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Biodiversity and apomixis: Insights from the East-Asian holly ferns in *Polystichum* section *Xiphopolystichum*

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1. Introduction

Polyploidy, apomixis, and hybridization are common across many plant lineages and are important catalysts for evolution and speciation. For pteridophytes in particular, these three evolutionary processes are often closely linked and, together, are responsible for the complex patterns of reticulate evolution found in an array of fern genera (Otto and Whitton, 2000; Soltis et al., 2004; Grusz, 2016). Thus, understanding sources of diversity in ferns, especially in world centers of diversity such as western China and the tropical Andes, rests on deciphering how polyploidy, hybridization, and apomixis interact to shape patterns of pteridophyte speciation.

Polyploidy, which is characteristic of up to 95% of fern species (Love et al., 1977; Grant, 1981) and accompanies 31% of fern speciation events (Wood et al., 2009), may arise via chromosomal doubling (autopolyploidy) or hybridization followed by doubling of chromosomes (allopolyploidy—Manton, 1950; Stebbins, 1950). These hybrids may either enhance or compromise genetic diversity depending on the nature of the hybrid or parental species involved (Barrington et al., 1989). Many fern polyploids, notably allopolyploids, are capable of ordinary sexual reproduction. However, both auto- and allopolyploids may have an unbalanced set of genomes, e.g. a genome from at least one parent is represented with an odd number of copies (ABB), or one in which genomes from each parent is evenly represented but are still unbalanced (AABBBB). The result is that chromosomes in such a genome are left either with multiple homologs with which to pair during meiosis, or none at all. One means of overcoming sterility imposed by an unbalanced genome is apomictic reproduction (Cosendai et al., 2011). There is a strong association between polyploidy, both auto- and allopolyploidy, and apomixis, some estimates suggest that as many as three quarters of apomictic ferns are triploid (Wagner and Wagner, 1980; Asker and Jerling, 1992). It should be noted, however, that some autotetraploid fern lineages are apomictic, such as *Pellaea glabella* Mett. ex Kuhn and *P. occidentalis* Rydb. (Gastony, 1988), and some diploid apomicts are known, including *Dryopteris wallichiana* (Spreng.) Hyl., *Cheilanthes leucopoda* Link, and *Pteris cretica* L. (Verma and Khullar, 1965; Knobloch, 1967; Fraser-Jenkins, 2007). In general, apomixis may be adaptive in relation to environmental factors such as aridity or insularity (Haufler et al., 2016), and hence, as a means of reproduction, it may confer an adaptive advantage.

Unlike vegetative sexual reproduction, apomixis, by including spore production, maintains the benefit of spore dispersal and also allows for the accumulation of genetic variation through recombination of novel mutations (when meiosis persists) (van Baarlen et al., 2002). In fern apomixis, unreduced spores are produced at the end of meiosis and syngamy does not occur. In the Döpp-Manton (Manton, 1950) scheme of fern apomixis, successful sporogenesis includes three mitotic divisions followed by chromosomal replication but not mitotic division, resulting in a restitution nucleus with each chromosome represented by two newly produced homologues. A final complete meiotic division results in 32 spores— a spore number characteristic of apomictic ferns. Importantly, in the Döpp-Manton scheme of apomixis, gametophytes usually develop only antheridia (the male reproductive structures), and either lack archegonia (the female reproductive structures) or produce archegonia containing abortive eggs. Therefore Döpp-Manton apomicts are most often paternal progenitors when they are involved in hybridization.

The relationship between apomixis and reticulation makes it important to decipher the relative contribution of sexually reproducing progenitors and their derivative apomicts to the total pattern of diversity in ferns. Reticulate evolutionary histories can be inferred when two or more alleles are sequenced from a single individual, and end up phylogenetically sister to sequences derived from two or more distinct species. Such multi-allele phylogenies can be generated through the use of vector cloning, next-generation sequencing, and/or careful analysis of chromatographic reads generated from direct sequencing. The last technique can specifically reveal double peaks resulting from summation of multiple alleles at a single position in the nucleotide sequence, as would be expected from a heterozygote or allopolyploid (Zhidkov et al., 2011; Chang et al., 2012; Lyons et al., 2017). Molecular

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phylogenies constructed from sequences generated by direct Sanger sequences that do not utilize such analyses may be misleading in taxonomic groups predisposed to hybridization, as phylogenies are strictly bifurcating representations of speciation (Soltis and Soltis, 2009).

Several prominent recent investigations have employed an integrated systematic approach to disentangling the evolutionary history and delimiting species in highly reticulate fern species complexes in which polyploidy, hybridization, and apomixis all play a role in speciation. Grusz et al. (2009) demonstrated that two closely related North American apomicts — *Myriopteris wootonii* (Maxon) Grusz & Windham and *M. yavapensis* (T. Reeves ex Windham) Grusz & Windham —both unbalanced allopolyploids—actually share diploid progenitors. Similarly, in the Aspleniaceae, four unbalanced polyploid apomictic lineages in the *Asplenium monanthes* L. complex likely share a sexual diploid progenitor, but differ in their ploidy and genomic contributions (Dyer et al., 2012). Hori et al. (2014) used plastid and nuclear sequence data to determine the genomic composition of several apomicts in the East Asian *Dryopteris varia* (L.) Kuntze complex. Nuclear *pgiC* sequences suggested that apomicts in the complex each resulted from unique hybridization events among a group of closely related sexual diploid species. Other notable work in this area has been done in *Pellaea* (Gastony, 1988), *Cornopteris* (Park and Kato, 2003), and *Phegopteris* (Driscoll et al., 2003). Many of these studies found strong evidence from nuclear markers of hybridization between apomictic species and their progenitors, resulting in new apomictic lineages. However, elucidation of the patterns of genomic inheritance as well as the identity and relationships of lineages within these complexes can open the door to other more confounding issues in the study of fern apomixis. For example, Dyer et al. (2012) and Hori et al. (2014) both found evidence of multiple hybrid origins and multiple cytotypes, in the *A. monanthes* and the *D. varia* complex, respectively. Findings such as these often complicate taxonomy as well as efforts to consistently apply criteria with the goal of defining species in these complexes.

Species can be defined by criteria relying on the mechanism of speciation, processes or biological forces maintaining cohesion and isolation from other species, and unique molecular or morphological characteristics (Cracraft, 1990; Nixon and Wheeler, 1990; de Queiroz, 1998). The biological processes inherent in apomicts can cause problems with each of these approaches to characterizing species. Indeed, the species concepts that are currently most widely applied, including the biological, phenetic, and some versions of the phylogenetic species definitions (Mayr, 1942, 1963; Mishler, 1985; Nixon and Wheeler, 1990), fail to recognize apomictic lineages as distinct species. For instance, apomictic ferns, by undergoing primarily or exclusively asexual reproduction, defy the biological species definition's tenet that a species remains cohesive through interbreeding (Gastony, 1988, Gastony and Windham, 1989; Grusz et al., 2009). Similarly, one implication of the tendency of apomictic ferns to arise from multiple origins, or hybridization, is that many potentially evolutionarily and phylogenetically distinct apomictic lineages are taxonomically united as one species on the basis of morphology under the phenetic species concept (Barrington et al., 1989; Takamiya et al., 2001). In contrast, some apomictic species may belong to a single lineage with a single evolutionary origin, but exhibit more intra- to inter-specific morphological variation. The result is accumulating mutations that become fixed in the absence of sexual reproduction within various populations, leading to taxonomic categorization as multiple species as defined by morphology. The phylogenetic species criterion has three variants, each espoused by different authors: (1) "…the smallest diagnosable cluster of individual organisms within which there is a parental pattern of ancestry and descent" (Nixon and Wheeler, 1990), (2) "a population or group of populations defined by one or more apomorphous features" (Rosen, 1979), and (3) "…that set of organisms between two speciation events, or between one speciation event and one extinction event" (Ridley, 1989). Apomictic lineages often meet criterion versions (1) and (2), in that they are often genetically and phylogenetically distinct units, each with unique apomorphies—molecular, morphological, and reproductive. The third variant of the phylogenetic species criterion is much more difficult to apply to apomictic lineages because they may be the products of, and participants in, repeated hybridization.

Given the shortcomings of applying just one of these definitions to the problem of species delimitation in apomictic ferns and their allies, we support the use of a species concept that incorporates phylogeny, morphology, ecology, and reproduction, as incorporated in the general lineage concept of de Queiroz (1998, 1999). De Quieroz has advocated for a more inclusive approach to species definition and delimitation, one that accommodates both sexual and asexual species. He argues that the species concept should be considered in the context of *lineages*, where the discrete *taxon* is part of a "series of entities forming a single line of direct ancestry and descent" (de Queiroz, 1998). Under his *General Lineage Concept*, a species is considered a *segment* of a lineage, in that a species is temporally separated from its ancestors and descendants on an evolutionary time scale. Of central importance to us, this more unified species concept treats other species concepts as *criteria* for species delimitation, all relevant to delimiting species under the general lineage concept. Each species in an apomictic complex should be defined, at a minimum, by one accepted species criterion.

The monophyletic *Polystichum* Roth sect. *Xiphopolystichum* Daigobo of *Polystichum* (Dryopteridaceae) (Daigobo, 1972; Zhang, 1996; Kung et al., 2001; Lu et al., 2007; Li et al., 2008), known commonly as holly ferns, currently includes both sect. *Xiphopolystichum sensu stricto* (*s.s.*; Kung et al., 2001) and section *Duropolystichum* Fraser-Jenkins (Zhang and Barrington, 2013). Virtually all sect. *Xiphopolystichum* species are native to East and Southeast Asia, with most being endemic to the region. Species in this group are most abundant in mountainous regions surrounding the Sichuan Basin (Zhang and Barrington, 2013), including the majority of known apomictic lineages. Several named species within sect. *Xiphopolystichum s.s.* have been identified as triploid apomicts: *Polystichum tsus-simense* (Hook.) J. Sm, *P. xiphophyllum* (Baker) Diels, *P. mayebarae* Tagawa, and *P. sinotsus-simense* Ching & Z. Y. Liu (Daigobo, 1973; Gibby, 1985), and have an array of leaf dissections between once and twice pinnate, most typically once pinnate-pinnatifid. *Polystichum pseudoxiphophyllum* Ching ex H. S. Kung is highly morphologically similar to *P. xiphophyllum*, though cytological and reproductive data are lacking for this taxon. Given the tendency for hybrid ferns to exhibit a morphology intermediate between their parents (e.g., Barrington, 1986), we posit that apomictic lineages with a once pinnate-pinnatifid laminar dissection are likely to have originated as hybrids of once-pinnate and twice-pinnate progenitors (Wagner, 1983). *Polystichum herbaceum* Ching & Z. Y. Liu and *P. revolutum* P.S. Wang are proposed to be sexually reproducing diploids (Gibby, 1985) belonging to sect. *Xiphopolystichum sensu stricto* (*s.s.*). Among these candidate diploid progenitors, only one, *P. revolutum*, is once-pinnate.

Here, we establish phylogenetic relationships among taxa currently circumscribed in sect. *Xiphopolystichum s.s.* to elucidate the evolutionary history of apomictic lineages in the group and to delimit species using a combination of criteria appropriate for apomicts. In sect. *Xiphopolystichum*, we contend that delineating species is best based on the phylogenetic, phenetic, and evolutionary species criteria considered together. Our dataset lends itself to evaluating each of these criteria both independently and synthetically toward defining species.

We explore the contribution of the once-pinnate *Polystichum revolutum* to the ancestry of apomictic lineages in sect. *Xiphopolystichum* through a study of nuclear markers in the light of ploidy levels, breeding systems, and morphology. Specifically, we test the hypothesis that *P. revolutum* is the once-pinnate progenitor that contributed the less-dissected morphology to the apomicts. Further, we address three major questions regarding the genomic composition of the apomicts in relation to their morphology. Are the genomes in balance? Do the identities and proportions of the contributed genomes relate to the variation in lamina dissection so prominent in the complex? Do these

genetic profiles yield insights into the delimitation of taxonomic species in the section?

2. Materials and methods

2.1. Taxon sampling and species delimitation

We assembled a broad sample of species in *Polystichum* sect. *Xiphopolystichum s.s.*, as well as select members of sect. *Xiphopolystichum s.l.* as circumscribed in Zhang and Barrington (2013). *Polystichum* sect. *Xiphopolystichum s.l.* includes members of *Duropolystichum. Polystichum otophorum,* included in sect. *Xiphopolystichum s.s.* (Zhang and Barrington, 2013) is here considered synonymous with *P. xiphophyllum* given morphological and molecular similarity (Le Péchon et al., 2016). The single prominent exception is *P. pseudosetosum* Ching & Z. Y. Liu, for which we had no material. Sampling of sect. *Xiphopolystichum s.s.,* comprised 32 accessions collected across western China including Yunnan, Guizhou, and Sichuan provinces during two field trips by the authors, one in 2006 and one in 2015. We chose to sample accessions from as wide a geographic and morphological range as possible. In general, three to five accessions for each ingroup species were analyzed to account for morphological variation and potential population variation within species. Herbarium vouchers for all collections were deposited at the Pringle Herbarium (VT), University of Vermont, Burlington, VT, USA, or the Yunnan University Herbarium (PYU). Choice of *Polystichum* outgroups was guided by previous phylogenetic analyses (Little and Barrington, 2003; Li et al., 2008) and included representation both from the sections of *Polystichum* most closely related to sect. *Xiphopolystichum*, as well as from genera most closely related to *Polystichum*. A complete list of taxa used in the study including voucher information and GenBank accession numbers is provided in Appendices A and B.

2.2. Flow cytometry

Flow cytometry analysis was conducted for 11 accessions in order to estimate ploidy. Each accession included samples of each taxon in sect. *Xiphopolystichum s.s.* (Appendix B). Approximately one to three grams of tissue of each accession were dried in silica in the field and later stored at 10 °C for two to seven months before preparation for flow cytometry. Tissue preparation for flow cytometry followed the protocol of Bainard et al. (2011) with some modifications. Some coarse tissues were subject to an incubation period in digestive enzymes according to Naill and Roberts (2005) prior to staining. At least two replicates were run on the flow cytometer for each accession on different days, depending on availability of field-collected tissue. Samples were analyzed using a Coulter Epics XL flow cytometer (Beckman Coulter Genomics), equipped with a UV lamp located in the Plant Biology Dept., University of Vermont. Histograms were analyzed using FlowPy (http://flowpy.wikidot.com) software. Fresh tissue of *Pisum sativum* L., cultivated in the laboratory, was used as a genome-size standard, with a known 2C value of 4.88 pg (Bennett and Smith, 1976). For accessions with peaks overlapping with *Pisum sativum,* findings were cross validated using *Hordeum vulgare* L. var. morex, with a 2C genome size of 11.1 pg (Bennett and Smith, 1976). All sample G1 peaks were adjacent but not overlapping with G1 peaks of the standard, allowing for comparison and estimation of sample 2C values using the formula, Sample 2C value (DNA pg) = Reference 2C value x (sample 2C mean peak position/reference 2C mean peak position). The 2C (pg) values of named sect. *Xiphopolystichum* species were expected to be multiples of the 1C (pg) value of known diploid *Polystichum acrostichoides*, 7.75 pg (Bainard et al., 2011). Ploidies were therefore estimated by comparison to these hypothesized values.

2.3. Spore measurements

For each species, sporangia and spores from one to two specimens (details, Appendix B) were mounted in Hoyer's medium on glass slides and imaged at 100x using a compound microscope. The sporangia and spores were imaged in order to count spores per sporangium (to assess reproductive biology) as well as measure the length of spores (as an indication of ploidy). One to two sporangia per specimen were counted: plants with spore counts of 32 or fewer were inferred to be apomictic; those with more than 32 per sporangium were inferred to be sexual. Twenty to 30 spores per specimen were measured to calculate mean length and standard deviation for each species. Spore length was measured from the images using ImageJ (Schneider et al., 2012). The external spore membrane (*perispore*), which is pronounced among the species examined, was excluded from measurements.

2.4. DNA Extraction, amplification and sequencing

Total genomic DNA was extracted from fresh (1 g) or silica-dried (0.5 g) leaves using a cetyl trimethyl-ammonium bromide (CTAB) procedure (Porebski et al., 1997) with some modifications. Leaves were ground with a bead-beating machine using glass beads. CTAB buffer was supplemented with polyvinyl pyrrolidone (PVP), and crushed leaf tissue was precipitated in chloroform. Samples were then subjected to washes in 70% and 90% ethanol and re-suspended in Tris-EDTA buffer. The plastid DNA sequences *rbcL*, *trnL-trnF* spacer region, and *trnS-rps4* spacer region were PCR amplified under standard conditions using previously published primers, with modifications for *rbcL* (Taberlet et al., 1991; Little and Barrington, 2003; McHenry and Barrington, 2014), as were the nuclear DNA sequences *gapCp* (exons 8–11) and *PgiC* (exons 14–16) (Table 1), with primers modified from Schuettpelz et al. (2008), Koenemann et al. (2011), and Lyons et al. (2017). The marker *trnS-rps4* was amplified using an initial denaturation step of 94 °C for 3 min, followed by 30 cycles of 94 °C for 45 s, 53 °C for 45 s, and 72 °C for 2 min, and a final extension at 72 °C for 10 min. Amplification of *rbcL*, *trnL-trnF,* and the nuclear markers was done using an initial denaturation step of 94 °C for 10 min, followed by 35 cycles of 94 °C for 45 s, 58 °C for 1 min, and 72°C for 1 min. Resulting PCR products were cleaned using ExoSAP-IT (USB Corporation, Cleveland, Ohio, USA), and sequenced on an ABI PRISM 3730*x* automated sequencer (Beckman Coulter Genomics, Danvers, MA, USA). Each plastid and nuclear region was sequenced in both the forward and reverse direction using the amplification primers.

2.5. Sequence analysis

Consensus sequences were generated from assemblies of forward and reverse reads, and aligned using MUSCLE (Edgar, 2004) with minor manual adjustments. Assembly, consensus generation, and alignment

Table 1
Primers modified from named references.

Marker	Primer sequence	Reference
trnL-trnF	F 5′GGTTCAAGTCCCTCTATCCC3′ R 5′ATTTGAACTGGTGACACGAG3′	Taberlet et al. (1991)
rps4-trnS	F 5′TTACCGAGGGTTCGAATCCCTC3′ R 5′GAGTATTACTCCCGCAAAG3′	McHenry and Barrington (2014)
rbcL	F 5′TTCATGCGTTGGAGAGATC3′ R 5′GGACTCCACTTACWAGCTTC3′	Little and Barrington (2003)
gapCp	F 5′CCAAGTTCAACTGGTGCT3′ R 5′TGCTWCATCTGCAGACAACC3′	Schuettpelz et al. (2008)
pgiC	F 5′TTTGCTCCTCACATTCAAC3′ R 5′CTTAGTATGGAAAGCAATGGAAAGG3′	Lyons et al. (2017); Koenemann et al. (2011)

Table 2
Primers with the total aligned length indicated. The number of informative characters is given for each marker, including gaps.

Marker	Length	Informative Characters	Nucleotide Substitution Model
trnL-trnF	432	30	GTR + G
rbcL	1338	54	SYM + I
rps4-trnS	1060	63	GTR + G
Pgi-C	648	46	HKY + G
gapCp	657	39	HKY + G

were implemented in Geneious version 9.0 (Kearse et al., 2012); for species represented by multiple accessions, consensus sequences were generated for phylogenetic analysis. Indels were coded simply as single characters with binary states (simple gap coding; Simmons and Ochoterena, 2000). The resulting indel data were appended to the end of sequences for use in all subsequent model testing and phylogenetic inference analysis. Initially, data sets for each marker were aligned and analyzed individually using Bayesian Inference (BI) approaches. The tree topologies generated from the individual analyses were inspected for discordance among topologies. Few differences were observed among topologies and differences were poorly supported; consequently, plastid sequences and nuclear sequences were each concatenated into one plastid and one nuclear data set for further analysis.

Bayesian Inference was applied to both the nuclear and plastid datasets using MrBayes version 3.2.6 (Ronquist et al., 2012) on the CIPRES Science Gateway server (Miller et al., 2010). For BI analysis of plastid sequences, the alignment was partitioned by markers and optimal evolutionary models discerned from jModeltest 2 (Table 2; Guindon and Gascuel, 2003; Darriba et al., 2012) using the Akaike Information Criterion (AIC). BI for both the plastid and nuclear dataset was run for 50,000,000 generations. Log files from both analyses were analyzed in Tracer c 1.5 (Rambaut and Drummond, 2007) to determine the proportion of generation for burn-in. For the BI analysis of the plastid dataset, the first 500,000 trees were discarded as burn-in iterations, the remainder were used to generate a 50% majority-rule consensus tree. For BI analysis of the nuclear dataset, the first 1,000,000 generations were discarded as burn-in and the remainder were used to generate a 50% majority rule consensus tree. Posterior probabilities were obtained from MrBayes, and the phylogenetic tree including branch lengths was visualized using FigTree Version 1.4 (http://tree.bio.ed.ac.uk/software/figtree/).

2.6. Mixed nucleotide signals and reticulation

The aligned chromatograms of the original forward and reverse sequences for the nuclear markers *gapCp* and *pgiC* were examined for multiple nucleotide peaks at informative positions. Following Tate et al. (2006), Jorgensen and Barrington (2017), and Lyons et al. (2017), these multiple peaks were taken as evidence of different allelic variants retrieved for the marker in question, summed in the single chromatogram generated from direct Sanger sequencing (Fig. S2).

2.7. Morphological analysis

For all 43 accessions representing the breadth of morphological variation in sect. *Xiphopolystichum,* including the 20 for which ploidy was estimated using flow cytometry and spore length, five morphological characters (Table S1) were scored. A principal component analysis (PCA) was then conducted using the R package *gg biplot* (http://ggplot2.tidyverse.org/). Each combination of pairs PC1, PC2, and PC3 (Table S2) was plotted for interpretation. The PCA (Fig. S1) was used as a heuristic tool for determining the most important morphological characters in distinguishing sect. *Xiphopolystichum* species.

3. Results

3.1. Flow cytometry reveals multiple ploidies within and between Polystichum species

Flow cytometry analysis of accessions of sect. *Xiphopolystichum* revealed only diploid accessions of *Polystichum revolutum* and *P. herbaceum*, and only triploid accessions of *P. mayebarae, P. sinotsus-simense*, and *P. pseudoxiphophyllum.* On the other hand, *P. tsus-simense* accessions included both triploid and diploid genome sizes and *P. xiphophyllum* accessions included both triploid and tetraploid genome sizes. For each of these named species with multiple ploidies, the more common DNA ploidy was triploid (Fig. 3B, Table S3).

3.2. Spore data corroborate flow cytometry ploidy estimates

Spore counts and measurements corroborated findings from flow cytometry. Spore counts for named species *Polystichum xiphophyllum, P. tsus-simense, P. mayebarae, P. sinotsus-simense,* and *P. pseudoxiphophyllum* consistently revealed between 20 and 32 spores per sporangium, suggesting apomictic reproduction. Spore counts of *P. revolutum* and *P. herbaceum* each yielded more than 45 spores per sporangium, suggesting sexual reproduction. These two species had mean spore lengths of approximately 30 μm, which in the light of the flow-cytometry data we take to be a typical diploid spore length for the section. The mean spore length of most apomictic species was about 40 μm, consistent with their being triploid, as inferred from the flow-cytometry data. However, some accessions of *P. xiphophyllum* had a higher mean spore length, and some accessions of *P. tsus-simense* had a lower mean spore length. These accessions include those with atypical results in the flow-cytometry data. One *P. xiphophyllum* accession with a larger genome size than other accessions of the species had larger spores; one *P. tsus-simense* accession with a lower genome size than other accessions of the same species had smaller spores (Fig. 3A, Table S3).

3.3. Plastid and nuclear datasets support two major clades in Xiphopolystichum s.s.

The length of all plastid and nuclear markers, as well as the models implemented in Bayesian phylogenetic analysis, are given in Table 2. We chose to use concatenated plastid and nuclear phylogenies generated from BI analyses for hypothesis testing. Comparison of the plastid and nuclear phylogenies demonstrated general congruency in the topology of the sect. *Xiphopolystichum s.s.* clade (Figs. 1 and 2), except that *Polystichum revolutum* and the triploid *P. xiphophyllum* (3x) were sister in the former and unresolved in the latter, and *P. pseudoxiphophyllum* belongs to clade A in the plastid phylogeny and clade B in the nuclear phylogeny. In both 50% majority-rule trees, sect. *Xiphopolystichum s.s.* was recovered as a monophyletic group, with two major clades: Clade A including *P. revolutum, P. mayebarae,* both sampled cytotypes of *P. xiphophyllum*, and Clade B including *P. herbaceum, P. sinotsus-simense,* and both sampled cytotypes of *P. tsus-simense.* Unfortunately, between-species relationships in Clade B are unresolved in both phylogenies (Figs. 1 and 2). Within *Duropolystichum*, sister relationships between *P. garhwalicum* and *P. hillebrandii* and between *P. latilepis* and *P. neolobatum* were supported by the plastid data, with *P. latilepis* and *P. hillebrandii* being supported as sister in the nuclear phylogeny. In general, the topology for sect. *Xiphopolystichum s.s.* is congruent with findings from Le Péchon et al. (2016), in which sect. *Xiphopolystichum s.s.* is a monophyletic group. Final alignments and phylogenetic trees for both plastid and nuclear datasets are available in the Treebase database (treebase.org) under submission numbers 22411 and 22579, respectively.

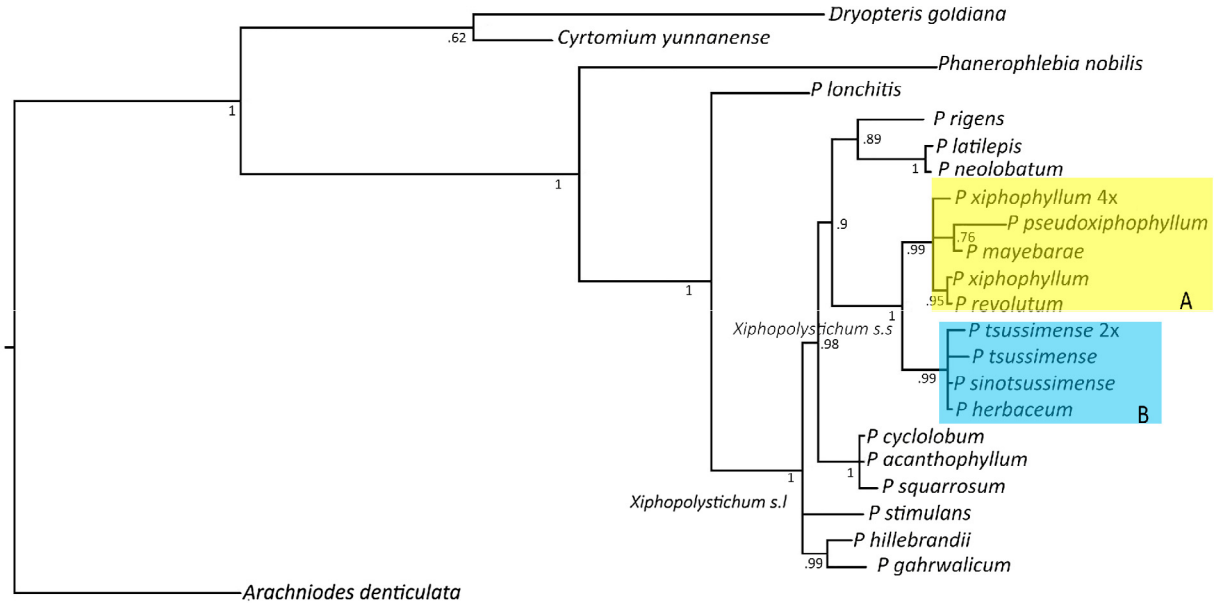

Fig. 1. Phylogeny of *Polystichum* sect. *Xiphopolystichum* based on the combined analysis of plastid markers, *trnL-F, rps4-trnS,* and *rbcL.* Sect. *Xiphopolystichum s.s.* is comprised of clades A and B. The tree is the 50% majority rule Bayesian Inference (BI) phylogram of sect. *Xiphopolystichum*. Posterior Probability values are given at each node.

3.4. Nucleotide summation as evidence for reticulation

Alignments of chromatographic sequences for *gapCp* and *PgiC* in our dataset revealed seven and nine sites with two nucleotide calls, respectively (Table 3a and 3b). Within the sect. *Xiphopolystichum s.s.* clade, chromatograms of nuclear sequences with multiple nucleotide calls were retrieved from four species, *Polystichum sinotsus-simense, P. xiphophylum* (both 3x and 4x)*, P. mayebarae,* and *P. pseudoxiphophyllum.* All of the two-nucleotide calls included two nucleotides common at that same site in other sequences. For the majority of sites with double-nucleotide calls, the signal combines nucleotides shared with sexual diploids *P. revolutum* in Clade A and *P. herbaceum* in Clade B (Figs. 1 and 2). However, the relative strength of nucleotide signals combined at these sites varied among the apomictic polyploids *P. xiphophyllum* (3x and 4x), *P. mayebarae, P. sinotsus-simense,* and *P. pseudoxiphophyllum*. The diploid species *P. revolutum* and *P. herbaceum* did not have mixed nucleotide signals, nor did triploid apomict *P. tsus-simense.*

Polystichum mayebarae, and both cytotypes of *P. xiphophyllum* belong to Clade A (Figs. 1 and 2), along with sexual diploid *P. revolutum.* All of the two-nucleotide calls in the polyploid apomicts *P. mayebarae* and *P. xiphophyllum* (3x and 4x) share one nucleotide with *P. revolutum.* At most of these sites for *P. mayebarae* and *P. xiphophyllum* (3x and 4x), the higher peak is shared with *P. revolutum,* and the lower peak is shared with *P. herbaceum* and *P. tsus-simense* in Clade B. For *P. pseudoxiphophyllum,* resolved in clade A in the plastid phylogeny and clade B in the nuclear phylogeny, the stronger peak is shared with *P.*

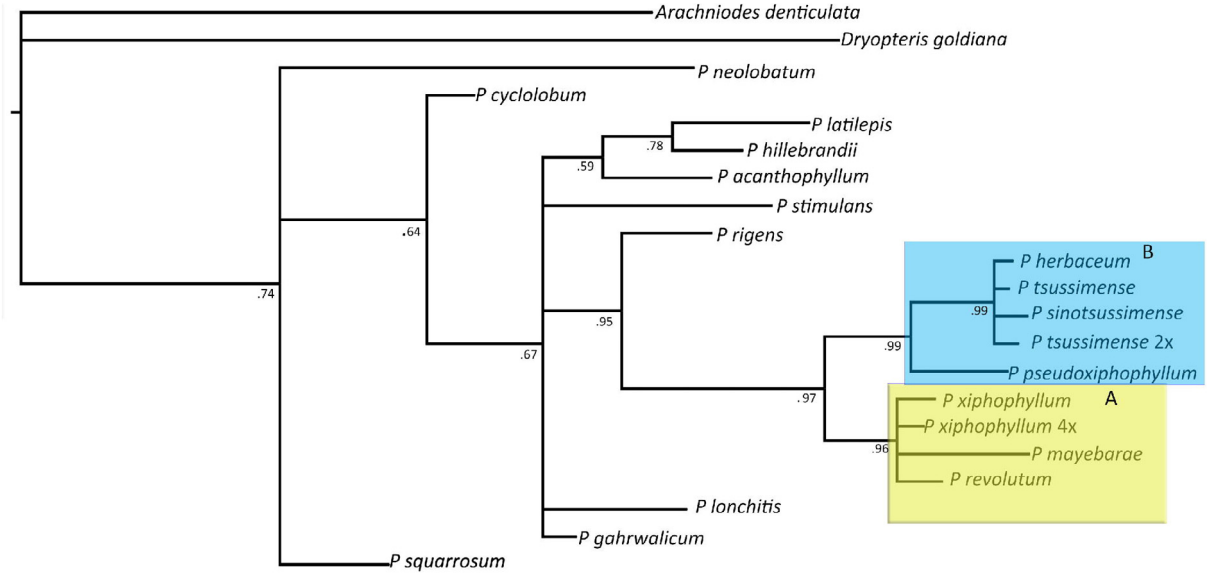

Fig. 2. Phylogeny of sect. *Xiphopolystichum* based on the combined analysis of nuclear markers, *gapCp* and *PgiC.* The tree is the 50% majority-rule phylogram from the Bayesian Inference (BI) analysis. BI Posterior Probability values are given at each node.

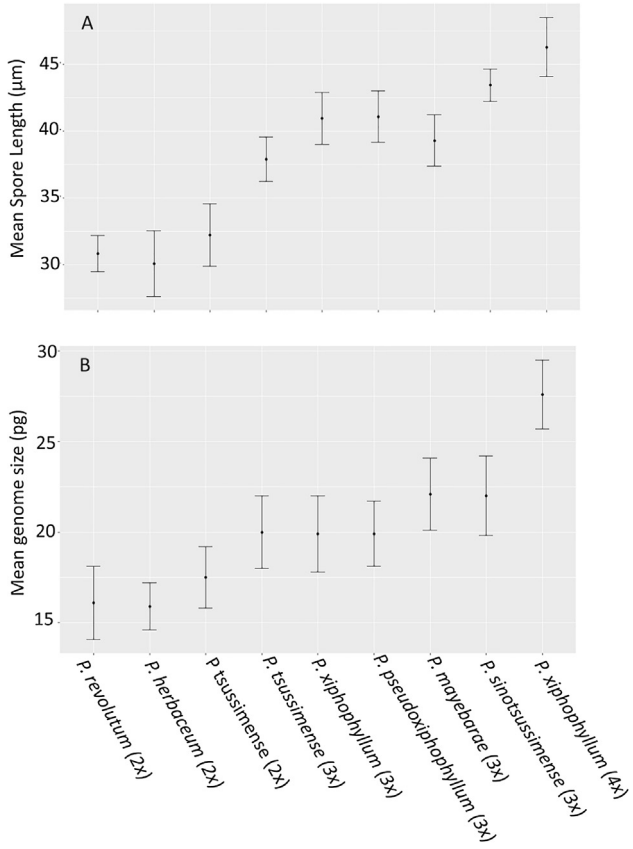

Fig. 3. a, b. Mean spore length (A) for each species in sect. *Xiphopolystichum s.s.* and cytotype; bars indicate 95% confidence intervals (CI). Mean genome size (B) for each species and cytotype; bars indicate 95% CI.

Table 3
a,b. Base positions in an alignment of chromatograms of *gapCp* (A) and *PgiC* (B) with multiple peaks. For species with double peaks in the chromatograms, the nucleotide giving a stronger signal is shown in bold.

a.

	110	238	247	256	268	302	526
P. revolutum	C	A	A	T	C	A	T
P. xiphophyllum	**C**/A	**A**/T	**A**/T	**T**/C	**C**/A	T/**A**	**T**/C
P. mayebarae	**C**/A	**A**/T	**A**/T	**T**/C	**C**/A	T/**A**	**T**/C
P. sinotsussimense	A	A/**T**	A/**T**	T/**C**	**A**/C	**T**/A	**C**/T
P. tsussimense	A	T	T	T	A	T	C
P. herbaceum	A	T	T	T	A	T	C

b.

	106	127	213	323	331	337	404	467	508
P. revolutum	T	A	T	C	A	G	G	T	T
P. xiphophyllum	C/**T**	**A**/C	**C**/T	**C**/T	**A**/C	**G**/A	**G**/T	**T**/A	**T**/C
P. xiphophyllum (4x)	C/**T**	**A**/C	**C**/T	**C**/T	**A**/C	**G**/A	**G**/T	**T**/A	**T**/C
P. pseudoxiphophyllum	–	A/**C**	C/**T**	**T**/C	A/**C**	G/**A**	G/**T**	**A**/T	T/**C**
P. mayebarae	C/**T**	**A**/C	**C**/T	**C**/T	**A**/C	**G**/A	**G**/T	**T**/A	**T**/C
P. sinotsussimense	C	C	C/**T**	**T**/C	**C**/A	G/**A**	T	A	C
P. tsussimense	C	C	C	T	C	A	T	A	C
P. tsussimense (2x)	–	C	C	T	C	A	T	A	C
P. herbaceum	C	C	C	T	C	A	T	A	C

herbaceum and the weaker peak is shared with *P. revolutum*. *Polystichum sinotsus-simense* belongs to Clade B along with *P. tsus-simense* and *P. herbaceum*. Most of the two-nucleotide calls in the polyploid apomict *P. sinotsus-simense* share one nucleotide with *P. herbaceum*. At most of these sites, the higher peak is shared with *P. herbaceum*.

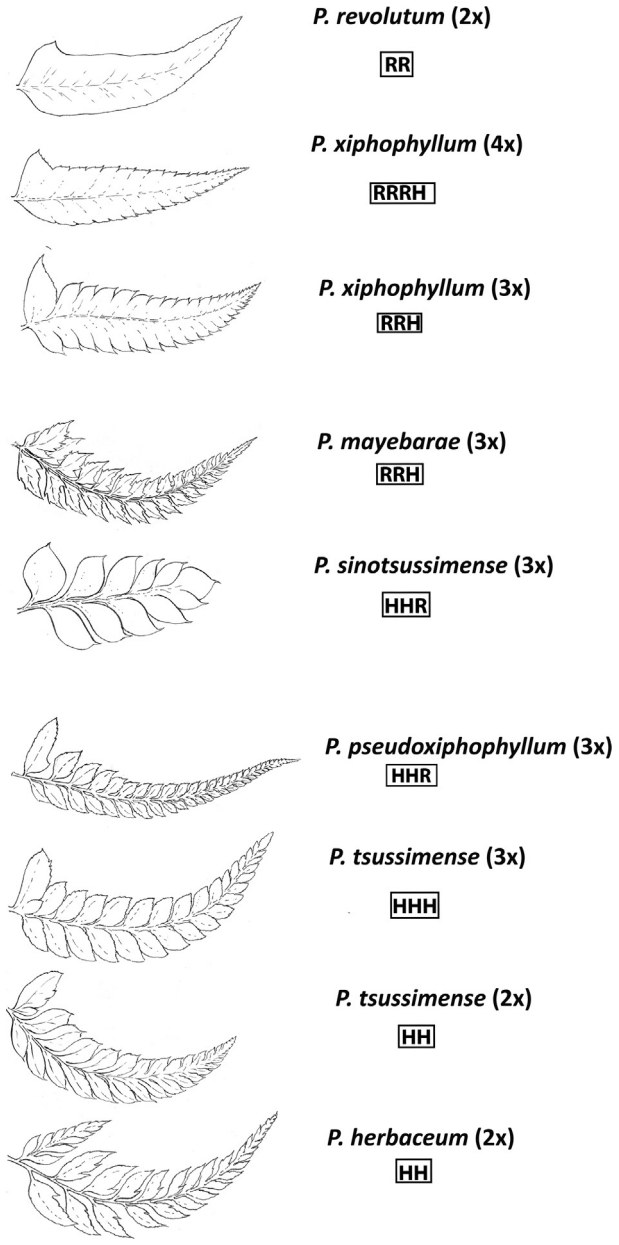

Fig. 4. Pinna morphology for species resolved in sect. *Xiphopolystichum s.s.*, as well as the tetraploid cytotype of *Polystichum xiphophyllum* and diploid cytotype of *P. tsus-simense,* which are morphologically distinct from the more common triploid cytotypes. Letters underneath each pinna diagram indicate the inferred genomic composition.

3.5. Morphological analysis

PCA based on five leaf measurements revealed that the first two principal components accounted for 74% of the variance across species. A plot of these two PC axes revealed largely overlapping clusters representing *P. herbaceum*, *P. pseudoxiphophyllum, P. revolutum, P. sinotsus-simense, P. tsus-simense,* and *P. xiphophyllum*. The diploid race of *P. tsus-simense* and the tetraploid race of *P. xiphophyllum* are each represented by only two accessions and hence are not represented by clusters (Fig. S1). The most important character in defining clusters on the first principal component was number of pinnules on the second pinna (a proxy for level of leaf dissection), whereas clusters were best resolved by the ratio of the length of first and second pinna (a proxy for overall frond shape) on the second principal component (Table S2). The level of leaf dissection in sect. *Xiphopolystichum s.s.* ranges from once-pinnate in

P. revolutum to fully twice-pinnate in *P. herbaceum* (Fig. 4). All other species and cytotypes recognized in the group are intermediate in level of dissection. The mean ratio of length of the basalmost pinna to the second basalmost pinna is highest in *P. revolutum* and lowest in *P. herbaceum.* All other species in the group have a ratio either identical to the two diploids, or intermediate. The value of this ratio is generally lower for species in Clade B than in Clade A (Figs. 1 and 2).

4. Discussion

Considering evidence from phylogeny, ploidy, reproductive mode, and the genome signatures in each hybrid taxon, we have discerned new apomictic cytotypes and established the ways in which each of the two sexual diploid genomes have contributed to the evolution of the known apomictic taxa in *Polystichum* sect. *Xiphopolystichum*.

4.1. Hybridization history of apomicts

4.1.1. Clade A

Based on both nuclear and plastid phylogenies, considered in the light of ploidy levels, sect. *Xiphopolystichum s.s.* comprises two clades, both of which include one sexual diploid and multiple apomicts. In clade A of the plastid phylogeny (Fig. 1), nucleotide polymorphisms for nuclear genes of apomictic triploid and tetraploid *Polystichum xiphophyllum,* triploid *P. mayebarae*, and triploid *P. pseudoxiphophyllum* indicate that they are allopolyploids derived from the clade A diploid *P. revolutum* and clade B diploid *P. herbaceum* (Fig. 5). Furthermore, relative peak heights in the chromatograms are consistent with triploid *P. xiphophyllum* and triploid *P. mayebarae* having incorporated two genomes from *P. revolutum* and one from *P. herbaceum*. We also propose that our newly discovered tetraploid cytotype of *P. xiphophyllum* incorporates three *P. revolutum* genomes with one *P. herbaceum* genome. One plausible scenario for the evolution of tetraploid *P. xiphophyllum* is one or more hybridization events between apomictic triploid *P. xiphophyllum* and sexual *P. revolutum* (Fig. 5). Conversely, relative peak heights in chromatograms for triploid apomict *P. pseudoxiphophyllum* suggest that it has inherited one genome from *P. revolutum* and two genomes from *P. herbaceum.*

4.1.2. Clade B

Unlike clade A apomicts, only one allelic form of the nuclear markers was found for triploid apomict *Polystichum tsus-simense* and diploid apomict *P. tsus-simense* in clade B (Fig. 1); in both cases the sequence is identical to *P. herbaceum*. Based on these data, we suggest that triploid

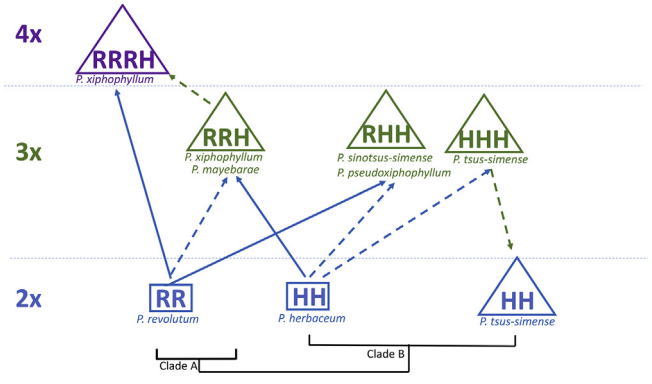

Fig. 5. Proposed scenarios yielding sect. *Xiphopolystichum s.s.* taxa. *R* represents a constituent genome inherited from *Polystichum revolutum. H* represents a constituent genome inherited from *P. herbaceum.* Solid lines represent a meiotically reduced genomic contribution; dotted lines linking named species represent an unreduced genomic contribution; Apomicts are in triangles, while sexual species are in squares. Ploidies are indicated on the left side of the figure as 2x (diploid), 3x (triploid), or 4x (tetraploid).

P. tsus-simense is an autopolyploid derived from *P. herbaceum*, whereas diploid *P. tsus-simense* is derived from the triploid apomict via a loss of chromosomal material, as reported for *Osmunda* apomicts by Manton (1950).

Understanding the heritage of triploid apomict *Polystichum sinotsus-simense* presents a greater challenge than the rest. Like apomicts in clade A, this taxon carries nucleotide polymorphisms at some positions exclusive to the *P. revolutum* and *P. herbaceum* genomes, with relative chromatograph peaks in the exons suggesting a larger genomic contribution from the latter parent (Fig. 5). However, unlike the remaining apomicts with signals of hybrid origin, nucleotides in the introns are generally homozygous for the *P. herbaceum* allele, possibly caused by reverse mutations due to higher mutation rates in introns relative to exons, fixation due to natural selection or drift, homoeologous recombination, or deletions/translocation events that are often easily tolerated by polyploids (Klekowski, 1973; Schubert and Lysak, 2011). Because the genomic heritage of *P. sinotsus-simense* has proven to be more difficult to understand using Sanger sequencing approach, the lineage is a good candidate for further investigation using next-generation sequencing approaches.

The species pairs *Polystichum mayebarae–P. xiphophyllum* and *P. pseudoxiphophyllum–P. sinotsussimense* present the whole array of problems complicating effective evolutionary categorization. Notably, each pair results from a hybridization event, but with each lineage arising from the same progenitors having the same reproductive anomaly and the same balance of contributed genomes. Further, in spite of sharing a pair of progenitors with each parent contributing the same proportion of genetic material to these triploid pairs, they are morphologically distinct from each other. Hence these two species pairs present a case of multiple hybrid origins, and one in which the use of multiple species criteria are required to resolve the two lineages as separate species.

4.2. Parental genome dosage effects

We hypothesized that the leaf division of hybrid apomicts would be intermediate between those of their progenitors based on an additive genetic model; indeed we found this pattern for clade A *Polystichum xiphophyllum* (both 3x and 4x), *P. mayebarae, P. sinotsussimense*, and *P. pseudoxiphophyllum.* Level of dissection and the ratio of the length of basalmost pinna to second to basalmost pinna loaded most strongly on the first and second PCA axes. Also consistent with this hypothesis was leaf dissection in *P. tsus-simense,* which nuclear-sequence data suggest is an autopolyploid derived from *P. herbaceum*; *P. tsus-simense* has a similar level of dissection to *P. herbaceum*. The level of dissection among clade B species *P. tsus-simense, P. herbaceum,* and *P. sinotsus-simense* was generally greater than that of species in clade A, with the exception of *P. mayebarae*. This finding is in line with our genetic data revealing that the twice-pinnate *P. herbaceum* makes the greatest or only genomic contribution to lineages in clade B. On the other hand, the less-dissected species in clade A have a stronger genetic signal indicating contribution from the once-pinnate *P. revolutum* (Fig. 6).

Morphological intermediacy in hybrids is well documented in ferns (Barrington, 1986). All of the allopolyploid apomictic ferns in sect. *Xiphopolystichum* are intermediate in leaf division between their proposed progenitors. However, the hypothesized genomic composition of *P. mayebarae* in clade A would predict a lower level of dissection than that observed. There are several scenarios that may independently, or in concert, account for this finding. Potentially, the unexpected morphology of *P. mayebarae* could be accounted for by an unsampled twice-pinnate progenitor closely related to the lineages comprising clade A. Alternatively, the unexpectedly high level of leaf division in *P. mayebarae* could be explained by non-additive (i.e. transgressive) behavior of genes regulating leaf dissection (McDade, 1990; Rieseberg et al., 1995; Hegarty and Hiscock, 2005; Soltis and Soltis, 2009). Finally, reciprocal hybridization should be considered in understanding morphological differences between *P. mayebarae* and *P. xiphophyllum.* Often

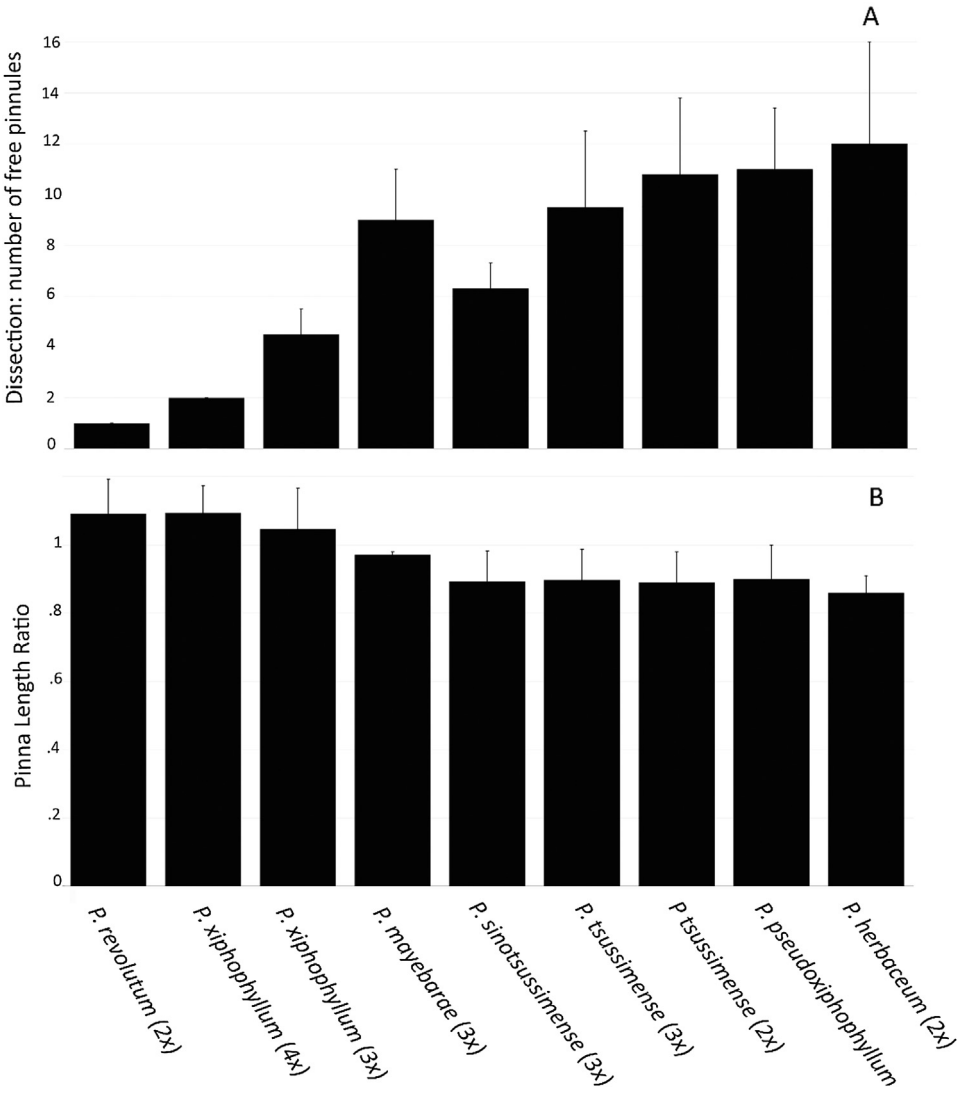

Fig. 6. (a) The number of pinnules on the acroscopic side of the second basal most pinna for each sect. *Xiphopolystichum s.s.* taxon (a quantitative proxy for level of dissection). Inferred ploidy levels as in Figs. 4 and 5. (b) The ratio of the first to the second pinna length for each taxon.

hybrids with multiple origins have both progenitors serving as the maternal or paternal parent (Stein and Barrington, 1990; Vogel et al., 1998; Sigel et al., 2014; Jorgensen and Barrington, 2017). If *P. xiphophyllum* and *P. mayebarae* do in fact have different maternal and paternal parents among their hypothesized progenitors, *P. revolutum* and *P. herbaceum,* then we would expect each to behave differently in the nuclear and chloroplast phylogenies. In the chloroplast phylogeny, we find that *P. xiphophyllum* is more closely related to *P. revolutum* than *P. mayebarae,* suggesting that the two may have different maternal progenitors. *Polystichum pseudoxiphophyllum* and *P. sinotsus-simense* present a similar problem in that although they have the same genome composition, *P. revolutum* is likely the maternal progenitor of *P. pseudoxiphophyllum,* and the paternal progenitor of *P. sinotsus-simense,* particularly given differences in the resolution of *P. pseudoxiphophyllum* in the nuclear and plastid phylogenies (Figs. 1 and 2).

4.3. Species concepts

The named taxa in our sect. *Xiphopolystichum s.s.* study set stand as species when tested using the phylogenetic, phenetic, and evolutionary criteria together. Although events in the evolution of lineages constituting sect. *Xiphopolystichum* and their sequelae (hybridization, multiple origins, and the concomitant morphological complexity) are confounding to the goal of categorizing any lineage in this group as a species, some characteristics of sect. *Xiphopolystichum* apomicts and their relatives are stable and can be used reliably to distinguish evolutionary lineages from each other. Each named species in sect. *Xiphopolystichum s.s.* has one or more distinct alleles for each genetic marker, both plastid and nuclear, sampled in the present study. Accordingly, they are phylogenetically distinct. Similarly, ploidy and reproductive mode (sexual or apomictic) are stable characters that help to delineate groups of individuals as distinct lineages in sect. *Xiphopolystichum*. For instance, all of the individuals sampled that are morphologically identifiable as *P. revolutum* are sexual diploids, as evidenced by flow cytometry and spore counts. Some lineages included in the present study are morphologically indistinct from closely related lineages, but are phylogenetically distinct. *Polystichum mayebarae* is phylogenetically very similar to *P. xiphophyllum,* and we hypothesize that they are products of hybridization between the same progenitors. However, *P. mayebarae* is morphologically distinct and identifiable. Similarly, *P. herbaceum*, *P. sinotsus-simense*, and *P. tsus-simense* are phylogenetically unresolved (Figs. 1 and 2), but are morphologically distinct (Fig. 4). We found that new apomictic cytotypes of *P. xiphophyllum* and *P. tsus-simense* are morphologically and genetically highly similar to the more common triploid apomictic cytotypes of each. Although these cytotypes are single evolutionary lineages that maintain

their integrity, we have chosen not to designate the various cytotypes of *P. tsus-simense* and *P. xiphophyllum* as distinct species. Recent phylogenetic and systematic works on East Asian ferns involving apomictic complexes have taken a similar approach to ours, combining molecular and morphological data to define more biologically realistic taxonomic groups (Chang et al., 2013; Chen et al., 2014; Hori et al., 2014). In these East Asian apomictic complexes, cytotypes differing in ploidy and reproduction, but that are morphologically and phylogenetically indistinguishable from one another, are included in single species as multiple variants or cytotypes.

4.4. Conclusion

Each of our findings on delineation of species in *Polystichum* sect. *Xiphopolystichum* is relevant to understanding species diversity of ferns in China. Species diversity is dependent on how species are defined and, in ferns, these definitions are complicated by the likes of apomixis, polyploidy, and hybridization. Currently, the *Flora of China* includes 34 species in *Polystichum* sect. *Xiphopolystichum s.l.* (Zhang and Barrington, 2013). In large part, we find that the diversity of the plants we sampled for this project is best represented taxonomically as it is in the Flora of China. At least two other apomictic lineages, *P. neolobatum* and *P. rigens,* exist in the broader terrain of sect. *Xiphopolystichum s.l.* Given the potential of apomictic lineages to either inflate or underrepresent species diversity, it will be important to understand these lineages and their potentially reticulate relationships to other species.

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Appendix A

Voucher information given as: Accession number, collector, collection location, herbarium code (data sets). Datasets are coded as follows: *rps4-trnS* – R, *rbcL*- B, *trnL-trnF* – T, *PgiC* – P, *gapcp* –G

Arachniodes denticulata: ESAJ 56, Jorgensen, Costa Rica, VT (P = MG97622); 12322, Sigel, Costa Rica, VT (R = KX768068); 10457, Barrington, Costa Rica, VT (T = MG97623); 10551, VT (B = MH179981) **Dryopteris goldiana:** 10431-G60, Barrington, Lamoille County, Vermont, VT (P = MG97624); 10508, Conant, Lamoille County, Vermont, VT (R = MH175165, B = AF537228.1) **Cyrtomium yunnanense:** 1922-N17, Jorgensen, Costa Rica, VT (T = DQ202418.1) **Phanerophlebia nobilis:** 10404, Yatskievych, Mexico, VT (R = EU03178, B = MH179980, T = MH09285) **Polystichum lepidocaulon:** 10534, McAlpin, Sichuan, China, VT (B = AF537224, T = MH09286) **Polystichum lonchitis:** 10413, Zika, Washington, USA, VT (R = MH101452, B = MH159209, T = MH09287); 12402, Schneller, Switzerland, Z (G = KX866669.1) **Polystichum latilepis:** 11909, S.G Liu, Sichuan, China, PYU (R = MH175166, B = MH159210, T = DQ202428, P = MH101454) **Polystichum neolobatum:** 10523, M. Kato, Nagano, Japan, VT (R = MH175167, B = AF537252); 11944, S.G. Liu, Sichuan, China, PYU (P = MH101425) **Polystichum acanthophyllum:** 11900, S. G. Liu, Sichuan, China, PYU (R = MH175168, B = MH159211, T = MH09288); 12281, S.G. Liu, Yunnan, China, VT (P = MH101426); 11768, Barrington, Yunnan, China, VT (G = MH101440) Polystichym gahrwalicum: 710, Barrington, Yunnan, China, VT (R = MH175169, B = MH179982, T = MH179983, P = MH179984, G = MH179985) **Polystichum squarrosum:** 11656, Little, Kaski, Nepal, VT (R = MH175170, B = EF177339, T = EF177309.1, G = MH101441) **Polystichum cyclolobum:** 11656DL28, Little, Nepal, VT (R = MH175171, B = MH159212, T = MH101449); 12287DL43, Little, Nepal, VT (G = MH198048) **Polystichum stimulans:** 11907 K48, S.G. Liu, Sichuan, China, PYU (R = MH175172, B = MH159213); 10375, R. L. Viane, Yunnan, China, VT (P = MH101427, G = MH101442) **Polystichum hillebrandii:** 10274, Driscoll, Hawaii, United States, VT (R = MH175173, B = MH179976, T = MH101423, G) **Polystichum sinotsussimense:** 11904, S.G. Liu, Yunnan, China, PYU (R = MH175174, B = KC878857, T = DQ150416, P = MH101428, G = MH101447); SY62811, N.R. Patel, Sichuan, China, VT (P = MH101429); **Polystichum herbaceum:** 11905, S.G. Liu, Yunnan, China, PYU (R = MH175175, B = MH159214, T = DQ150405.1, P = MH101430, G = MH101445); JO6287, N.R. Patel, Yunnan, China, VT (R = MH175177) **Polystichum rigens:** R92, M. Kato, Nishimomo, Japan, VT (R = MH175176, P = MH101431) **Polystichum xiphophyllum:** 11903, S.G. Liu, Yunnan, China, PYU (R = MH175178, B = MH159215,T = DQ150421); 11728, D.S. Barrington, Sichuan, China, VT (P = MH101432, G = MH101446) **Polystichum xiphophyllum (4x):** 62223, N.R. Patel, Sichuan, China, VT (R = MH175179, P = MH101433)); **Polystichum tsussimense:** 11906, S.G. Liu, Sichuan, China, PYU (R = MH175180, B = MH179977, T = DQ150419); 11794, D.S. Barrington, Yunnan, China, VT (P = MH101434, G = MH101447); 12353, S.G. Liu, Yunnan, China, PYU (P = MH101435); JO62872, N.R. Patel, Sichuan, China, VT (R = MH175181); **Polystichum tsussimense (2X):** DU6232, N.R. Patel, Sichuan, China, VT (R = MH175182, P = MH101436); **Polystichum mayebarae:** 11887, S.G. Liu, Yunnan, China, VT (B = MH159216, T = DQ150408.1); 11790, D.S. Barrington, Yunnan, China, VT (P = MH101437, G = MH101448); **Polystichum pseudoxiphophyllum:** 707, N.R. Patel, Sichuan, China, CIB (R = KU244858, T = KU244943.1); CQ6259, VT (P = MH179978) **Polystichum revolutum:** 11727, D.S. Barrington, Sichuan, China, VT (R = MH175183, B = MH159217, T = MH101424, P = MH179979, G = MH101451).

Appendix B

Voucher information given as: Accession number, collector, collection location. herbarium code (data sets). Datasets are coded as follows: Flow Cytometry – F, Spore Length – L, Spore Counts – C

Polystichum sinotsussimense: SY62811, N.R. Patel, Sichuan, China, VT (F); SY62812, N.R. Patel, Sichuan, China, VT (L, C) **Polystichum herbaceum:** HX62714, N.R. Patel, Yunnan, China, VT (F, L); HX6273, N.R. Patel, Yunnan, China, VT (L, C); **Polystichum xiphophyllum:** DU62311, N.R. Patel, Sichuan, China, VT (F, C); 622–10, N.R. Patel, Sichuan, China, VT (L); 622EX, N.R. Patel, Sichuan, China, VT (L); EM 12617, N.R. Patel, VT (C) **Polystichum xiphophyllum (4x):** 62223, N.R. Patel, Sichuan, China, VT (L, C, F); DU 6237, N.R. Patel, Sichuan, China, VT (F) **Polystichum tsussimense:** HX6272, N.R. Patel, Sichuan, China, VT (C, L); SH6261, N.R. Patel, Hubei, China, VT (L); DU 623–3, N.R. Patel, Sichuan, China, VT (F) **Polystichum tsussimense (2X):** DU6232, N.R. Patel, Sichuan, China, VT (L, C); JO6283, VT (L, F) **Polystichum mayebarae:** 11887, S.G. Liu, Yunnan, China, VT (F) **Polystichum pseudoxiphophyllum:** CQ6259, N.R. Patel, Sichuan, China, VT (F, C, L) **Polystichum revolutum:** EM1617, N.R. Patel, Sichuan, China, VT (P, C, L); EM4617, N.R. Patel, Sichuan, China, VT (F); EM6617, N.R. Patel, Sichuan, China, VT (F).

Appendix C. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ympev.2018.05.003.

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