

PHYLOGENY AND BIOGEOGRAPHY OF EXINDUSIATE ANDEAN *POLYSTICHUM* (DRYOPTERIDACEAE)¹

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- **Premise of the study:** Uplift of the tropical Andes had a significant impact on the diversification of South American flora and fauna. Recent biogeographic inquiries have established patterns of Andean divergence, but investigations on ferns are scant. The fern genus *Polystichum* Roth (Dryopteridaceae) combines widespread geographic and elevational distribution with a large number of species to form an ideal system for investigation of the origin and diversification patterns of a fern lineage in the tropical Andes.
- **Methods:** The relationships among 42 *Polystichum* species, including taxa from all major biogeographic regions, were analyzed with 2591 aligned nucleotides from four plastid markers using maximum parsimony and Bayesian inference. The resulting phylogeny was then used to estimate divergence times and reconstruct both ancestral areas and ancestral elevations.
- **Key results:** Tropical Andean South American polystichums that lack an indusium (sori exindusiate) were confirmed to form a monophyletic group. This exindusiate Andean *Polystichum* clade diverged from a middle-elevation forest lineage now rich in species endemic to Mexico during the middle Miocene (13.12 million years ago). The majority of diversification that followed took place in the montane regions of the central Andes with radiations to the northern Andes, southeastern Brazil, and alpine regions.
- **Conclusions:** The monophyletic exindusiate Andean *Polystichum* lineage diverged from a Mexican lineage in the middle Miocene and diversified in the central Andes before dispersing northward. This south-to-north dispersal pattern, documented for many other Andean lineages, corresponds with episodes of uplift in the tropical Andes.

Key words: Pteridophyta; ferns; Dryopteridaceae; *Polystichum*; neotropics; Andean diversification; biogeography.

The uplift of the tropical Andes has impacted climate, topography, and watercourses (Hoorn et al., 2010), triggering explosive radiations into the newly available habitats (e.g., see Hughes and Eastwood, 2006). Recent research on the evolutionary and biogeographic histories of Andean lineages has shown that uplift patterns of the Andes and diversification patterns of Andean taxa correspond (e.g., see Chaves et al., 2011). Consequently, resolving both the dynamic geologic history of the tropical Andes and the divergence histories of organisms that inhabit this area is central to explaining the origins of the remarkable modern biodiversity characteristic of the tropical Andes.

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The tropical Andes—a renowned biodiversity hotspot—reached elevations that could support their cool, wet habitats toward the end of the Neogene, two to four million years ago (mya). While the central Andes (Bolivia and Peru) began uplifting 85 mya and the northern region (Ecuador, Colombia, and Venezuela) 55 mya, the entire mountain range had reached less than fifty percent of modern elevations by the beginning of the Oligocene (Gregory-Wodzicki, 2000). The central Andes reached half of their modern elevation in the late Oligocene (25 mya, Western Cordillera) to late Miocene (10 mya, Eastern Cordillera; Gregory-Wodzicki, 2000; Graham et al., 2001; Garzzone et al., 2008). Additional uplift in the central Andes began in the early Pliocene (Graham et al., 2001) or late Miocene (Gregory-Wodzicki, 2000) and brought the mountains to their modern elevation by the late Pliocene. The northern Andes reached half of their modern elevation by the late Miocene (10 mya, Eastern Cordillera; Gregory-Wodzicki, 2000) with an additional rapid increase (0.6 mm/yr) in elevation in the Pliocene, achieving their modern elevation in the Pleistocene (2.5 mya; Gregory-Wodzicki, 2000; Hooghiemstra and Van der Hammen, 2004). Between the northern and central Andes lies a lowland corridor here called the Equatorial Region (southern Colombia, and Ecuador). Numerous marine incursions from the Pacific impacted the Equatorial Region until the middle Miocene (Hoorn et al., 1995; Santos et al., 2008), when uplift of the tropical Andes raised this region. The uplift ended the marine incursions and raised the Equatorial Region to modern elevations, which remain lower than the abutting northern and central Andes (Hoorn et al., 1995).

Numerous lineages exploited the newly opened montane habitats during and immediately after the Neogene uplift of the central and northern Andes (e.g., Weir et al., 2008; Scherson et al., 2008; Hughes and Eastwood, 2006; Cadena et al., 2007;

Pérez-Emán, 2005). The prominent south-to-north pattern of diversification within lineages that reached the Andes during the Miocene to early Pliocene corresponds spatiotemporally to the sequence of Andean uplifts, earlier in the south (e.g., Chaves et al., 2011; Kreier et al., 2008; Brumfield and Edwards, 2007; Doan, 2003; Bell and Donoghue, 2005; Emswiler, 2002; Hagen and Kadereit, 2001, 2003). An increasing number of analyses have integrated phylogenetic relationships, divergence-time estimates, and ancestral-area reconstructions (as documented in Hoorn et al., 2010) to interpret the correspondence between geologic and divergence histories in the tropical Andes. However, investigations of South American lineages in underrepresented groups such as the ferns are scant. The fern genus *Polystichum* Roth (Dryopteridaceae) is a good candidate for inquiry, i.e., in the Andes it combines high species diversity with widespread geographic and elevational distribution to make for a system with potential to provide insight into the origins and patterns of diversification of fern lineages in the tropical Andes.

Polystichum is one of the most species-rich fern genera (Smith et al., 2006) and comprises at least 260 species distributed worldwide with centers of diversity in eastern Asia (Zhang and Barrington, 2013) and tropical America (Barrington, 2011). Phylogenetic analyses conducted by Little and Barrington (2003), Li et al. (2004), Driscoll and Barrington (2007), Lu et al. (2007), and Li et al. (2008) support *Polystichum* as monophyletic as long as *Cyrtomium* C. Presl sensu stricto is excluded. *Polystichum* in the neotropics constitutes a monophyletic group (Driscoll and Barrington, 2007) with three centers of diversity: 1) the Greater Antilles (31 species, Mickel, 1997); 2) Mexico and Guatemala (18 species, Mickel and Smith, 2004; Stolze, 1981); and 3) the northern and central Andes (26 species, Kessler et al., 2005; McHenry, 2012). Within the neotropical clade is a lineage with a geographic distribution centered in the tropical Andes (from Venezuela to Bolivia); it is sister to a clade of species most diverse in southern Mexico (Driscoll and Barrington, 2007). Evolutionary relationships within the Andean *Polystichum* clade are unknown. Andean polystichums have never been studied in a phylogenetic context, except for three species previously included to represent the region in a phylogenetic analysis of the genus (Driscoll and Barrington, 2007). These three sampled Andean polystichums share a morphological synapomorphy in that they lack an indusium, otherwise present in all but two neotropical *Polystichum* species so far sampled.

We used a phylogenetic and biogeographic inquiry into the exindusiate Andean *Polystichum* clade to examine patterns of biodiversity in the Andes with the following objectives: (1) to elucidate evolutionary relationships within the exindusiate Andean *Polystichum* clade and test previously proposed relationships across the genus and beyond; (2) to infer the provenance of the clade; and (3) to infer divergence times and historical biogeography of exindusiate Andean *Polystichum* in the context of Andean geologic events.

MATERIALS AND METHODS

Taxon sampling and species delimitation—Included in the sample were all the exindusiate *Polystichum* taxa in the tropical Andes recognized by McHenry (2012), except for three species known only from type specimens from which we were unable to extract DNA (*P. bachii* M. Kessler & A. R. Sm., *P. chaparensis* M. Kessler & A. R. Sm., and *P. congestum* M. Kessler & A. R. Sm.). The ingroup comprised 56 *Polystichum* accessions from across the northern and central Andean region, Argentina, Uruguay, and Brazil. In constructing the sample we

chose: (1) accessions that represented different geographic localities for each taxon; and (2) fertile accessions that adequately represented morphological diversity in the group. Up to four accessions for each species were analyzed, including a more robustly sampled *P. orbiculatum* (Desv.) J. Rémy & Fée, *P. gelidum* (Kunze ex Klotzch) Fée, and *P. platyphyllum* (Willd.) C. Presl to represent their widespread distributions and morphological variation. Samples selected for our analyses did not show signs of hybridity (intermediate morphology between two species, and malformed spores). To estimate divergence times and reconstruct ancestral geographic distributions, we used a reduced data set with one accession representing each taxon. Data for geographic and elevational species distributions were obtained from our own observations on collection trips to the Andes in 2010 and 2011, and from a review of herbarium specimens from AAU, B, K, LPB, MA, MO, NY, P, QCA, UC, US, and VT (abbreviations follow Thiers, 2012). Herbarium vouchers for all of our new collections were deposited at the Pringle Herbarium (VT), University of Vermont, Burlington, Vermont, USA. Choice of *Polystichum* outgroups was guided by previous phylogenetic analyses (e.g., see Li et al., 2008) and included representation from each major clade identified in Driscoll and Barrington (2007). Additional genera were included as calibration points following Schuettelpelz and Pryer (2009). A complete list of taxa used in the study including taxon authorities, voucher information, and GenBank accession numbers is provided in Appendix 1.

DNA Extraction, Amplification and Sequencing—Total genomic DNA was extracted from fresh (0.1 g) or silica-dried (0.02 g) material collected during three field trips to the Andes or donated by collaborators. Leaf material collected in the field was preserved fresh in plastic bags at 4°C or in silica desiccant gel and stored at -80°C until extraction. When fresh or silica material was not available, DNA was extracted from herbarium specimens. Total genomic DNA was extracted from pinnules following a modified CTAB protocol (Doyle and Doyle, 1987). Four plastid DNA sequences were amplified using the polymerase chain reaction (PCR): (1) the plastid gene *trnS*; (2) the plastid gene *rbcL*; (3) the region between *trnL* and *trnF* (*trnLF*); and (4) the region between *trnS* and *rps4* (*trnS-rps4*). Primers for amplification and sequencing were taken from the literature as follows: (1) *rbcL* (Little and Barrington, 2003); (2) *trnS* (Shaw et al., 2005); (3) *trnLF* (Taberlet et al., 1991); and (4) *trnS-rps4* (Souza-Chies et al., 1997). In addition, six new internal primers were developed to amplify and sequence difficult accessions (reported in Table 1). Amplification by the polymerase chain reaction was performed in a TC-312 or TC-3000 thermal cycler (Techne, Burlington, New Jersey, USA) in 25 µL aliquots with the following components: 150 ng of genomic template; 0.1 µM of each primer; 1× ExTaq Buffer (TaKaRa, Gene Clone, Madison, Wisconsin, USA); 200 µM/L of each dNTP; and 0.625 U Ex Taq polymerase (TaKaRa). All sequences were amplified as follows: (1) initial denaturation at 94°C for 7 min; (2) followed by 40 cycles (94°C for 30 s, 58°C for 1 min, 72°C for 1 min); and (3) a final extension at 72°C for 7 min. PCR products were cleaned using ExoSAP-IT (USB Corporation, Cleveland, Ohio, USA). Sequencing of the cleaned PCR products employed a cycle sequence reaction using the BigDye Terminator Cycle Sequence Ready Reaction Kit version 3.1 (Perkin-Elmer/Applied Biosystems, Foster City, California, USA). Sequences were resolved on an ABI Prism 3100-Avant Genetic Analyzer (Vermont Cancer Center DNA Analysis Facility, Burlington, Vermont, USA). The raw chromatographs from each amplified PCR product sequenced were aligned using both the forward and the reverse sequences, and consensus sequences were assembled for each gene using Sequencher 4.5 (Genes Code Corporation, Ann Arbor, Michigan, USA) or Geneious Pro version 5.0.3 (Drummond et al., 2007). The chromatograms were then inspected to review and manually edit the automated base calls.

TABLE 1. Primers developed for use in this study to amplify *trnS-rps4* and *rbcL*.

Primer	5'-primer sequence-3'
<i>trnS-rps4</i>	
MAMtrnS-rps41F	TTACCGAGGGTTCGAATCCCTC
MAMtrnS-rps41R	GAGTATTACTCCCGCAAAG
MAMtrnS-rps42F	GCTTTGGGGAGTAATAC
MAMtrnS-rps42R	ACCGATCAATCAGCTTCC
<i>rbcL</i>	
CXLrbcL631F	TTCATGCGTTGGAGAGATC
CXLrbcL1369R	GGACTCCACTTACWAGCTTC

Sequence Alignment and Phylogenetic Analysis—Consensus sequences were aligned using MUSCLE (Edgar, 2004) as implemented in Geneious Pro version 5.0.3 (Drummond et al., 2007). All phylogenetically informative indels were coded manually following “the simple gap coding” of Simmons and Ochoterena (2000) and added as additional binary characters at the end of the NEXUS file. The six data sets (one for each of our four markers, one for indels, and one for the combined data set) were constructed using Mesquite version 2.75 (Maddison and Maddison, 2011).

All data sets were analyzed separately, and the consensus topologies between them were visually compared to check for possible conflicts among the data sets. Since we visually detected minimal topological discordance between the data sets, we concatenated sequences from all four cpDNA regions using Geneious Pro version 5.0.3 (Drummond et al., 2007).

For Maximum Parsimony (MP), the concatenated cpDNA sequences were analyzed using TNT version 1.1 (Goloboff et al., 2008). All characters were equally weighted and coded as unordered. We used a heuristic search with 1000 parsimony-ratchet replicates (Nixon, 1999) with 200 ratchet iterations, the up and down weights set to 5% each, and held 20 trees per ratchet, followed by tree-bisection-reconnection (TBR) branch-swapping. Bootstrap values (Felsenstein, 1985) were obtained for our MP nodes with a bootstrap support analysis (BS) using 1000 replicates doing 10 ratchets per replicate, holding 20 trees per ratchet, and keeping only the strict consensus tree. The amount of homoplasy within the data sets was determined using the consistency index (CI) and the retention index (RI). MP trees were viewed using the program Winclada (Nixon, 2004).

For Bayesian Inference (BI) the concatenated cpDNA sequences were analyzed using MrBayes version 3.2 (Ronquist et al., 2012). The data were partitioned by plastid region, and optimal evolutionary models (Table 2) were selected for each partition using jModeltest 2 (Darriba et al., 2012) under the Akaike Information Criterion (AIC). BI using MrBayes was run for 4 million generations in two independent analyses with trees sampled every 1000 generations. Stationarity was determined using the log-likelihood scores for each run plotted against generation in the program Tracer version 1.5 (Rambaut and Drummond, 2007). The first 400,000 trees were discarded as the burn-in phase, and a 50% majority rule consensus tree was calculated for the remaining trees. Posterior probabilities were obtained using MrBayes version 3.2 (Ronquist et al., 2012). The program FigTree version 1.3 (Rambaut, 2008) was used to view a 50% majority rule consensus tree with posterior probabilities.

Divergence time estimates—Divergence times were estimated using a Bayesian method implemented in the program BEAST version 1.6.2 (Drummond and Rambaut, 2007) with the relaxed phylogenetic method of Drummond et al. (2006). Data were partitioned by plastid region, i.e., an optimal model for each of the molecular partitions was applied. As fossils for the genus *Polystichum* are not available, the input data were compiled using the program BEAUTi version 1.6.2 with two calibration points based on the estimates in Schuettpelz and Pryer (2009): (1) the most recent common ancestor of *Phanerophlebia*, *Cyrtomium*, and *Polystichum* (no less than 34.9 mya); and (2) the most recent common ancestor of *Cyrtomium* and *Polystichum* (no less than 30.8 mya). The node constraints were assigned a lognormal distribution, with a standard deviation = 1.0. A Yule process speciation prior and an uncorrelated lognormal model of rate variation were implemented. The analyses ran for 10 million generations and were sampled every 1000 generations. The program Tracer version 1.5 (Rambaut and Drummond, 2007) was used to examine the estimated sample sizes (ESS), the coefficients of variation, and the 95% highest posterior density (HPD) intervals to determine if the parameter space had been sufficiently sampled and if the appropriate model of rate variation was chosen. To obtain a maximum clade credibility (MCC) chronogram, the tree sets and the parameter estimates produced by BEAST were summarized using the program Tree-Annotator version 1.6.2 (Drummond and Rambaut, 2007). The program FigTree

version 1.3 (Rambaut, 2008) was then used to visualize the chronogram with 95% HPD intervals at each node.

Reconstruction of ancestral areas of geographic distribution and elevation—Definition of biogeographic areas was modified from Antonelli et al. (2009) to better fit the geographic distributions of *Polystichum*. Eight areas were defined for the biogeographic analysis: (1) Old World; (2) North America; (3) Mexico and Central America; (4) the West Indies; (5) the northern Andes (Venezuela and northern Colombia); (6) the Equatorial Region (southern Colombia and Ecuador); (7) the central Andes (Peru, Bolivia, northern Argentina, Uruguay, and Paraguay); and (8) southeastern South America (Brazil). Geographic distributions were scored as unordered character states using collection localities, and multiple states were scored for taxa with widespread distributions. Outgroups were coded according to their documented geographic distributions.

Reconstruction of ancestral geographic distributions was carried out on the reduced sample using both MP and BI methods. The MCC tree was used to implement Bayesian Binary MCMC [Markov Chain Monte Carlo] (BBM) in RASP (Yu et al., 2010), and a MP tree was used to implement MP in Mesquite version 2.75 (Maddison and Maddison, 2011). The BBM was run in parallel for 5 million generations with 10 chains under the Jukes-Cantor fixed-state frequencies model and among-site variation (Gamma) set to equal.

Ancestral elevation was reconstructed on the same reduced-taxon sample MP tree with MP implemented in Mesquite version 2.75 (Maddison and Maddison, 2011). The median elevation to the nearest 100 meters was scored for each taxon based on calculations from label data with herbarium specimens and personal observations in the field.

RESULTS

Sequence characteristics—The concatenated data matrix totaled 2596 characters of which 262 (10%) were parsimony informative. Length, number of informative characters, and evolutionary model are reported for the four markers in Table 2. When analyzed separately, the four data sets showed some topological variation at distal nodes, but were broadly congruent (trees not shown). The combined plastid regions resulted in a more resolved phylogeny with improved support when compared to any plastid region separately or in different combinations (trees not shown).

Phylogenetic analysis—The MP analysis of the combined plastid data sets recovered 48 equally parsimonious trees 942 steps long. These shortest trees had a consistency index (CI) of 69 and a retention index (RI) of 81.

The 50% majority rule phylogram from the BI analysis was entirely congruent with the MP strict consensus; the trees differed only in resolution and clade support values (Fig. 1). *Polystichum* was recovered as monophyletic and sister to *Cyrtomium* (0.91 PP/99% BS; Fig. 1). Within *Polystichum*, species from the continental neotropics resolved as sister to our sample of *Polystichum* from the West Indies (1.0 PP/100% BS; *WI* in Fig. 1). Sister to the remainder of the continental neotropical lineage was *P. speciosissimum* (A. Braun ex Kunze) R. M. Tryon & A. F. Tryon (0.99 PP/100% BS; Fig. 1), a Mexican species with a disjunct population in Costa Rica. The continental neotropical lineage was resolved into two well-supported species-rich clades: (1) the indusiate lineage rich in endemics from Mexico and Central America (1.0 PP/99% BS; labeled *Mexican* in Fig. 1); and (2) the exindusiate Andean lineage (0.97 PP/100% BS; labeled *Andean* in Fig. 1). Resolved within the exindusiate Andean clade were three monophyletic groups (*Clades I, II, and III*; Fig. 1).

Clade I is well-supported (1.0 PP/100 BS; Fig. 1); it comprises all three accessions of one species, *Polystichum stuebelii* Hieron. This species is endemic to the premontane (elevation

TABLE 2. Characteristics of the cpDNA markers used in the phylogenetic analysis.

Marker	Model (AIC)	Marker length	Parsimony informative characters
<i>rbcL</i>	TIM1+I+G	1169	66 (6%)
<i>trnLF</i>	TPM3uf+G	349	66 (19%)
<i>trnS-rps4</i>	TIM1+G	576	72 (13%)
<i>rps4</i>	HKY+G	486	42 (9%)

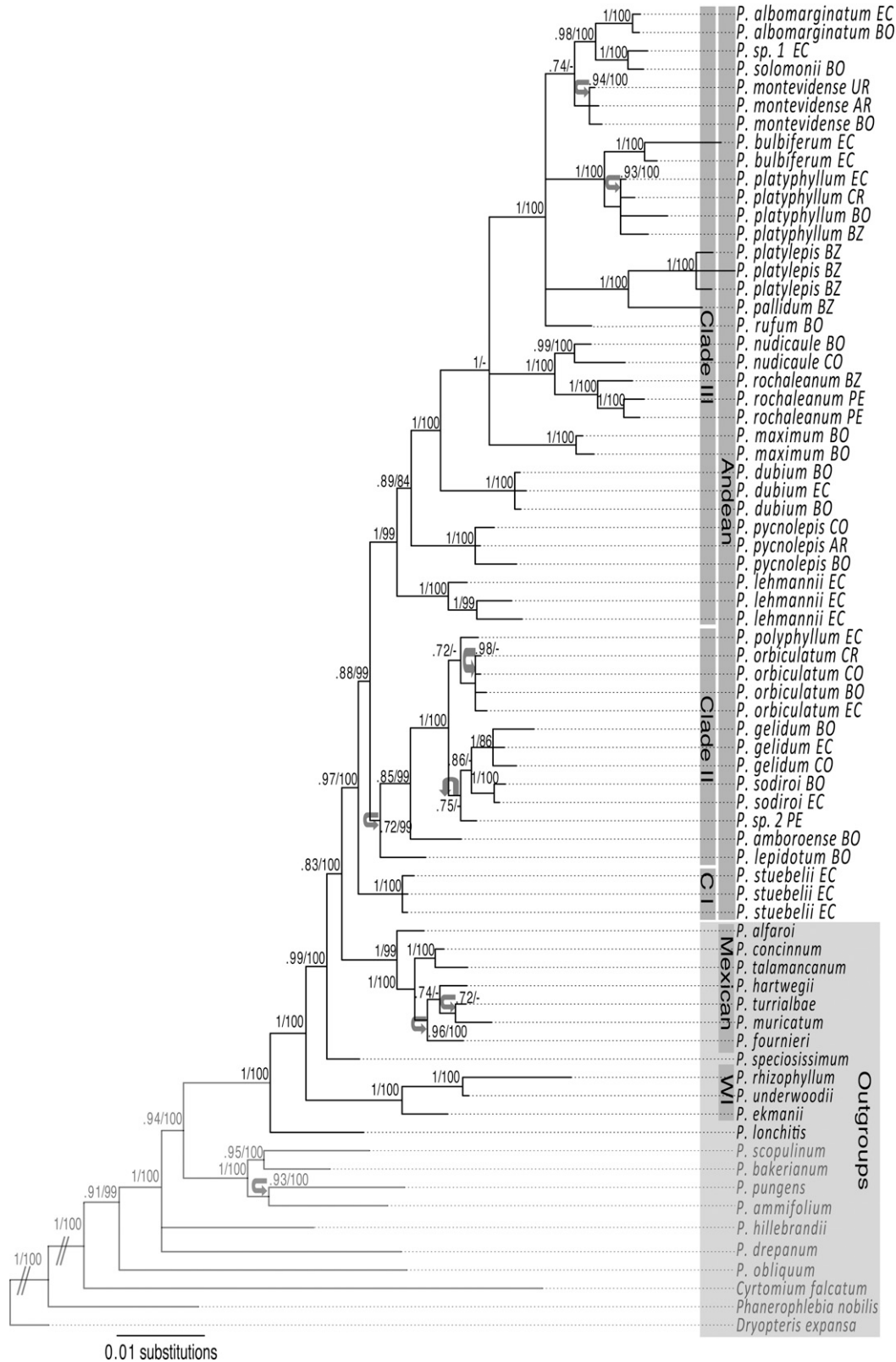


Fig. 1. Phylogeny of *Polystichum* based on the combined analysis of plastid markers. The tree is the 50% majority rule phylogram from the Bayesian Inference (BI) analysis with nodes collapsed that do not concur with the Maximum Parsimony (MP) strict consensus or the maximum clade credibility (MCC) chronogram. BI Posterior Probability (PP)/MP Bootstrap Support (BS) given at each node, - represents nodes not resolved in the MP analysis. Accessions in the neotropical clade are black and other terminals are shaded gray. Abbreviations : AR, Argentina; BO, Bolivia; BZ, Brazil; CO, Colombia; CR, Costa Rica; EC, Ecuador; PE, Peru; UR, Uruguay; WI, West Indies.

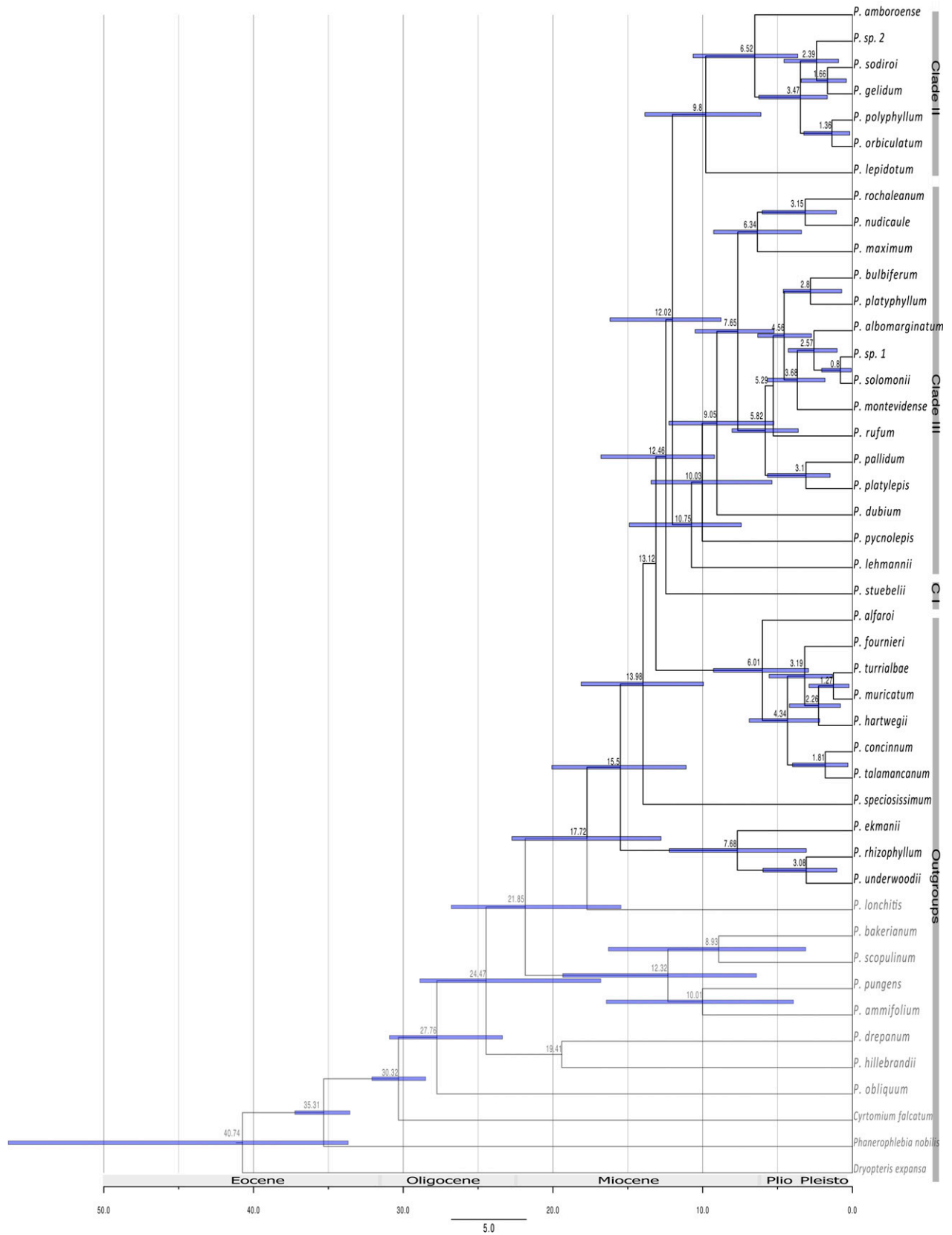


Fig. 2. MCC chronogram for exindusiate Andean *Polystichum*. Estimated divergence dates are displayed at each node. Node bars indicate 95% confidence intervals of node ages estimated from 1000 trees randomly sampled from the Bayesian stationary distribution. Branches are proportional to relative ages (in millions of years, see scale at bottom of tree).

classification follows Holdridge, 1987, throughout) western slope of the Equatorial Region, where it is found at one of the lowest elevations (median elevation 1750 m) for species of the exindusiate Andean clade.

Clade II comprises seven species that are found throughout the tropical Andes as well as Central America and Mexico: *Polystichum lepidotum* M. Sundue & M. Kessler, *P. ambo-roense* M. Kessler & A. R. Sm., *P. gelidum* (Kunze ex Klotzsch) Fée, *P. sodiroi* H. Christ, *P. orbiculatum* (Desv.) J. Rémy & Fée, *P. polyphyllum* C. Presl, and one undescribed species (Fig. 1). The earliest-diverging lineages (*Polystichum lepidotum* and *P. ambo-roense*), recently described by Kessler et al. (2005), are known from only a few accessions found in remote localities mostly in Bolivia. *Polystichum lepidotum* is a plant of pristine alpine habitats (median elevation 3800 m) while *P. ambo-roense* is found in montane forests (median elevation 2500 m) in Bolivia's Parque Nacional Amboró. The remaining members of the clade are a notoriously difficult group comprising the widespread and morphologically protean *P. orbiculatum* and its segregates. We resolved *P. orbiculatum* as monophyletic with low sequence divergence between different geographic localities and morphologically divergent accessions (Fig. 1). We found that *P. orbiculatum* was nested within a clade that comprises four high-elevation taxa often synonymized with *P. orbiculatum* (e.g., see Tryon and Stolze, 1991). The taxa are: (1) *P. polyphyllum*, a rare alpine lineage; (2) an undescribed species found in alpine habitats; (3) *P. gelidum*, a prominent subpáramo and *Polylepis* (Rosaceae) forest species that has a widespread distribution throughout the tropical Andes; and (4) *Polystichum sodiroi*, a lineage restricted to alpine habitats on the western slope of the Equatorial Region.

Clade III is also well-supported (1.0 PP/99% BS; Fig. 1), and comprises 15 species: *Polystichum albomarginatum* M. Kessler & A. R. Sm., *P. solomonii* M. Kessler & A. R. Sm., *P. montevidense* (Spreng.) Rosenst., *P. platyphyllum*, *P. bulbiferum* Barrington, *P. rufum* M. Kessler & A. R. Sm., *P. pallidum* Gardn., *P. platylepis* Fée, *P. nudicaule* Rosenst., *P. rochaleanum* Fée, *P. maximum* M. Kessler & A. R. Sm., *P. dubium* (H. Karst.) Diels, *P. pycnolepis* (Kunze ex Klotzsch) T. Moore, *P. lehmannii* Hieron., and an undescribed species (Fig. 1). The earliest-diverging lineage in this clade comprises a well-supported clade of *Polystichum lehmannii* accessions (1.0 PP/100% BS; Fig. 1). Though *P. lehmannii* has been reported from Venezuela (Smith, 1985) to Bolivia (Kessler et al., 2005), all accessions classified by McHenry (2012) as *P. lehmannii* are restricted to montane forests of the western slope in the Equatorial Region. Previous *P. lehmannii* determinations by Smith (1985) and Kessler et al. (2005) have been reclassified as *P. gelidum*. The next two divergence events yield one alpine (to subalpine) lineage, *P. pycnolepis*, and one montane-forest lineage, *P. dubium*. The remaining species constitute a group of plants with a broad array of elevational and geographic distributions. The first divergence in this clade yields a polytomy comprising three clades: (1) *P. maximum*, a montane species recently described by Kessler et al. (2005), known only from two populations in Bolivia; (2) *P. nudicaule* and *P. rochaleanum*, species restricted to alpine rock outcrops from Peru and Bolivia with two disjunct populations, *P. nudicaule* in the northern Andes and Central America, and *P. rochaleanum* in southeastern South America; and (3) nine taxa in four unresolved clades. Among the nine taxa in the poorly resolved clade are three species recently described by Kessler et al. (2005)—*P. rufum*, *P. solomonii*, *P. albomarginatum*—and one taxon not yet described. We found *P. rufum* and *P. solomonii* to

be restricted to montane forests in Bolivia, but *P. albomarginatum* has been collected in premontane forests in both Bolivia and the Equatorial Region. The nine-taxon clade also includes two premontane species geographically restricted to Brazil (Fig. 1)—*P. platylepis* and *P. pallidum*. Recent phylogenetic investigations on both ferns (e.g., see Kreier et al., 2008; Sánchez-Baracaldo, 2004) and vascular plants (Safford, 1999, and references therein) have found a similar disjunct distribution between the Andes and the Brazilian Highlands. The remaining three taxa (*P. platyphyllum*, *P. bulbiferum*, and *P. montevidense*) are found on premontane (to montane) forested slopes. *Polystichum platyphyllum* extends north through Central America, the West Indies and into Mexico, whereas its sister species, *P. bulbiferum*, is found only in the Equatorial Region. *Polystichum montevidense* is distributed from Peru through Argentina to southern Brazil and Uruguay (Condack et al., 2013).

Divergence time estimates—The chronogram obtained from the divergence-time estimate summarized as a maximum clade credibility tree (Fig. 2) found a similar but a better-resolved topology than from the phylogeny (Fig. 1).

Our divergence-time estimate for the origin of the neotropical clade from *Polystichum lonchitis* (L.) Roth is the early Miocene (mean estimate 17.72 mya; Fig. 2). The next event, also in the early Miocene (mean estimate 15.5 mya; Fig. 2), was the divergence of plants currently found in the West Indies (WI) from the continental neotropical lineage. The exindusiate Andean lineage diverged from the Mexican lineage in the middle Miocene (mean estimate 13.12 mya; Fig. 2); the earliest divergence within the exindusiate Andean clade was also in the middle Miocene (mean estimate 12.46 mya; Fig. 2). The diversification that followed in the exindusiate Andean clade occurred in the late Miocene, throughout the Pliocene, and into the Pleistocene (mean estimates 12.02 mya to 0.8 mya; Fig. 2).

Ancestral Areas Reconstruction—The BI and MP reconstructions of ancestral geographic distributions placed the most recent common ancestor of the continental tropical-American *Polystichum* clade in the Mexican geographic region (Fig. 3 MP; Table 3 BI). An early divergence in the exindusiate Andean clade that divided the lineage into Clade II and Clade III was reconstructed to have its most recent common ancestor in the southern portion of the tropical Andes, either in the Equatorial Region or in the central Andes. The ancestral area of Clade II resolved as the central Andes and Clade III resolved as either the Equatorial Region or the central Andes (Fig. 3 MP; Table 3 BI). The remaining internal nodes in both Clades II and III are reconstructed to have a central Andean ancestral distribution with multiple secondary dispersal events to the northern Andes and southeastern South America, as well as back to Central America and Mexico (Fig. 3 MP; Table 3 BI).

Elevation transitions—The parsimony reconstruction of median elevation for the exindusiate Andean *Polystichum* clade resolved the most recent common ancestor of the exindusiate Andean clade to be a premontane species (Fig. 3) with one of the lowest median elevations encountered in the clade. The earliest divergence within the exindusiate Andean clade isolated a premontane lineage with one extant species (median elevation scored 1500 m to 2000 m) from the remainder. The ancestral median elevation for the latest diverging members of Clade II

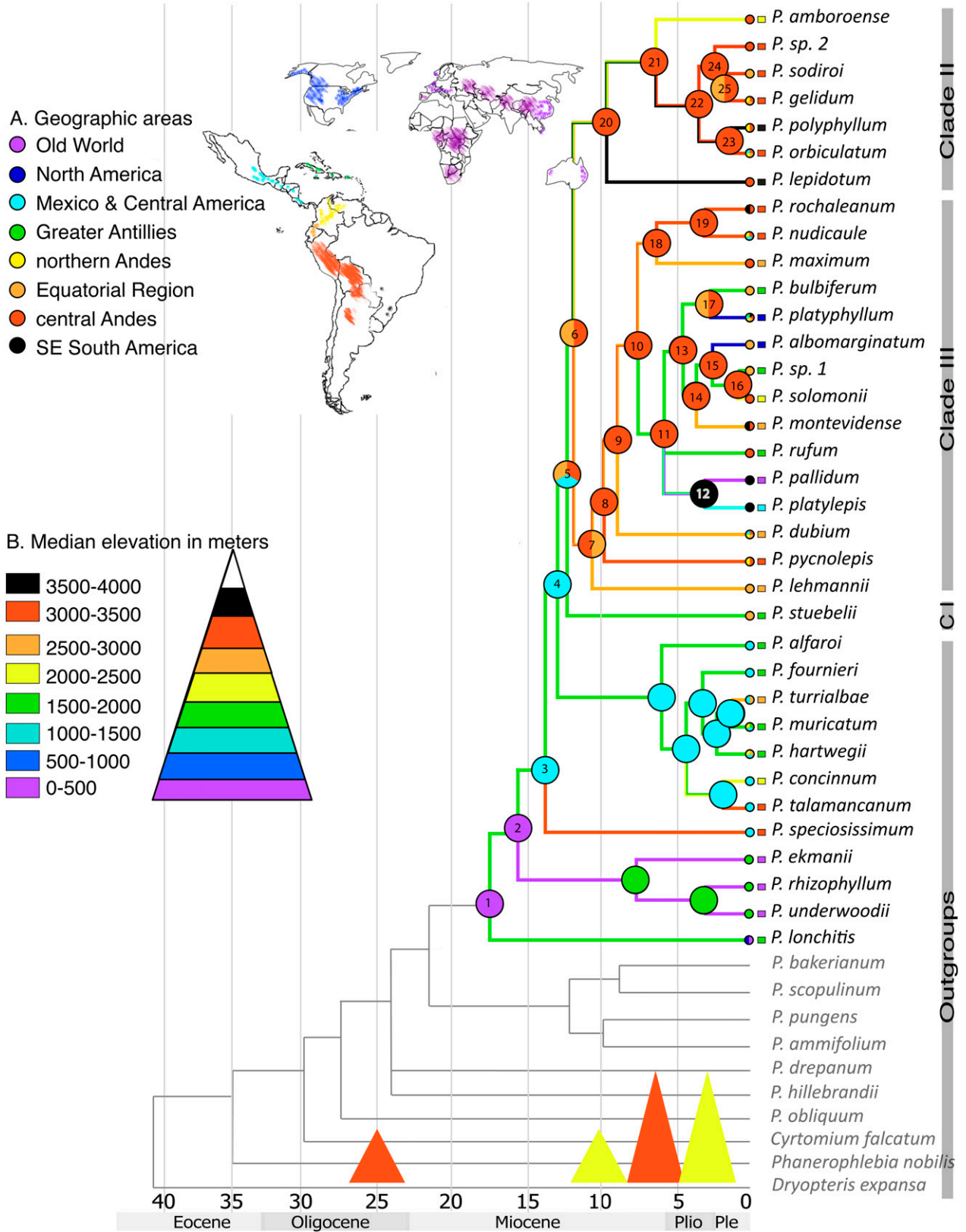


Fig. 3. Combined MCC chronogram and ancestral reconstruction of exindusiate Andean *Polystichum*. Nodes are collapsed that do not concur between the MP and the MCC. Pie graphs at nodes indicate reconstructed MP ancestral distributions, colors correspond to map and legend (A). Numbers within pie charts at node correspond to Table 3 (the numeric output from BBM [RASP] analysis). Branch color indicates reconstructed MP ancestral elevation, colors correspond to elevation chart and legend (B). Triangles at base of chronogram represent approximate phases of Andean uplift, color-coded for region.

TABLE 3. Numeric output from Bayesian Binary MCMC [Markov Chain Monte Carlo] (BBM) in RASP (Yu et al., 2010) analysis. Node numbers correspond to Fig. 3. Geographic area codes are as follows: A = Old World, B = North America, C = Mexico and Central America, D = Greater Antilles, E = northern Andes, F = Equatorial Region, G = central Andes, H = southeastern South America.

Node number	Most likely ancestral distribution	Relative probability
1	A	0.83
2	A	0.41
3	C	0.96
4	C	0.91
5	F	0.69
6	G	0.36
7	F	0.33
8	EG	0.29
9	EFG	0.34
10	G	0.56
11	G	0.5
12	H	0.93
0	G	0.55
13	FG	0.32
14	FGH	0.28
15	FH	0.29
16	F	0.65
17	FG	0.25
18	G	0.78
19	G	0.76
20	G	0.81
21	G	0.74
22	FG	0.32
23	EFG	0.41
24	FG	0.38
25	EFG	0.46

resolved as alpine (above 3000 m) and the majority of taxa in Clade III as montane (median elevations 2500 to 3500 m) with transitions to both premontane (median elevations 1500 to 2000 m) and sub-alpine (median elevations 3000 m and above) during more recent divergences.

DISCUSSION

Evolutionary Relationships—The phylogeny retrieved for exindusiate Andean *Polystichum* provides increased resolution of relationships both within the genus *Polystichum* and among its allied genera. The combination of plastid markers used in our analysis provided improved clade support relative to previous *Polystichum* analyses, which have used the same one or two markers in different combinations (Little and Barrington, 2003 [*rbcL*]; Perrie et al., 2003 [AFLPs, *trnS-rps4*]; Li et al., 2004 [*rbcL*]; Driscoll and Barrington, 2007 [*trnL-F*, *rbcL*]; Lu et al., 2007 [*rbcL*]; Li et al., 2008 [*trnL-F*, *trnS-rps4*]). With the use of four plastid markers for 56 accessions, the monophyly of *Polystichum* is sustained, and there is strong support for *Cyrtomium* as sister to *Polystichum* (0.91 PP/99% BS; Fig. 1), a relationship retrieved by Li et al., (2008) but poorly resolved in most past analyses (Little and Barrington, 2003; Li et al., 2004; Driscoll and Barrington, 2007; Lu et al., 2007).

In agreement with Driscoll and Barrington (2007), neotropical *Polystichum* and exindusiate Andean *Polystichum* are both resolved as monophyletic with our broader sample of nucleotides and taxa. The exindusiate Andean *Polystichum* clade is

sister to the Mexican *Polystichum* clade (Fig. 1), and the Mexican *P. speciosissimum* is sister to both.

Provenance of the exindusiate Andean *Polystichum* lineage—The northern origin for exindusiate Andean *Polystichum* supported by our results has been proposed for other groups that have radiated in the Andes (e.g., see Pérez-Emán, 2005; Hughes and Eastwood, 2006; Cadena et al., 2007; Weir et al., 2008; Scherson et al., 2008). Chronologically, our results suggest that the exindusiate Andean *Polystichum* lineage diverged from a Mexican ancestor during middle-Miocene uplift episodes. At this time the northern and central Andes are likely to have been at less than half of their modern elevations (Garzzone et al., 2008; Graham et al., 2001; Gregory-Wodzicki, 2000), suggesting that the mountains reached no more than 2000 m (Garzzone et al., 2008). The ancestral elevation (median elevations from 1500 to 2000 m) reconstructed for the exindusiate Andean *Polystichum* lineage places it in premontane habitats that had just become available.

Divergence times and historical biogeography of exindusiate Andean *Polystichum*—Within the exindusiate Andean *Polystichum* clade, divergence events developed in concert with patterns of historical geologic events during the middle Miocene and throughout the Pliocene as documented for other Andean lineages that have undergone recent radiation in the Andes (e.g., see Emshwiller, 2002; Hagen and Kadereit, 2001, 2003; Bell and Donoghue, 2005; Brumfield and Edwards, 2007; Kreier et al., 2008; Chaves et al., 2011). At the outset, for example, the first divergence in the exindusiate Andean *Polystichum* clade, *Polystichum stuebelii*, a premontane species endemic to the Equatorial Region (Clade I, median elevation scored 1500 to 2000 m; Fig. 3), diverged from the common ancestor of the entire remaining exindusiate Andean *Polystichum* lineage. Time of origin for *P. stuebelii* is in the middle Miocene (mean estimate 12.46 mya; Fig. 2), a time of initial uplift in the northern and central Andes (Gregory-Wodzicki, 2000; Hooghiemstra and Van der Hammen, 2004) when suitable premontane habitats would have just become available. The species thus gives insight into the likely habitat preference of the exindusiate Andean lineage near its inception.

Correspondence between exindusiate Andean *Polystichum* divergence and tropical Andean uplift is further evidenced by our divergence-time estimates, which date divergence events in the clade from the late Miocene to the Pleistocene (mean estimates 12.46 to 0.8 mya; Fig. 2). The late Miocene to Pleistocene is a period of intensified Andean uplift that triggered numerous montane (1500 to 3000 m) Andean lineages to diversify rapidly (Hoorn et al., 2010, and references therein). As suggested by Gregory-Wodzicki (2000), Graham et al. (2001), Hooghiemstra and Van der Hammen (2004), and Garzzone et al. (2008), montane habitat was first available in the central Andean mountains; subsequent elevation gains followed in a south-to-north pattern through the tropical Andes during the Pliocene. Diversification of the two major clades in exindusiate Andean *Polystichum* follows this pattern; most divergence events are reconstructed to be in the central Andes in the Pliocene followed by multiple secondary dispersal events to the north, presumably as suitable habitat became available (Clades II and III; Figs. 2 and 3).

Additional support for the south-to-north diversification hypothesis is higher diversity in the southern tropical Andes, i.e., 10 of the 23 species (43%) in our study set of exindusiate

Andean polystichums are endemic to the Equatorial Region and central Andes. In contrast, the northern Andes have no endemic species—suggesting that the habitats in the northern Andes have provided less time for divergence, presumably because they have not been available as long (in agreement with Scherson et al., 2008). Similarly, southeastern South America has only two (9%) endemic species in the exindusiate Andean *Polystichum* lineage, suggesting that southeastern South America was only recently colonized by Andean lineages (in agreement with Safford, 2007) that have just begun to diversify.

By the Pleistocene, the tropical Andes had gained their modern elevations, and alpine habitats (over 3000 m) were newly opened—triggering some of the most impressive plant radiations known (e.g., Hagen and Kadereit, 2001; Luteyn, 2002; Bell and Donoghue, 2005; Hughes and Eastwood, 2006). In concert with the opening of this alpine habitat, the exindusiate Andean *Polystichum* lineage transitioned to alpine habitats two to four times (Fig. 3)—similar to the pattern in the fern genus *Jamesonia* (Sánchez-Baracaldo, 2004)—yielding species with the highest-elevation distributions in our study set (e.g., *P. lepidotum*, *P. nudicaule*, *P. orbiculatum*, *P. polyphyllum*, and *P. pycnolepis*). As different lineages transitioned to higher elevations, a suite of similar morphological transformations also occurred, including an increase in spore size (not due to ploidy increase) and simpler, reduced perispore ornamentation (imperforate perispores with short, broad ridges at high elevations compared to fenestrate perispores with tall, delicate ridges in mid-elevations; McHenry, 2012).

CONCLUSION AND FUTURE DIRECTIONS

Although biogeographic analyses of the fern genera *Eriosorus*, *Jamesonia* (Sánchez-Baracaldo, 2004), and *Serpocaulon* (Kreier et al., 2008) have recently yielded insights into geographic and elevational dimensions of Andean fern diversification, they do not provide a context for geologic events because they lack estimated divergence times. Here we report the first insight into the timing of diversification of Andean fern lineages in the context of the known sequence of geologic events, including our investigation into their origin and their spatiotemporal patterns of diversification. In summary, we found that the exindusiate Andean *Polystichum* lineage reached South America from the North; from our results we infer that the exindusiate Andean *Polystichum* clade dispersed from a boreotropical Mexican ancestor, reaching the tropical Andes in the middle Miocene. Based on our results, we hypothesize the successful migrant rapidly diversified and spread throughout the montane central Andes in the late Miocene and Pliocene, in concert with tropical Andean uplift, thus exploiting the newly opened montane and alpine habitats. Expansion into the northern Andes and southeastern Brazilian highlands followed in the late Pliocene and Pleistocene.

This study represents the first step in understanding evolution of *Polystichum* in the neotropics using plastid markers. Hybridization is common in *Polystichum* (Barrington, 1990; 2003); however, most *Polystichum* phylogenies published to date (Little and Barrington, 2003; Li et al., 2004; Driscoll and Barrington, 2007; Lu et al., 2007; Li et al., 2008; work presented here) have relied on plastid markers that are maternally inherited in ferns (Gastony and Yatskievych, 1992; Vogel et al., 1998). Thus, phylogenetic analyses of *Polystichum* that incorporate biparentally inherited markers will be useful in elucidating reticulate relationships and their impact on the evolutionary history of the genus.

Additionally the rampant morphological homoplasy witnessed in the genus (summarized in Barrington, 2011), also documented in the exindusiate Andean clade, warrants further investigation. Suites of morphological characters shared among distant *Polystichum* lineages seem to correspond with the habitat of the species rather than to their evolutionary history. For instance, morphological character transformations appear to correspond with transition to higher elevations in Andean polystichums. This morphological trend should be investigated further to (1) determine the isolating environmental mechanisms and their molecular counterparts; and (2) prevent redundant description of species with phenotypic variation due to environmental factors.

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APPENDIX 1. Collection information for voucher specimens. Taxa are arranged alphabetically by genus. Sequences that were not amplified successfully are indicated by a dash (–).

Taxon; Voucher specimen, Herbarium (Herbaria acronyms follow Index Herbariorum (Thiers, 2012); GenBank accession number (sequence is *rbcL*, *trnLF*, *trnS-rps4*, *rps4*).

- Cyrtomium falcatum* (L.) C. Presl; *D. P. Little* 342, VT; AF537226, EF177268, KF020385, KF020442. *Dryopteris expansa* (C. Presl) Fraser-Jenk. & Jermy; *Nelson* 7921, COLO; AY268844, AY268775, KF020383, KF020440. *Phanerophlebia nobilis* (Schldtl. & Cham.) C. Presl var. *nobilis*; *G. Yatskiyevych et al.* 85–211, IND; AF537231, EF177269, KF020384, KF020441. *Polystichum sp. 1*; *M. A. McHenry* 10-30, VT; KF020326, KF020350, KF020429, KF020478. *Polystichum sp. 2*; *C. Rothfels* 3966, VT; KF020327, KF020351, KF020430, KF020465. *Polystichum albomarginatum* M. Kessler & A. R. Sm.; *C. Buchtien* 97, UC; –, KF020352, KF020405, KF020446; *M. Lehnert* 1257, VT; KC819964, KC819949, KC819979, KC819994. *Polystichum alfaroi* (Christ) Barrington; *D. Barrington* 1213, VT; –, –, KF020398, KC890808; *D. Barrington* 1978, VT; AF537236, EF177271, –, –, *Polystichum amoroense* M. Kessler & A. R. Sm.; *M. Sundue* 773, VT; KF020328, KF020353, KF020406, KF020447. *Polystichum ammfolium* (Poir.) C. Chr.; *T. A. Ranker* 1537, VT; AF537237, EF177287, KF020391, KC890811. *Polystichum bakerianum* (Atk. ex C. B. Clarke) Diels; *D. Barrington* 2246 (VT); KC878851, KC878859, KF020392, KC890809. *Polystichum bulbiferum* Barrington; *M. A. McHenry* 10-38, VT; KF020329, KF020355, KF020408, KF020449; *M. A. McHenry* 10-33, VT; KF020322, KF020354, KF020407, KF020448. *Polystichum concinnum* Lellinger ex Barrington; *J. Kluge* 1441, VT; EF177320, EF177276, KF020399, KC890810. *Polystichum drepanum* (Sw.) C. Presl; *Chelsea Physic Garden accession* 8864, VT; KC878852, KC878861, KF020387, KC890811. *Polystichum dubium* (H. Karst.) Diels; *M. Lehnert* 1277, VT; KF020330, KF020357, KF020410, KF020450; *M. A. McHenry* 11-23, VT; KF020320, KF020356, KF020409, KF020475; *M. A. McHenry* 10-85, VT; KF020319, KF020358, KF020411, KF020476. *Polystichum ekmanii* Maxon; *P. Wiczorek* 215, VT; AF537242, EF177272, KF020396, KC907700. *Polystichum fournieri* A. R. Sm.; *M. A. McHenry* 08-16, VT; KF020331, KF020362, KF020401, –, *Polystichum gelidum* (Kunze ex Klotzsch) Fée; *M. Sundue* 1328, VT; KF020334, KF020361, KF020425, KF020462; *M. Lehnert* 1561, VT; KF020333, KF020360, KF020424, KF020461; *M. A. McHenry* 10-87, VT; KF020332, KF020359, KF020423, KF020460. *Polystichum hartwegii* (Klotzsch) Hieron.; *T. S. Quedensley* 4936, VT; KF020335, KF020363, KF020400, KF020444. *Polystichum hillebrandii* Carruth.; *H. Driscoll* 315, VT; EF177323, EF177279, KF020389, KC890812. *Polystichum lehmannii* Hieron.; *M. Lehnert* 1276, VT; –, KF020366, KF020414, KF020452; *M. A. McHenry* 10-64, VT; KF020337, KF020365, KF020413, KF020474; *M. A. McHenry* 10-61, VT; KF020336, KF020364, KF020412, KF020451. *Polystichum lepidotum* M. Sundue & M. Kessler; *M. A. McHenry* 10-94, VT; KF020338, KF020367, KF020415, KF020477. *Polystichum lonchitis* (L.) Roth; *D. P. Little* 344, VT; AF537247, KC878862, KF020393, KC890813. *Polystichum maximum* M. Kessler & A. R. Sm.; *M. A. McHenry* 10-84, VT; KF020318, KF020368, KF020416, KF020453; *I. Jiménez* 1292, LPB; KC819965, KC819950, KC819980, KC819995. *Polystichum montevidense* (Spreng.) Rosenst.; *R. Morero* RM 344, VT; KC819968, KC819953, KC819998, KC819983; *M. A. McHenry* 10-100, VT; KC819967, KC819952, KC819982, KC819997; *J. Condam* 687, R; KC819966, KC819951, KC819981, KC819996. *Polystichum muricatum* (L.) Fée; *D. P. Little* 349, VT; AF537251, EF177275, KF020404, KF020445. *Polystichum nudicaule* Rosenst.; *M. Sundue* 1285, VT; KC819969, KC819954, KC819984, KC819999; *M. A. McHenry* 10-88, VT; KF020321, KF020369, KF020417, KF020454. *Polystichum obliquum* (D. Don) T. Moore; *D. Barrington* 2090, VT; EF177328, EF177284, KF020386, KC890823. *Polystichum orbiculatum* (Desv.) J. Rémy & Fée; *D. Barrington* 2143, VT; KC878853, KC878863, KF020419, KF020456; *A. Vasco* 598, NY; KF020339, KF020371, KF020420, KF020457; *M. A. McHenry* 11-04, VT; KF020341, KF020370, KF020422, KF020459; *M. A. McHenry* 10-77, VT; KF020340, KF020372, KF020421, KF020458. *Polystichum pallidum* Gardn.; *J. Condam* 662, VT; KC819971, KC819956, KC819986, KC820001. *Polystichum platylepis* Fée; *J. Condam* 636, VT; KC819974, KC819959, KC819989, KC820004; *J. Condam* 579, VT; KC819972, KC819957, KC819987, KC820002; *J. Condam* 588, VT; KC819973, KC819958, KC819988, KC820003. *Polystichum platyphyllum* (Willd.) C. Presl; *D. Barrington* 2099, VT; EF177329, KF020427, KF020463; *M. A. McHenry* 10-55, VT; KC878854, KC878864, KF020426, KC890815; *I. Jiménez* 1501, UC; KF020325, KF020373, KF020428, KF020464; *J. Condam* 651, VT; KC819970, KC819955, KC819985, KC820000. *Polystichum polyphyllum* C. Presl; *M. A. McHenry* 10-51, VT; KF020342, KF020374, KF020418, KF020455. *Polystichum pungens* (Kaulf.) C. Presl; *J. Roux* 2370, VT; AF537253, EF177295, KF020390, KC890816. *Polystichum pycnolepis* (Kunze ex Klotzsch) T. Moore; *R. Morero* 343, VT; KF020343, KF020375, KF020431, EF177285; *M. A. McHenry* 10-81, VT; KF020324, KF020376, KF020432, KF020467; *M. Sundue* 1275, VT; KC819975, KC819960, KC819990, KC820005. *Polystichum rhizophyllum* (Sw.) C. Presl; *M. Sundue* 2069, VT; KC878855, KC878867, KF020394, KC890821. *Polystichum rochaleanum* Fée; *C. Rothfels* 3990, VT; KF020345, –, KF020434, KF020468; *J. Condam* 516, VT; KC819976, KC819961, KC819991, KC820006; *Galiano* 4507, UC; KF020344, KF020377, KF020433, KF020480. *Polystichum rufum* M. Kessler & A. R. Sm.; *I. Jiménez* 1078, UC; KC819977, KC819962, KC819992, KC820007. *Polystichum scopulinum* (D. C. Eaton) Maxon; *P. Zika* 18579, VT; KC878856, KC878860, KF020388, KC890817. *Polystichum sodiroi* Christ; *M. Sundue* 1060, VT; KF020346, KF020378, KF020435, KF020469; *M. A. McHenry* 10-07, VT; KF020347, KF020379, KF020436, KF020470. *Polystichum solomonii* M. Kessler & A. R. Sm.; *M. Sundue* 775, VT; KC819978, KC819963, KC819993, KC820008. *Polystichum speciosissimum* (A. Braun ex Kunze) Copel.; *D. P. Little* 297, VT; AF537255, EF177317, KF020397, KF020443. *Polystichum stuebelii* Hieron.; *M. A. McHenry* 11-17, VT; KF020348, KF020382, KF020437, KF020471; *M. Lehnert* 1149, VT; KF020349, KF020381, KF020439, KF020473; *M. Sundue* 1109, VT; KF020323, KF020380, KF020438, KF020472. *Polystichum talamancanum* Barrington; *D. P. Little* 299, VT; EF177335, EF177335, KF020402, KF020479. *Polystichum turrialbae* Christ; *D. P. Little* 295, VT; AF537259, KC878865, KF020403, KC890818. *Polystichum underwoodii* Maxon; *P. Wiczorek* 258, VT; AF537260, KC878868, KF020395, KC890819.