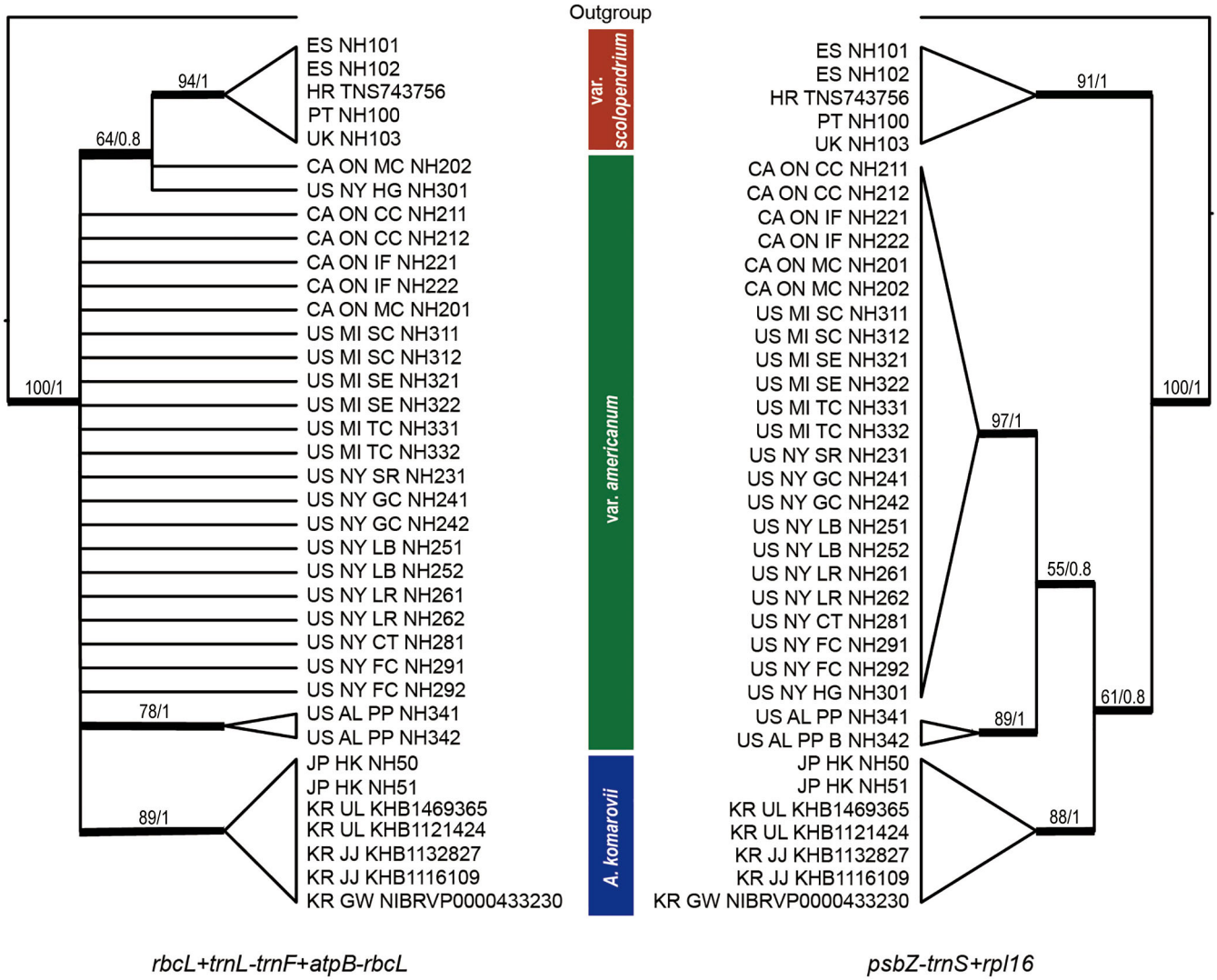


Fig. 2. Comparison of the phylogenetic trees constructed based on the concatenated dataset of the universal markers *rbcL*, *trnL-trnF*, and *atpB-rbcL* (left) and the novel infraspecific markers *psbZ-trnS* and *rpl16* (right). Numbers above branches represent the bootstrap support (BS) value and posterior probability (PP), respectively. The branches with greater than BS 50% and PP 0.5 are bolded. The codes on each terminal node follow the population code and collection number of voucher specimen in Appendix 2.

subsp. *japonicum*, which is consistent with the taxonomic treatment of Viane & Reichstein (1991).

Validation of infraspecific plastid markers for the *Asplenium scolopendrium* complex. — The balance between high resolution and standardization is essential for selecting infraspecific plastid DNA markers (Moritz & Cicero, 2004). Despite the general concern that the plastid genome has low intrinsic evolutionary rates (Chase & al., 2007; Kress & Erickson, 2007), this study found several hypervariable regions where a small number of single-base substitutions and indels had accumulated at the infraspecific level. In particular, the SSC region was the most variable region based on *Pi* and the number of SNPs was consistent with the patterns found in other ferns (Gao & al., 2018; Fan & al., 2021). Concerning mutation rates, synonymous sites, where a mutation does not change the amino acid sequence, are known to have a higher average divergence rate (Zhu & al., 2014). Mutations in *Asplenium scolopendrium* plastid genome also followed this general trend, showing that most SNPs found in genic regions had greater synonymous substitution rates than non-synonymous substitution rates.

Several sites such as *ycf2*, *rpl16*, *psbZ-trnS*, and *clpP-psbB* were identified as particularly hypervariable in the *Asplenium scolopendrium* complex, and the first three markers showed sufficiently high resolution and accuracy to delineate the members of the *A. scolopendrium* complex and assigned each taxon into its appropriate lineage. *Ycf2* is the largest plastid gene reported in plants and is related to ATP production in chloroplasts (Kikuchi & al., 2018). The similarity of *ycf2* nucleotide sequences among land plants is less than 50% among bryophytes, ferns, and seed plants (Wicke & al., 2011), suggesting that *ycf2* is one of the fastest evolving genes in the plastid genome and has elevated substitution rates (Kim & Lee, 2004; Wicke & al., 2011). Although debate remains regarding whether this region can serve as a supplementary DNA marker given that it appears to be influenced by positive selection pressure from environmental conditions and thus less likely represents neutral molecular variations (Wu & al., 2020), the *ycf2* marker developed in this study consistently differentiated *A. scolopendrium* taxa at the infraspecies level.

The *rpl16* gene, which encodes the ribosomal protein L16, is also one of the most divergent regions of the plastid genome and has high rates of sequence change (Downie & al., 2000). A marker targeting the *rpl16* intron has been developed based on plastid genomes from *Adiantum* and *Psilotum*, and used to address phylogenetic relationships in *Botrychium*, *Hiya*, and *Dicksonia* (Small & al., 2005; Williams & Waller, 2012; Shang & al., 2018). However, this marker has limited application to other fern taxa as it generates weak or double bands in PCR amplification including in genus *Asplenium* (Small & al., 2005). Alternatively, the *rpl16* marker developed in this study, which included the *rpl16* intron and exon, the *rpl16-rps3* intergenic spacer, and the partial *rps3* gene, consistently discriminated between *A. scolopendrium* taxa at the infraspecies level. The sequence variations in this marker were relatively stable regardless of sample size, implying that the informative variations were invariant across conspecific groups. The stability of the *rpl16* intron has also been supported in angiosperm taxa (Kelchner & Clark, 1997; Downie & al., 2000; Li & al., 2010).

Table 1. Features of four candidate markers applied to a testing group (14 representative populations).

		rpl16	*ycf2*	*psbZ-trnS*	*clpP-psbB*
Aligned length [bp]		756	709	912	814
Conserved sites [bp]		747	703	899	808
Nucleotide diversity (*Pi*)		0.0043	0.0036	0.0051	0.0024
Polymorphic sites	Single nucleotide polymorphisms (SNPs)	9	6	13	6
	Indels	–	–	2	–
Parsimony-informative sites		7	5	11	4
PCR success [%]		100	100	100	100
Mean genetic distance between groups	Overall	0.0044	0.0036	0.0053	0.0024
	European – North American	0.0079	0.0061	0.0077	0.0040
	European – East Asian	0.0072	0.0032	0.0034	0.0010
	North American – East Asian	0.0043	0.0060	0.0080	0.0038
Mean genetic distance within group	European	0.0000	0.0007	0.0007	0.0012
	North American	0.0004	0.0000	0.0025	0.0007
	East Asian	0.0009	0.0000	0.0012	0.0007
Discrimination accuracy [%]		100	100	94	41

Note: *rpl16* includes the *rpl16* intron (471 bp) and 5′ exon (exon 1, 9 bp), the *rpl16-rps3* intergenic spacer (IGS, 113 bp), and the partial *rps3* gene (163 bp); *psbZ-trnS* includes the partial *trnG-psbZ* IGS (42 bp), the complete *psbZ* gene (189 bp), the *psbZ-trnS* IGS (412–413 bp), the complete *trnS* gene (89 bp), and the partial *trnS-psbC* IGS (179 bp); *clpP-psbB* includes the partial *clpP* intron (88 bp), the *clpP* 5′ exon (exon 1, 71 bp), the complete *clpP-psbB* IGS (449 bp), and the partial *psbB* gene (206 bp).

Sequence variations in *psbZ-trnS* and *clpP-psbB* have been identified through the comparison of plastid genome sequences such as those of *Solanum* and *Dendrobium* (Särkinen & George, 2013; Niu & al., 2017; Liu & al., 2019), but few studies have targeted these regions for phylogenetic analysis, and the primers used (e.g., *Dioscorea*, Xia & al., 2019; *Lepisorus*, Zhao & al., 2020) were not compatible to the taxa examined in our study. The *psbZ-trnS* marker developed in this study demonstrated a higher resolution to separate taxa at the infraspecific and population levels. However, the sequence variation in *psbZ-trnS* increased in proportion to the sample size because some SNPs appeared to be autapomorphic. In the case of *clpP-psbB*, most of its molecular variation was less informative for phylogenetic analysis, suggesting that substitutions occurred independently at the individual level, rather than arising from common ancestor. Thus, two of the infraspecific markers (i.e., *rpl16* and *psbZ-trnS*) were considered optimal candidates due to their high resolution, stability, and accuracy in revealing relationships among the members of the *Asplenium scolopendrium* complex. Resolution and standardization were greatly improved by concatenating these two markers, but further improvement ceased with inclusion of additional markers. Given that using multiple markers raises the risk of creating autapomorphies or sequencing errors (Hollingsworth

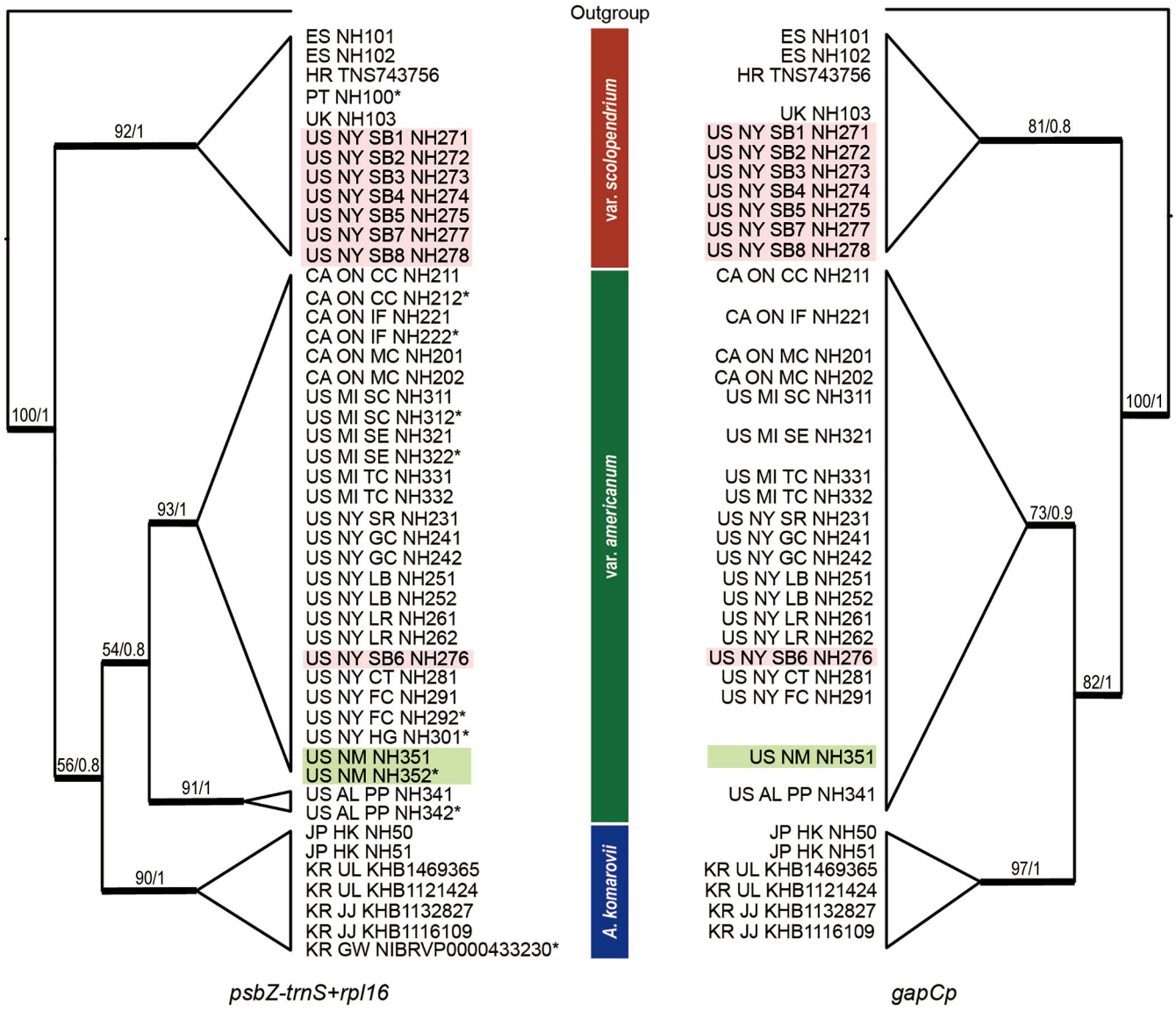


Fig. 3. Infraspecific phylogenetic relationships of three major taxa in the *Asplenium scolopendrium* complex, including the taxonomically uncertain Sentinel Basin (SB, New York, U.S.A., 8 individuals, pink boxes) and New Mexico (NM, U.S.A., 2 individuals, green boxes) populations. Phylogenetic trees are based on the concatenated plastid sequences of *psbZ-trnS* and *rpl16* (left) and nuclear *gapCp* (right). Numbers above branches represent the bootstrap support (BS) value and posterior probability (PP), respectively. The branches with above BS 50% and PP 0.5 are bolded. Asterisks indicate the samples with plastid sequences only. The nuclear *gapCp* sequences of SB2, SB6, and SB8 contained 16 polymorphic sites designated according to the IUPAC nucleotide codes, as shown in Table 2.

& al., 2011) or causing discrepancies between a gene tree and species boundaries (Fazekas & al., 2009), *rpl16* alone or the combination of *rpl16* and *psbZ-trnS* were optimal for delimiting taxa in the *A. scolopendrium* complex and assigning each taxon into their corresponding lineages or populations.

Evolutionary significant units of the *Asplenium scolopendrium* complex. — Overall, there was congruence between phylogenetic trees/networks based on maternally (plastid DNA) and biparentally inherited (nuclear DNA) sequences for the three major taxa of the *Asplenium scolopendrium* complex, illustrating that reticulation among these three taxa did not likely occur. Despite potentials of long-distance dispersal in ferns, the three major taxa of *A. scolopendrium* appeared to have complete lineage sorting due to a high degree of geographic separation and a long history of lineage divergences, likely beginning from the Late Pliocene (Heo & al., 2022). Thus, the existing taxonomic treatment that separates them into three distinct subspecies accurately reflects their distinct evolutionary potentials.

However, *Asplenium scolopendrium* var. *americanum* was subdivided into two subclades (southern and northern

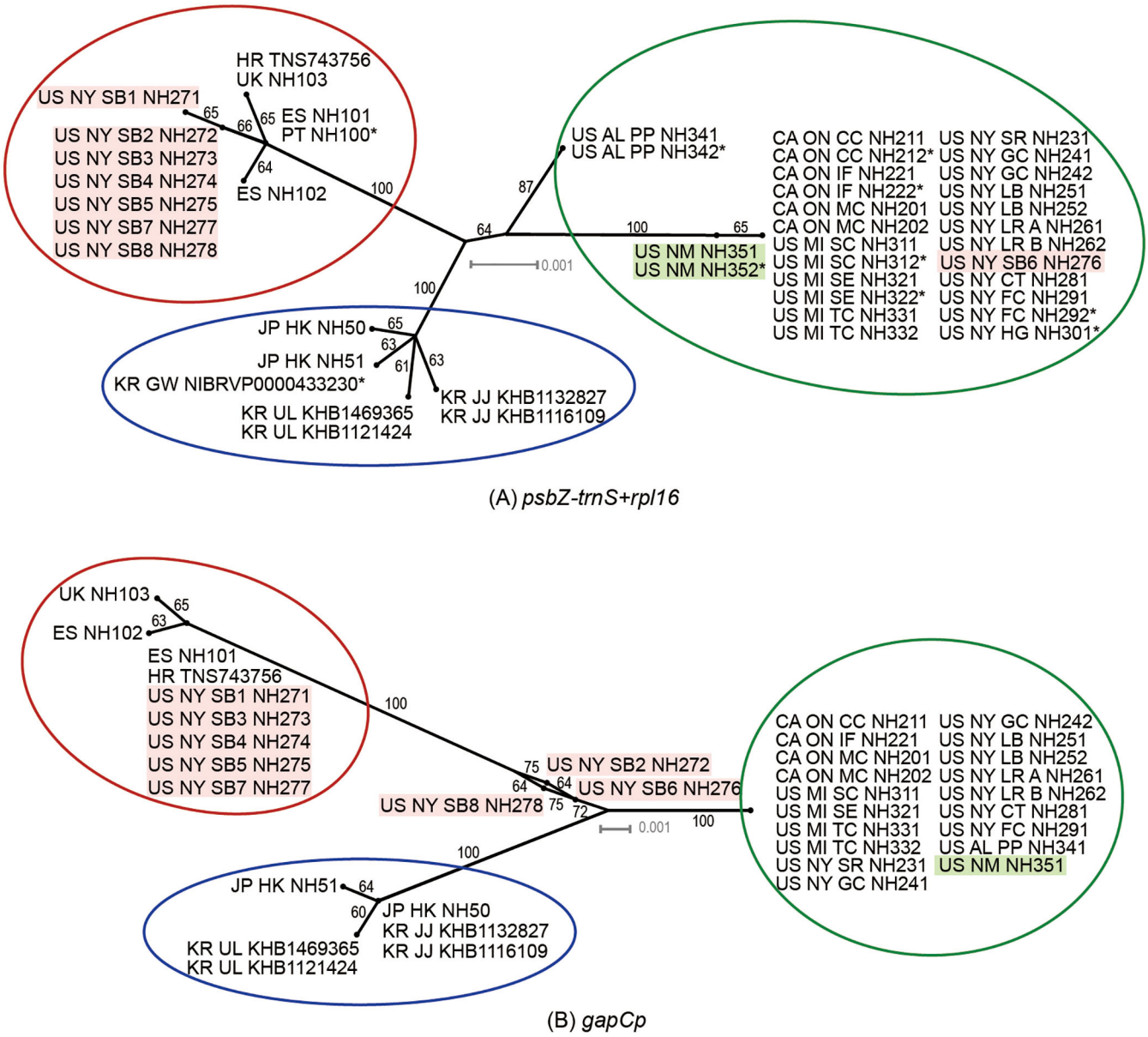


Fig. 4. Phylogenetic networks of three major taxa in the *Asplenium scolopendrium* complex. **A**, The network derived from the concatenated plastid sequences of *psbZ-trnS* and *rpl16*; **B**, The network based on the low-copy nuclear *gapCp* marker. The colored boxes indicate taxonomically uncertain samples (SB in pink; NM in green). The nuclear *gapCp* sequences of SB2, SB6, and SB8 contained 16 polymorphic sites designated according to the IUPAC nucleotide codes, as shown in Table 2. Note that US NY SB2, SB6, and SB8 are presumed hybrids between European and American taxa. Numbers above each branch represent bootstrap support (BS) value. The branches with above BS 50% are bolded. Asterisks indicate the samples with plastid sequences only. Each colored circle indicates geographic origin: red = Europe, blue = East Asia, and green = North America.

Table 2. Polymorphic sites of the *gapCp* region found in three individuals from the Sentinel Basin population (SB2, SB6, and SB8).

		Exon 9	Intron 9													Intron 10	
		64	202	290	291	344	380	428	431	461	465	480	550	564	567	571	746	Note
Asplenium scolopendrium var. *scolopendrium*		T	A	C	G	G	A	C	A	T	A	C	G	G	A	A	T	–
Asplenium scolopendrium var. *americanum*		C	G	T	A	C	G	G	G	C	T	A	A	T	G	G	A	Other than Sentinel Basin
Asplenium komarovii		C	A	C	A	C	G	G	A	T	T	A	A	T	G	G	A	–
Sentinel Basin (New York, U.S.A.)	SB1	T	A	C	G	G	A	C	A	T	A	C	G	G	A	A	T	Likely introduced *Asplenium scolopendrium* var. *scolopendrium*
	SB3	T	A	C	G	G	A	C	A	T	A	C	G	G	A	A	T	
	SB4	T	A	C	G	G	A	C	A	T	A	C	G	G	A	A	T	
	SB5	T	A	C	G	G	A	C	A	T	A	C	G	G	A	A	T	
	SB7	T	A	C	G	G	A	C	A	T	A	C	G	G	A	A	T	
	SB2	Y	R	Y	R	S	R	S	R	Y	W	M	R	K	R	R	W	Putative hybrids between *Asplenium scolopendrium* var. *scolopendrium* and var. *americanum*
	SB6	Y	R	Y	R	S	R	S	R	Y	W	M	R	K	R	R	W	
	SB8	Y	R	Y	R	S	R	S	R	Y	W	M	R	K	R	R	W	

Other samples are also included to illustrate the additive pattern that indicates hybridization between *Asplenium scolopendrium* var. *scolopendrium* and var. *americanum*. IUPAC ambiguity nucleotide codes are used to describe polymorphisms (K = G + T, M = A + C, R = A + G, S = G + C, W = A + T, Y = C + T). Listed sequences for *A. scolopendrium* var. *scolopendrium* and var. *americanum*, and *A. komarovii* were shared by all sampled individuals.

populations) in the phylogenetic tree/network based on plastid sequences (Figs. 2, 3), whereas nuclear phylogenetic relationships did not separate *A. scolopendrium* var. *americanum* into subclades. Although this difference can be explained as nuclear markers coalesce four times more slowly than plastid markers due to biparental inheritance (Moore, 1995), gene flow between these groups has likely continued due to artificial transplanting as a means of population augmentation (USFWS, 1993). Nevertheless, the lineage separation based on plastid sequences likely resulted from the highly disjunctive and fragmented distributions of American populations as they have persisted in geographically isolated climatic refugia since the Pleistocene (Watkins & Farrar, 2005; Heo & al., 2022). Genetic drift, particularly in the small southern populations (e.g., Alabama), has likely facilitated divergences and thus necessitates more geographically refined ESUs as the operational conservation units for reintroduction or augmentation programs to preserve long-term genetic distinctiveness (Moritz, 1994). In contrast, the northern populations (e.g., New York and Michigan in the U.S., and Ontario, Canada) formed a single subclade and thus could be treated as a single ESU within which individual translocations would be unlikely to degrade genetic diversity.

Although East Asian populations formed a single clade in both phylogenetic trees, several divergent branches were found in the phylogenetic networks (Fig. 4). Given that mainland endangered populations (e.g., China and the Korean Peninsula) are fragmented and small (Korea National Arboretum, 2008; Shiyong & al., 2017), whereas island populations (e.g., Japan) are abundant and highly radiated (Murakami, 2020), both natural and anthropogenic conditions appear to have increased divergence within the East Asian lineage, and thus the potential to generate multiple ESUs remains high.

Genetic admixture between European and American taxa. — Our results revealed the presence of genetically different individuals in the SB population. Surprisingly, only one out of the eight sampled individuals (SB6) was nested in the American clade in the plastid phylogenetic trees, while the remaining seven samples were positioned in the European clade. The phylogenetic network analysis based on the nuclear DNA sequences additionally revealed that three samples (SB2, SB6, SB8) formed a distinct phylogenetic position from the other SB individuals. These three samples exhibited additive polymorphisms, which completely included both European and American *gapCp* copies within the same individuals, indicating that hybridization had occurred between tetraploid American and diploid European taxa in the SB population. These patterns explained previous findings of occurrences of triploid individuals and irregularly shaped spores in the SB population (Heo, 2021). Since SB6 is positioned in the American clade and SB2 and SB8 are positioned in the European clade in the plastid phylogenetic trees/networks (Figs. 2–4), this implies that these individuals respectively had American and European maternal origins given that cpDNA is maternally inherited.

Determining the origin of hybridization is crucial for conservation (Allendorf & al., 2001). Although natural hybridization may contribute to increasing biodiversity, anthropogenically facilitated hybridization and introgression with closely related taxa have often been documented as possible causes of genetic extinction (Rieseberg & al., 1993; Rhymer & Simberloff, 1996; Hegde & al., 2006; Gómez & al., 2015). In addition, hybridization between different cytotypes can lead to direct demographic extinction due to sterile hybrid offspring and reduced opportunity for cytologically consistent mating. Despite geographic isolation among the three major taxa and complete lineage sorting based on our data, the SB population likely exemplifies a case of artificial hybridization as several sources have reported human-mediated introduction of the European taxon to North America through spore sowing by private individuals earlier in the 20th century (Benedict, 1927; Wherry, 1936; Faust, 1960; The Nature Conservancy, 1990; Parker, 2009).

Along with the presence of introduced European individuals, complete additive polymorphisms suggest that the putative hybrids (SB2, SB6, SB8) are likely first-generation (F_1). Thus, the SB population likely comprises mixtures of pure individuals of introduced European and native American taxa, as well as F_1 hybrids. If the SB population contains an adequate number of American individuals, they could be recovered by selective removal of introduced European individuals and hybrids. In this case, the nuclear *gapCp* and novel plastid markers developed in this study would facilitate the detection of these European individuals and hybrids. This process could instigate timely conservation actions that offset the negative impacts of anthropogenic introduction and subsequent hybridization.

■ TAXONOMIC TREATMENT – RECOGNIZED TAXA

Asplenium scolopendrium L. subsp. ***scolopendrium***, Sp. Pl.: 1079. 1753.

Asplenium scolopendrium subsp. ***americanum*** (Fernald) N.Heo, **nomencl. novelty [Art. 6 Ex. 13]** ≡ *Phyllitis scolopendrium* (L.) Newman var. *americana* Fernald in Rhodora 37(438): 220. 1935 ≡ *Phyllitis fernaldiana* Á.Löve in Svensk Bot. Tidskr. 48: 214. 1954 ≡ *Phyllitis japonica* subsp. *americana* (Fernald) Á.Löve & D.Löve in Acta Bot. Acad. Sci. Hung. 19: 205. 1973 ≡ *Asplenium scolopendrium* var. *americanum* (Fernald) Kartesz & Gandhi in Phytologia 70(3): 196. 1991 – Holotype: Canada, Ontario, County of Grey, Inglis Falls, 19 Jun 1934, *M.L. Fernald, R.B. Thomson & J.G. Wright 3040* (GH barcode 00021763!).

Asplenium scolopendrium subsp. ***japonicum*** (Kom.) Rasbach, Reichst. & Viane in Biol. Jaarb. 59: 162. 1992 ("1991") ≡ *Phyllitis japonica* Kom. in Izv. Bot. Sada Akad. Nauk S.S.S.R. 30: 192. 1932 ≡ *Asplenium komarovii* Akasawa in Bull. Kochi Women's Univ., Ser. Nat. Sci. 10: 26. 1962, non Thunb. 1784 – Holotype: Russia, Primorskiy Krai, 10 Mar 1929, *N. Rastorguev s.n.* (LE barcode 01006139!).

■ CONCLUSION

Asplenium scolopendrium is geographically segregated over a wide range and subsequently forms a species complex composed of very similar and closely related subspecies. One of the major taxonomic uncertainties in this complex relates to its controversial sister species *A. komarovii*. Despite their great geographical separation, all our phylogenetic analyses support the monophyly of *A. komarovii* and *A. scolopendrium*, indicating that *A. komarovii* is a sister to *A. scolopendrium* var. *americanum*, with *A. scolopendrium* var. *scolopendrium* as the basal group in the clade. We thus propose that *A. komarovii* should be subsumed into *A. scolopendrium* as the subspecies *japonicum*. In addition, the novel plastid markers developed in this study further revealed that the American taxon consisted of two subclades with potential to be treated as distinct ESUs. Our results also resolved taxonomic ambiguities by classifying a newly discovered population from New Mexico (U.S.A.) as a member of var. *americanum* and identifying a genetically admixed population in New York (U.S.A.) which contained putative hybrids. Our results illustrated that well-defined taxonomy and ESUs can greatly improve the implementation of tailored conservation actions for the *A. scolopendrium* complex.

■ AUTHOR CONTRIBUTIONS

NH and DDF conceived the ideas. NH designed the experiments and performed sample collection in collaboration with SY. NH and SY conducted wet lab components and analyzed data. NH wrote the manuscript, and all authors revised the manuscript. — NH, https://orcid.org/0000-0001-8644-7011; SY, https://orcid.org/0000-0002-4961-7189; DDF, https://orcid.org/0000-0001-9478-2629

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Appendix 1. Accession numbers of whole plastid genomes used for phylogenomic analysis. Names that have been reclassified throughout this study are indicated in parentheses.

ASPLENIACEAE: ***Asplenium komarovii*** Akasawa (***Asplenium scolopendrium*** subsp. ***japonicum*** (Kom.) Rasbach, Reichst. & Viane), MZ064529, ***Asplenium nidus*** L., MK002975, ***Asplenium prolongatum*** Hook., KY427332, ***Asplenium sarelii*** subsp. ***pekinense*** (Hance) Fraser-Jenk., Pangtey & Khullar, KY427331, ***Asplenium scolopendrium*** var. ***scolopendrium*** L. (***Asplenium scolopendrium*** subsp. ***scolopendrium*** L.), MZ329815, ***Asplenium scolopendrium*** var. ***americanum*** (Fernald) Kartesz & Gandhi (***Asplenium scolopendrium*** subsp. ***americanum*** (Fernald) N.Heo), MZ329814, ***Hymenasplenium unilaterale*** (Lam.) Hayata, KY427350. — ATHYRIACEAE: ***Athyrium devolii*** Ching, KY419703, ***Athyrium sinense*** Rupr., KY427333, ***Deparia lancea*** (Thunb.) Fraser-Jenk., KY427338, ***Deparia pycnosora*** (Christ) M.Kato, KY427339, ***Diplazium dilatatum*** Blume, KY427344, ***Diplazium striatum*** (L.) C.Presl, KY427346. — BLECHNACEAE: ***Blechnum melanocaulon*** (Brack.) T.C.Chambers & P.A.Farrant, KY427334. — CYSTOPTERIDACEAE: ***Cystopteris chinensis*** (Ching) X.C. Zhang & R.Wei, KY427337. — DIPLAZIOPSIDACEAE: ***Diplaziopsis cavaleriana*** (Christ) C.Chr., KY427341, ***Diplaziopsis javanica*** (Blume) C.Chr., KY427342, ***Homalosorus pycnocarpos*** (Spreng.) Pic.Serm., KY427349. — ONOCLEACEAE: ***Matteuccia struthiopteris*** (L.) Tod., KY427353, ***Onoclea sensibilis*** L., KY427354. — RHACHIDOSORACEAE: ***Rhachidosorus consimilis*** Ching, KY427356. — THELYPTERIDACEAE: ***Macrothelypteris torresiana*** (Gaudich.) Ching, MH500230, ***Phegopteris aurita*** (Hook.) J.Sm., KY427355, ***Thelypteris mordorica*** Christenh., KY427357, ***Thelypteris procera*** (D.Don) Fraser-Jenk., KY427336. — WOODSIACEAE: ***Woodsia macrochlaena*** Mett. ex Kuhn, KY427358, ***Woodsia polystichoides*** D.C.Eaton, KY427359.

Appendix 2. Accession numbers of partial plastid and nuclear DNA sequences used for intraspecific phylogenetic analyses.

Voucher information is provided using the following order: taxon, locality (population code), collector and collection number (herbarium) of voucher specimen, and GenBank accession numbers of *atpB-rbcL*, *rbcL*, *trnL-trnF*, *clpP-psbB*, *psbZ-trnS*, *rpl16*, *ycf2*, and *gapCp*. A dash (–) indicates missing data. An asterisk (*) indicates newly sequenced data from this study; [1] = samples used to validate infraspecific markers; [2] = taxonomically uncertain samples.

Asplenium scolopendrium subsp. ***americanum*** (Fernald) N.Heo, Canada (CA-ON-CC), *Weber-Townsend J.R. & al. NH211* (SYRF), OM675066, OM675285, OM675504, –, OM994594*, OM994641*, –, OP023334*; ***Asplenium scolopendrium*** subsp. ***americanum***, Canada (CA-ON-CC), *Weber-Townsend J.R. & al. NH212* (SYRF), OM675067, OM675286, OM675505, –, OM994595*, OM994642*, –, –; ***Asplenium scolopendrium*** subsp. ***americanum***, Canada (CA-ON-IF), *Weber-Townsend J.R. & al. NH221* (SYRF)[1], OM675179, OM675398, OM675617, OM994585*, OM994612*, OM994659*, OM994692*, OP023335*; ***Asplenium scolopendrium*** subsp. ***americanum***, Canada (CA-ON-IF), *Weber-Townsend J.R. & al. NH222* (SYRF), OM675180, OM675399, OM675618, –, OM994613*, OM994660*, –, –; ***Asplenium scolopendrium*** subsp. ***americanum***, Canada (CA-ON-MC), *Weber-Townsend J.R. & al. NH201* (SYRF)[1], OM675203, OM675422, OM675641, OM994586*, OM994618*, OM994665*, OM994691*, OP023336*; ***Asplenium scolopendrium*** subsp. ***americanum***, Canada (CA-ON-MC), *Weber-Townsend J.R. & al. NH202* (SYRF), OM675204, OM675423, OM675642, –, OM994619*, OM994666*, –, OP023337*; ***Asplenium scolopendrium*** subsp. ***americanum***, United States (US-NY-SR), *Weber-Townsend J.R. & al. NH231* (SYRF)[1], OM675252, OM675471, OM675690, OM994590*, OM994635*, OM994682*, OM994687*, OP023342*; ***Asplenium scolopendrium*** subsp. ***americanum***, United States (US-NY-GC), *Weber-Townsend J.R. & al. NH241* (SYRF), OM675122, OM675341, OM675560, –, OM994603*, OM994650*, –, OP023343*; ***Asplenium scolopendrium*** subsp. ***americanum***, United States (US-NY-GC), *Weber-Townsend J.R. & al. NH242* (SYRF), OM675123, OM675342, OM675561, –, OM994604*, OM994651*, –, OP023344*; ***Asplenium scolopendrium*** subsp. ***americanum***, United States (US-NY-LB), *Weber-Townsend J.R. & al. NH251* (SYRF), OM675187, OM675406, OM675625, –, OM994614*, OM994661*, –, OP023345*; ***Asplenium scolopendrium*** subsp. ***americanum***, United States (US-NY-LB), *Weber-Townsend J.R. & al. NH252* (SYRF), OM675188, OM675407, OM675626, –, OM994615*, OM994662*, –, OP023346*; ***Asplenium scolopendrium*** subsp. ***americanum***, United States (US-NY-LR), *Weber-Townsend J.R. & al. NH261* (SYRF), OM675195, OM675414, OM675633, –, OM994616*, OM994663*, –, OP023347*; ***Asplenium scolopendrium*** subsp. ***americanum***, United States (US-NY-LR), *Weber-Townsend J.R. & al. NH262* (SYRF), OM675196, OM675415, OM675634, –, OM994617*, OM994664*, –, OP023348*; ***Asplenium scolopendrium*** subsp. ***americanum***, United States (US-NY-SB), *Heo N. & Yun S. NH271* (SYRF)[2], –, –, –, –, OM994623*, OM994670*, –, OP023353*; ***Asplenium scolopendrium*** subsp. ***americanum***, United States (US-NY-SB), *Heo N. & Yun S. NH272* (SYRF)[2], –, –, –, –, OM994624*, OM994671*, –, OP023354*; ***Asplenium scolopendrium*** subsp. ***americanum***, United States (US-NY-SB), *Heo N. & Yun S. NH273* (SYRF)[2], –, –, –, –, OM994625*, OM994672*, –, OP023355*; ***Asplenium scolopendrium*** subsp. ***americanum***, United States (US-NY-SB), *Heo N. & Yun S. NH274* (SYRF)[2], –, –, –, –, OM994626*, OM994673*, –, OP023356*; ***Asplenium scolopendrium*** subsp. ***americanum***, United States (US-NY-SB), *Heo N. & Yun S. NH275* (SYRF)[2], –, –, –, –, OM994627*, OM994674*, –, OP023357*; ***Asplenium***

Appendix 2. Continued.

scolopendrium subsp. ***americanum***, United States (US-NY-SB), *Heo N. & Yun S. NH276* (SYRF)[2], –, –, –, –, OM994628*, OM994675*, –, OP023358* & OP023359*; ***Asplenium scolopendrium*** subsp. ***americanum***, United States (US-NY-SB), *Heo N. & Yun S. NH277* (SYRF)[2], –, –, –, –, OM994629*, OM994676*, –, OP023360*; ***Asplenium scolopendrium*** subsp. ***americanum***, United States (US-NY-SB), *Heo N. & Yun S. NH278* (SYRF)[2], –, –, –, –, OM994630*, OM994677*, –, OP023361*; ***Asplenium scolopendrium*** subsp. ***americanum***, United States (US-NY-CT), *Weber-Townsend J.R. & al. NH281* (SYRF), OM675074, OM675293, OM675512, –, OM994596*, OM994643*, –, OP023349*; ***Asplenium scolopendrium*** subsp. ***americanum***, United States (US-NY-FC), *Weber-Townsend J.R. & al. NH291* (SYRF)[1], OM675106, OM675325, OM675544, OM994578*, OM994600*, OM994647*, OM994699*, OP023350*; ***Asplenium scolopendrium*** subsp. ***americanum***, United States (US-NY-FC), *Weber-Townsend J.R. & al. NH292* (SYRF), OM675107, OM675326, OM675545, –, OM994601*, OM994648*, –, –; ***Asplenium scolopendrium*** subsp. ***americanum***, United States (US-NY-HG), *Weber-Townsend J.R. & al. NH301* (SYRF), OM675154, OM675373, OM675592, –, OM994608*, OM994655*, –, –; ***Asplenium scolopendrium*** subsp. ***americanum***, United States (US-MI-SC), *Weber-Townsend J.R. & al. NH311* (SYRF)[1], OM675228, OM675447, OM675666, OM994588*, OM994631*, OM994678*, OM994689*, OP023338*; ***Asplenium scolopendrium*** subsp. ***americanum***, United States (US-MI-SC), *Weber-Townsend J.R. & al. NH312* (SYRF), OM675229, OM675448, OM675667, –, OM994632*, OM994679*, –, –; ***Asplenium scolopendrium*** subsp. ***americanum***, United States (US-MI-SE), *Weber-Townsend J.R. & al. NH321* (SYRF)[1], OM675236, OM675455, OM675674, OM994589*, OM994633*, OM994680*, OM994688*, OP023339*; ***Asplenium scolopendrium*** subsp. ***americanum***, United States (US-MI-SE), *Weber-Townsend J.R. & al. NH322* (SYRF), OM675237, OM675456, OM675675, –, OM994634*, OM994681*, –, –; ***Asplenium scolopendrium*** subsp. ***americanum***, United States (US-MI-TC), *Weber-Townsend J.R. & al. NH331* (SYRF), OM675261, OM675480, OM675699, –, OM994636*, OM994683*, –, OP023340*; ***Asplenium scolopendrium*** subsp. ***americanum***, United States (US-MI-TC), *Weber-Townsend J.R. & al. NH332* (SYRF), OM675262, OM675481, OM675700, –, OM994637*, –, –, OP023341*; ***Asplenium scolopendrium*** subsp. ***americanum***, United States (US-AL-PP), *Weber-Townsend J.R. & al. NH341* (SYRF)[1], OM675058, OM675277, OM675496, OM994575*, OM994592*, OM994639*, OM994702*, OP023351*; ***Asplenium scolopendrium*** subsp. ***americanum***, United States (US-AL-PP), *Weber-Townsend J.R. & al. NH342* (SYRF), OM675059, OM675278, OM675497, –, OM994593*, OM994640*, –, –; ***Asplenium scolopendrium*** subsp. ***americanum***, United States (US-NM), *Baumann L. & al. NH351* (SYRF)[2], –, –, –, –, OM994620*, OM994667*, –, OP023352*; ***Asplenium scolopendrium*** subsp. ***americanum***, United States (US-NM), *Baumann L. & al. NH352* (SYRF)[2], –, –, –, –, OM994621*, OM994668*, –, –; ***Asplenium scolopendrium*** subsp. ***japonicum*** (Kom.) Rasbach., Reichst. & Viane, Japan (JP-HK), *Yun S. NH50* (SYRF)[1], OM675162, OM675381, OM675600, OM994582*, OM994609*, OM994656*, OM994694*, OP023370*; ***Asplenium scolopendrium*** subsp. ***japonicum***, Japan (JP-HK), *Yun S. NH51* (SYRF)[1], OM675163, OM675382, OM675601, OM994583*, OM994610*, OM994657*, OM994695*, OP023371*; ***Asplenium scolopendrium*** subsp. ***japonicum***, South Korea (KR-GW), *NIBRVP0000433230* (KB), OM675146, OM675365, OM675584, –, OM994607*, OM994654*, –, –; ***Asplenium scolopendrium*** subsp. ***japonicum***, South Korea (KR-JJ), *KHB1132827* (KH)[1], OM675130, OM675349, OM675568, OM994580*, OM994605*, OM994652*, OM994696*, OP023368*; ***Asplenium scolopendrium*** subsp. ***japonicum***, South Korea (KR-JJ), *KHB1116109* (KH)[1], OM675138, OM675357, OM675576, OM994581*, OM994606*, OM994653*, OM994697*, OP023369*; ***Asplenium scolopendrium*** subsp. ***japonicum***, South Korea (KR-UL), *KHB1469365* (KH)[1], OM675082, OM675301, OM675520, OM994576*, OM994597*, OM994644*, OM994686*, OP023366*; ***Asplenium scolopendrium*** subsp. ***japonicum***, South Korea (KR-UL), *KHB1121424* (KH)[1], OM675270, OM675489, OM675708, OM994591*, OM994638*, OM994685*, OM994701*, OP023367*; ***Asplenium scolopendrium*** subsp. ***scolopendrium*** L., Spain (ES), *Sloan E.T. NH101* (SYRF)[1], OM675090, OM675309, OM675528, OM994577*, OM994598*, OM994645*, OM994700*, OP023363*; ***Asplenium scolopendrium*** subsp. ***scolopendrium***, Spain (ES), *San José C. NH102* (SYRF), OM675091, OM675310, OM675529, –, OM994599*, OM994646*, –, OP023364*; ***Asplenium scolopendrium*** subsp. ***scolopendrium***, Croatia (HR), *TNS743756* (TI)[1], OM675170, OM675389, OM675608, OM994584*, OM994611*, OM994658*, OM994693*, OP023365*; ***Asplenium scolopendrium*** subsp. ***scolopendrium***, Portugal (PT), *Testo W. NH100* (SYRF)[1], OM675219, OM675438, OM675657, OM994587*, OM994622*, OM994669*, OM994690*, –; ***Asplenium scolopendrium*** subsp. ***scolopendrium***, United Kingdom (UK), *Fernando D.D. & al. NH103* (SYRF)[1], OM675114, OM675333, OM675552, OM994579*, OM994602*, OM994649*, OM994698*, OP023362*; ***Asplenium adiantum-nigrum*** L., JX475200; ***Asplenium nidus*** L., MK002975.