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Two Beringian Origins for the Allotetraploid Fern *Polystichum braunii* (Dryopteridaceae)

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Abstract—Although some polyploids in the genus *Polystichum* are well studied and have well-resolved evolutionary histories, the origin of the circumboreally distributed allotetraploid *Polystichum braunii* remains obscure. We use the chloroplast markers *rbcL*, *rps4-trnS*, and *trnL-F* as well as the nuclear markers *pgiC* and *gapCp* to demonstrate that *P. braunii* is a single allotetraploid with a minimum of two origins. The two variants isolated from the nucleus resolve with divergent clades, one eastern Asian and one North American. However, they do not have near allies among morphologically appropriate taxa in our sample; the North American progenitor appears to be extinct. A divergence-time analysis based on the cpDNA markers yielded evidence of an older time of origin for *P. braunii* than for an array of well-known allotetraploids in the eupolypod ferns. Niche modeling in the light of geological and paleontological evidence leads to the conclusion that the two origins were in Beringia. Since *P. braunii* is genetically undifferentiated but widely distributed, we argue that it has expanded to its circumboreal range in the recent past, though it has a relatively ancient origin.

Keywords—Allopolyploidy, Beringia, chloroplast DNA, nuclear DNA.

Allopolyploids incorporate genomes from different progenitors, usually different species (Stebbins 1947); in the ferns, unlike the angiosperms, they commonly arise from a hybridization event followed by a singular cellular event in which there is chromosome replication but not cell division (Wagner and Wagner 1980). The chromosome doubling predicated by the failed cell division allows restoration of fertility, each chromosome has a match in its newly duplicated genomic counterpart, and pairing at meiosis is successful. These fertile hybrids are in the broad sense morphologically intermediate between their progenitors (e.g. Manton 1950; Barrington 1986; Ekrt et al. 2010). The progenitors of hybrids and the allopolyploids derived from them have historically been inferred using a variety of methods, including morphology (Wagner 1954), chromosome counts and pairing behavior (Manton 1950; Manton and Reichstein 1961), and isozyme variation (Haufler 1987; Hunt et al. 2011). The current standard for inferring hybrid histories is to assemble information from DNA sequencing. Chloroplast genes and intergenic spacers are generally easy to amplify, but are less informative for inferring hybrid histories because they are inherited maternally in the ferns (Stein and Barrington 1990; Gastony and Yatskievych 1992). Genes from the nuclear compartment are useful for exploring allopolyploid origins, as they are inherited from both parents, but they are more challenging to amplify and interpret. The utility of an array of nuclear markers has been investigated, including the multiple-copy ITS (Widmer and Baltisberger 1999) and single-copy genes such as *gapCp* (Schuettpelz et al. 2008; Grusz et al. 2009), *pgiC* (Sessa et al. 2012b), and *LFY* (Shepherd et al. 2008). These nuclear markers, often assessed using vector cloning, have been used to infer hybrid histories by documenting the derivative allopolyploids to be additive, i.e. they show double-nucleotide calls at all positions where the progenitors differ (Tate et al. 2006). Most recently, Rothfels et al. (2013) have used transcriptome mining to provide a set of new single-copy markers, more than tripling the array of available tools for documenting allopolyploid origins.

The presence of multiple genomes in allopolyploids confers fixed heterozygosity, masking deleterious alleles and reducing inbreeding depression (Soltis and Soltis 2000; Husband et al. 2008; te Beest et al. 2012). Ferns produce large numbers of wind-dispersed spores and are free from the constraints of biotic factors like pollen and seed vectors (Barrington 1993), making them particularly good candidates for long-distance dispersal. In ferns, outcrossing is most common for the gametophytes of diploids; in contrast polyploid gametophytes commonly self-fertilize (Masuyama and Watano 1990; Testo et al. 2015). A single fern spore can travel a great distance to a new habitat, germinate to yield a gametophyte, and produce a sporophyte via gametophytic selfing (e.g. de Groot et al. 2012; Sessa et al. 2016). Hence, allopolyploid ferns combine increased capacity for selfing and the built-in genetic diversity inherent in their origins with the unusual dispersal capacity of spore-dispersed plants.

Stebbins (1940) championed the idea that allopolyploids expand to a wider geographical range than their progenitors over time, often eventually replacing them. This pattern may be driven by cycles of glaciation and deglaciation in boreal regions (Stebbins 1950). Especially in northern regions, the fixed-heterozygote genomes of allopolyploids may counter inbreeding and genetic drift during periglacial climate change (Brochmann et al. 2004). Ferns present an array of polyploids with patterns suggesting this history, from those with highly localized populations and widespread progenitors to those with continent-wide distributions and missing progenitors. Among likely recent polyploids are the tetraploids *Adiantum viridimontanum* Paris and *Asplenium tutwilarae* Keener and Davenport; they have highly restricted distributions and widespread diploid progenitors (Paris 1991; Wagner et al. 1993). Recent origin of *Adiantum viridimontanum* is also suggested by its current range having been entirely glaciated in the Pleistocene (Barrington and Paris 2007). In contrast, some polyploids have wide distributions and missing or disjunct progenitors suggesting older origins: three examples illustrate this point. The circumboreal *Gymnocarpium dryopteris* (L.) Newman has its progenitors restricted to western North America and the Appalachian Mountains (Pryer and Haufler 1993). The two widespread *Dryopteris* allotetraploids *D. carthusiana* (Villars) H. P. Fuchs and *D. cristata* (L.) A. Gray are derivative taxa that are more widespread than each of the extant progenitors and may have replaced their shared, missing progenitor, known as "*Dryopteris semicristata*" (Stein et al. 2010; Sessa et al. 2012a, b).

New Zealand *Asplenium* (Shepherd et al. 2008) presents a case in which the cycle has apparently begun again; there are no diploids among 19 species, and the tetraploids there have interacted to yield an array of eight octoploids.

The nearly cosmopolitan fern genus *Polystichum* Roth (Dryopteridaceae) comprises between 180 (Kramer and Green 1990) and 500 (Zhang and Barrington 2013) species. Hybridization is common in *Polystichum*; just about half of the species so far counted are polyploid (e.g. Löve et al. 1977), and many of these are known allopolyploids (e.g. Manton 1950; Wagner 1973; Barrington 1990). Among the polyploids in the genus whose origin is unknown is Braun's holly fern, *Polystichum braunii* (Spenner) Fée. Originally described from Europe, *P. braunii* has a circumboreal distribution across North America and Eurasia including northeastern North America, the Pacific Northwest, the Japan-Kamchatka-western Aleutian region, northeastern and western China, the Altai Mountains of Russia-Mongolia, and Europe (Hultén 1962; Fig. 1). It is one of the few strictly boreal species of *Polystichum*; the only other circumboreal species is the diploid *P. lonchitis* (L.) Roth. Throughout its range *P. braunii* is found in cool, wet, rocky woods, especially on calcareous talus slopes and natural sources of disturbance (e.g. slides and water courses). Chromosome counts from Europe, eastern and western North America, and eastern Asia (Manton 1950; Taylor and Lang 1963; Sleep 1966; Daigobo 1973; Barrington 1986) document the species as tetraploid (n = 82) throughout its range.

Work with artificially synthesized and natural hybrids in Europe in the 1960s established that *Polystichum braunii* is an allotetraploid. The key hybrid is *P.* × *luerssenii* Hahne, the tetraploid combining *P. braunii* and the allotetraploid *P. aculeatum* (L.) Roth, for which Manton and Reichstein (1961) documented virtually complete absence of pairing in meiosis I. From this work, it became clear that *P. braunii* was not an autopolyploid and that neither of its two genomes was homologous with the diploids whose genomes were incorporated in *P. aculeatum*, i.e. *P. lonchitis* and *P. setiferum* (Forssk.) Waynar (though Fraser-Jenkins (2008) claims that Sleep's hybridization work established *P. setiferum* as a progenitor of *P. braunii*). The hybrids of *P. braunii* with the two progenitor diploids yielded similar low levels of pairing (Manton and Reichstein 1961; Sleep and Reichstein 1967; Sleep 2014).

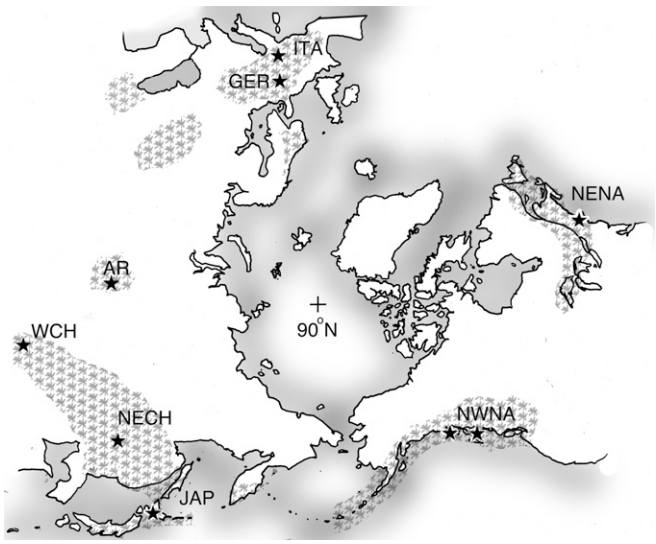

FIG. 1. Geographical distribution of *Polystichum braunii* accessions, north-polar view. Range of *P. braunii* indicated by tiled fern motif. AR = Altai, Russia. GER = Germany. ITA = Italy. JAP = Japan. NECH = northeastern China. NENA = northeastern North America. NWNA = northwestern North America. WCH = western China.

Also enlightening is the work on the hybrid of *Polystichum braunii* with the eastern North American diploid *P. acrostichoides* (Michx.) Schott (*P.* × *potteri* Barrington), for which both triploid and tetraploid cytotypes are known. Morzenti (1962) documented 41 pairs and 82 univalents in the tetraploid cytotype, again implying that when two homologous sets of chromosomes are present at meiosis I (here the *P. acrostichoides* chromosomes), the *P. braunii* chromosomes do not pair at all. In general, *Polystichum* hybrids have unusually high levels of pairing for ferns (Sleep and Reichstein 1967; Wagner 1973; Barrington 1990; Roux 1997; summarized in Sleep 2014). The anomalously low pairing of *P. braunii* chromosomes in hybrids with the European species and tetraploid *P.* × *potteri* suggests that its two component genomes are more divergent than other documented genome pairs in the genus.

Three infraspecific taxa recognized within *Polystichum braunii* during its history yield insights critical to understanding the origins and relationships of its two component genomes.

The taxon first described as *Polystichum andersonii* Hopkins based on a plant from Strathcona Park, Vancouver Island, and later treated as *P. braunii* subsp. *andersonii* (Hopkins) Calder & Taylor, is now recognized as a separate species (Wagner 1973; Wagner 1979). Morphological features and a hybrid with *P. munitum* with the equal numbers of univalents and bivalents suggesting a backcross led to the conclusion that *P. munitum* was one of the diploid progenitors of *P. andersonii* (Wagner 1973; Wagner 1979). D. H. Wagner (1979) proposed that a diploid with twice-pinnate fronds and a subapical rachis bulbil must be the other parent. A collection comprising fragments of leaves with these characteristics from British Columbia, proposed as the second progenitor, served as the type collection of *P. kwakiutlii* D. H. Wagner (Wagner 1990).

Polystichum setigerum (C. Presl) C. Presl was described from material collected along Nootka Sound on the west coast of Vancouver Island. Later named *P. alaskense* Maxon, the species for some years was taken as *P. braunii* subsp. *alaskense* Calder and Taylor. Dave Wagner's cytological work and morphological analysis led him to propose that *P. setigerum* is the hexaploid derivative of a cross between *P. braunii* and *P. munitum* (Wagner 1979).

The cool-temperate to boreal East Asian tetraploid species *P. microchlamys* (Christ) Kodama in Matsum. has been included in *P. braunii* twice: first in Hultén's (1927) *Flora of Kamchatka and the Adjacent Islands*, as *Polystichum braunii* var. *kamtschaticum* C. Chr. and second in Komarov's (1927) *Flora Peninsulae Kamtschatka* as *P. braunii* var. *subsessile* Komarov. Sleep (1971) reviewed the types of *P. microchlamys* and *P. braunii* var. *kamtschaticum* in the light of her own familiarity with *P. microchlamys* in Japan and argued persuasively that they were the same taxon, distinct from typical *P. braunii*. Fomin (1930) interpreted *P. braunii* var. *subsessile* as a synonym of *P. kamtschaticum*, so it probably pertains to *P. microchlamys* as well. Though Sleep (1971) makes a clear case for the morphological distinction of *P. braunii* and *P. microchlamys*, the repeated tendency to describe the latter as a variety of *P. braunii* suggests that the two may have a shared history, i.e. *P. microchlamys* may pertain to the lineage giving rise to one of the *P. braunii* genomes.

Two infraspecific taxa, by contrast, are not relevant to the present inquiry. First, Fernald (1928) distinguished American from European plants of *Polystichum braunii* s. s. (excluding the above varieties and subspecies) with the description of *P. braunii* var. *purshii* Fernald, based on a type from Smuggler's Notch in Vermont. Although Fernald presented an array of characters including leaf texture, spinule development, and petiole-scale histology to distinguish the two, D. H. Wagner (1979) found no basis for infraspecific divergence between European and North American populations. Second, the plant originally named *Aspidium braunii* Spenner var. *clarkii* Christ, but called *P. braunii* var. *clarkei* by Hultén (1962) is a synonym of the morphologically remote *P. lentum* (D. Don) T. Moore, based on our study of authentic material (US 00065509).

We are able to make inferences about the morphological features of *Polystichum braunii*'s progenitors from those of *P. braunii* with the exclusion of the infraspecific taxa formerly included with the species. Both progenitors are likely to be fully twice pinnate and have a well-developed indusium, as transgressive states for these characters are unknown in *Polystichum* hybrids. Distinctive features one or both of the progenitors must contribute are the lamina gradually reduced to about a third its maximum width at the base, herbaceous (vs. coriaceous) lamina texture, acuminate versus attenuate pinnae, spinulose pinnules attached at right angles to the pinna-rachis, and a dense indument of pale scales. There are two hypotheses for *P. braunii*'s progenitors in the literature. The first comes from Sleep (1966), who drew attention to the similarity between *Polystichum braunii* and *P. ohmurae* Sa. Kurata, a rare species endemic to central Honshu, Japan. Sleep was able to retrieve a meiotic count of 41 bivalents, but unable to include the species in her extensive breeding program. The striking similarity of this diploid species to *P. braunii*, with the exception of the submarginal as opposed to medial position of the sori, makes its inclusion in the search for progenitors of *P. braunii* important. The second comes from Fraser-Jenkins (2008) in his notes following the description of the Himalayan *Polystichum* × *pseudobraunii* Fras.-Jenk. for which he proposed *P. piceopaleaceum* Tagawa (section *Metapolystichum* Tagawa at the time) and *P. sinense* (Christ) Christ (sect. *Lasiopolystichum* Daigobo at the time) as progenitors. Fraser-Jenkins, impressed by the morphological similarity of his hybrid to *P. braunii*, proposed *P. piceopaleaceum* as one of its progenitors.

These ideas on origins, along with morphological considerations, suggest a working hypothesis. One progenitor was a robust, spinulose, 2-pinnate species typical of *Polystichum* section *Metapolystichum*, included in a more broadly circumscribed sect. *Hypopeltis* by Zhang and Barrington (2013). The other progenitor, which must contribute morphologically to the thinner lamina texture, narrow lamina base, and acute (vs. acuminate to attenuate) pinnae, was a member of the cool-temperate to alpine Asian sect. *Lasiopolystichum*, included in the more broadly circumscribed section *Sorolepidium* by Fraser-Jenkins (1997) and most recently in sect. *Hypopeltis* by Zhang and Barrington (2013).

In this study we investigate the genetic diversity and ancestry of *Polystichum braunii*. We used an array of coding and non-coding DNA sequences from the chloroplast to infer maternal ancestry. From the nucleus, we used two concatenated markers to assess genetic diversity and characterize the component genomes. We compared the genomic constituents of *P. braunii* from across its circumboreal range with an array of *Polystichum* species from eastern Asia and North America to: 1) test hypotheses for single versus multiple polyploid origins of *P. braunii*; 2) explore the affinities of the constituent genomes to extant lineages; and 3) explore the historical biogeography of *P. braunii*. We also provide insights into the western North American allopolyploids *P. andersonii* and *P. setigerum*.

Materials and Methods

***Taxonomic Sampling*—** A total of 55 accessions representing 45 taxa were included in the study group (listed in Appendix 1). The sample included 41 species of *Polystichum*, chosen to include: 1) likely allies of *P. braunii* based on morphology; and 2) representatives of all major clades of *Polystichum* identified in our previous studies (Little and Barrington 2003; Driscoll and Barrington 2007; Li et al. 2008). Candidate allies in Asia were those with fully twice-pinnate fronds that lacked bulbils. These candidate species largely lie in *Polystichum* sections *Hypopeltis* and *Sorolepidium* sensu Zhang and Barrington (2013). Although work on North American *Polystichum* has yielded a virtually complete understanding of species delimitation, ploidy, and ancestry for the polyploids, the situation in eastern Asia is different. There, much remains to be done in assessing ploidy levels, discovering progenitors of allopolyploids, and clearly delimiting species. Our sample of the East Asian species includes diploids, tetraploids, and species without cytological documentation (see Appendix 1 for cytological data by species), on the premise that discovering the relationships of *P. braunii* need not wait until all the taxonomic problems in China are solved.

For outgroups, we chose *Dryopteris intermedia* (Muhl. ex Willd.) A. Gray, *Arachniodes denticulata* (Sw.) Ching, *Phanerophlebia nobilis* (Schltdl. & Cham.) C. Presl, and *Cyrtomium macrophyllum* (Makino) Tagawa, because they represent the most closely allied genera in all previous studies (Little and Barrington 2003; Driscoll and Barrington 2007; Schuettpelz and Pryer 2007; Li et al. 2008).

We assembled an array of nine *Polystichum braunii* accessions from Vermont and Alaska, U. S. A.; Quebec and British Columbia, Canada; Hokkaido, Japan; Heilongjiang and Sichuan, China; the Altai Mountains of Russia; Baden-Württemberg, Germany; and Valsesia, Italy. Our own accessions of *P. braunii* were collected in Vermont, Quebec, British Columbia, and Western China, and stored in silica gel until extraction. Additional material of *P. braunii* and the other species studied was provided by generous collaborators from Alaska, Japan, China, Russia, Europe, and other locations (for full geographic distribution of the sample, see Fig. 1). cpDNA sequences from an extreme-western Aleutian population of *P. microchlamys* were generously shared by Sandra Talbot of the Alaska USGS.

***DNA Isolation, Amplification, Cloning, and Sequencing*—** Total genomic DNA was extracted from specimens using a modified version of the CTAB method (Doyle and Doyle 1987) as follows: Dried or fresh leaf tissue was ground in CTAB buffer (2% CTAB, 1.4 M NaCl, 0.25% beta-mercaptoethanol, 20 mM EDTA, 100 mM Tris HCl pH 8.0, 4% PVP) and incubated at 55°C for 1–24 hr, then extracted with 1 volume of 24:1 chloroform:isoamyl alcohol. DNA was precipitated with 0.08 volumes of cold potassium acetate and 0.54 volumes of cold isopropanol, pelleted by centrifugation, then washed with cold 70% ethanol followed by cold 95% ethanol. The pellets were dried for 20 minutes and resuspended in 20–50 μL sterile water.

***Chloroplast DNA Markers*—** The chloroplast markers comprised the *trnL-trnF* intergenic spacer (hereafter referred to as *trnL-F*), the *rbcL* gene, and the *rps4-trnS* intergenic spacer (hereafter referred to as *rps4-trnS*). Markers were amplified in 25 μL total volume including 50–150 ng genomic DNA, 1 × PCR buffer, 200 μM of each dNTP, 1 μM of each primer, and 0.65 U Ex Taq polymerase (TaKaRa Bio, Shiga, Japan). Previously published primers were used for *trnL-F* (Taberlet et al. 1991; primers e and f), *rbcL* (Little and Barrington 2003), and *rps4-trnS* (Souza-Chies et al. 1997; Li et al. 2008). The PCR products were purified with ExoSAP-IT (USB Corp., Cleveland, Ohio) and sequenced in both directions using an ABI Prism 3130x1 automated sequencer (DNA Analysis Facility, Vermont Cancer Center, Burlington, Vermont) with the ABI Prism BigDye Terminator cycle sequence ready reaction kit (Applied Biosystems, Foster City, California).

***Nuclear DNA Markers*—** The nuclear markers *gapCp* exons 8–10, *pgiC* exons 14–16, and their associated introns were amplified using the above conditions and previously published primer sets (Ishikawa et al. 2002; Schuettpelz et al. 2008). The PCR products were visualized on an agarose gel, then excised and purified using the Prep-Ease gel extraction kit (Affymetrix, Santa Clara, California). Purified fragments from *P. braunii*,

P. andersonii, and *P. setigerum* (but not the remaining species) were cloned using the TOPO TA cloning kit (Invitrogen, Grand Island, New York) following the manufacturer's instructions. Target fragments were amplified from purified plasmid DNA from 6–12 isolated colonies and sequenced as above.

Sequence Alignment and Phylogenetic Analysis—For both nuclear and chloroplast markers, raw chromatograms were edited by inspection, assembled into contigs with CodonCode Aligner version 4.0.2 (CodonCode Corporation, Dedham, Massachusetts), and aligned by hand in MacClade version 4.08 (Maddison and Maddison 2005). There were no areas of ambiguous alignment. Indels were coded using Simmons and Ochoterena's (2000) simple indel coding implemented by SeqState version 1.0 (Müller 2005).

Maximum parsimony (MP) analysis was carried out using TNT v1.5 (Willi Hennig Society; Goloboff et al. 2008; Goloboff and Catalano 2016). All characters were equally weighted and coded as unordered. A heuristic search was performed with 1,000 parsimony-ratchet replicates (Nixon 1999: 200 ratchet iterations, the up-and-down weights set to 5% each) with 20 trees held per ratchet, followed by tree-bisection-reconnection (TBR) branch swapping. Bootstrap support analysis (BS) was performed using 1,000 replicates doing 10 ratchets per replicate, holding 20 trees per ratchet, keeping only the strict consensus tree (Felsenstein 1985).

Bayesian inference (BI) was carried out using Mr. Bayes v. 3.2 (Ronquist et al. 2012). The Bayesian-analysis model selection for each marker was done using Modeltest v. 3.7 under the Akaike information criterion (Table 1; Posada and Crandall 1998; Posada and Buckley 2004; Posada 2006). The indels were given their own partition with a Jukes-Cantor model. A mixed-model Markov Chain Monte Carlo analysis was performed with each marker under its own best-fit model, with four independent Markov chains for 4,000,000 generations and trees sampled every 1,000 generations. Stationarity was determined by plotting the log-likelihood scores against generation for each run using the program Tracer 1.5 (Rambaut and Drummond 2007). Ten percent of the trees were discarded as the burn-in phase and a 50% majority rule consensus tree was calculated for the remaining trees. Preliminary analyses of each marker were largely congruent, so sequences for each data set within compartment were concatenated including indel data using SequenceMatrix (Vaidya et al. 2010).

The approach to analysis of the nuclear data required additional steps. Nuclear sequences generated by vector cloning were phased and concatenated as follows: Separately for *gapCp* and *pgiC*, we performed a phylogenetic analysis using just the sequences cloned from *Polystichum braunii*, *P. andersonii*, and *P. setigerum* yielding unrooted trees of all clones for each of the individuals. These unrooted trees display the relationships among an individual's cloned sequences. For each marker, the cloned sequences consistently formed two clusters (three in the case of *P. setigerum*), which we interpret as the signals from the component genomes. Consensus sequence for each cluster in each individual was generated; each individual is now represented by two (three in the case of *P. setigerum*) sequences or variants, the signatures of the component genomes. Then, for each marker, we performed an analysis of the variants (rooted with *P. hillebrandii* Carruth.) united with: a) the variants from *P. andersonii* and *P. setigerum*; and b) direct sequences from the remaining species. The yield was a gene tree of consensus clones and direct sequences for each marker. The two gene trees were congruent, so data from the two markers were concatenated into a combined matrix for further analysis. All of the phylogenetic analyses used in the construction of the concatenated nuclear-marker trees were performed with MrBayes.

Bayesian estimation of divergence times was conducted in BEAST 1.8.2 (Drummond et al. 2012) for the combined cpDNA data set. We used the same substitution models for each partition as in the Mr. Bayes analysis with four site-rate categories and base frequencies estimated for all partitions. We used an uncorrelated lognormal relaxed molecular clock model to estimate substitution rates with the birth-death process of speciation as the tree prior. As fossils for the genus *Polystichum* are not available, age estimates for two early divergence events were taken from dates developed for the analysis of Schuettpelz and Pryer (2009, supplemental data): 1) the most recent common ancestor of *Phanerophlebia*, *Cyrtomium*, and *Polystichum* (34.9 mya); and 2) the most recent common ancestor of *Cyrtomium* and *Polystichum* (30.8 mya). We applied a normal prior distribution to the two calibrations with the standard deviation set to 1. We ran the analysis for 30 million generations, sampling every 1,000 generations. Tracer 1.5 (Rambaut and Drummond 2007) was used to assess effective sample sizes and determine the burn-in period, which was 2.5 million generations. The consensus tree was compiled with TreeAnnotator 1.8.2 (Drummond and Rambaut 2007).

Material of the local Japanese endemic *P. ohmurae* was not available for this inquiry. However, an *rbcL* sequence for this species is available on Genbank (AB575210) as the result of work towards barcoding the Japanese fern flora (Ebihara et al. 2010). We performed an analysis of the *rbcL* sequences in our dataset with that for *P. ohmurae*, again using MrBayes, running the analysis for 1,000,000 generations, of which the first 100,000 were set aside as burn-in, with the same model for *rbcL* evolution as in the three-marker cpDNA analysis.

Inferring Climate Parameters for Polystichum braunii—We used georeferenced data from the Global Biodiversity Information Facility (GBIF, http://www.gbif.org) for 1,202 *Polystichum braunii* records with herbarium vouchers to develop a climate profile for the species with the package *dismo* (Hijmans et al. 2015) executed in R (R Core Team 2015). We plotted the geographic distribution of the specimens, then characterized climate for each collection area using data retrieved from the WorldClim database (Hijmans et al. 2005). From the whole sample, we then calculated mean annual temperature and annual temperature range.

Table 1. Characteristics of the chloroplast and nuclear DNA markers used in analysis of relationships of *Polystichum braunii*.

Marker (coverage)	Aligned length (bp)	Variable sites (percent)	Optimal model (AIC criterion)
gapCp (exons 8–11)	470	105 (14.0%)	GTR + G
pgiC (exons 14–16)	595	170 (25.2%)	TIM + G
rbcL	1190	96 (8.0%)	GTR+I+G
rps4-trnS	442	127 (28.8%)	GTR+I.
trnL-F	396	92 (24.2%)	GTR+G

Results

Chloroplast Phylogeny—The combined set of the three DNA sequences and their coded indels comprised 2,028 characters, 315 of which were variable, and 205 (9.8%) of which were parsimony informative (for details of length and variation of each marker, see Table 1). Bayesian and MP analyses of the combined data set retrieved consensus trees with congruent topologies (Fig. 2). Clades within *Polystichum* were numbered following Driscoll and Barrington (2007), as the structure of the phylogeny was similar. Clade IV is a large and well-supported (1.0 PP, 100% BS) assemblage of morphologically diverse and geographically widespread species. Clade IV and *P. lonchitis*, the single representative of Clade III (section *Polystichum*), are sister (0.99 PP, 98% BS); Clade II (section *Xiphopolystichum* s. l.) is sister to Clades III and IV (1.0 PP, 100% BS).

Polystichum braunii accessions from eastern North America, northwestern North America, Japan, northeastern China, the Altai Mountains, and Europe resolved in a strongly supported clade, the braunii clade (1.0 PP, 99% BS), which also included the Pacific Northwestern allopolyploids *P. setigerum* and *P. andersonii*, at an unresolved position at the base of Clade IV (Fig. 2). Short branch lengths in the braunii clade evidence minimal genetic divergence. A single accession of *Polystichum braunii*, from western China, was resolved without near relatives in a well-supported clade (1.0 PP, 100% BS) including all the sampled members of *Polystichum* sections *Hypopeltis* and *Sorolepidium* sensu Zhang and Barrington (2013). The northeastern Asian species *P. microchlamys* was retrieved sister to the austral *P. mohrioides* (Bory) C. Presl (0.9 PP, 100% BS). As a result we rejected a role for *P. microchlamys* in the history of *P. braunii*.

We also removed *Polystichum ohmurae* from consideration as a progenitor for *P. braunii* as the *rbcL* sequence resolved sister to *P. polyblepharum* among Japanese members of *Polystichum* section *Hypopeltis* distant from either of the two *rbcL* sequences recovered for *P. braunii*.

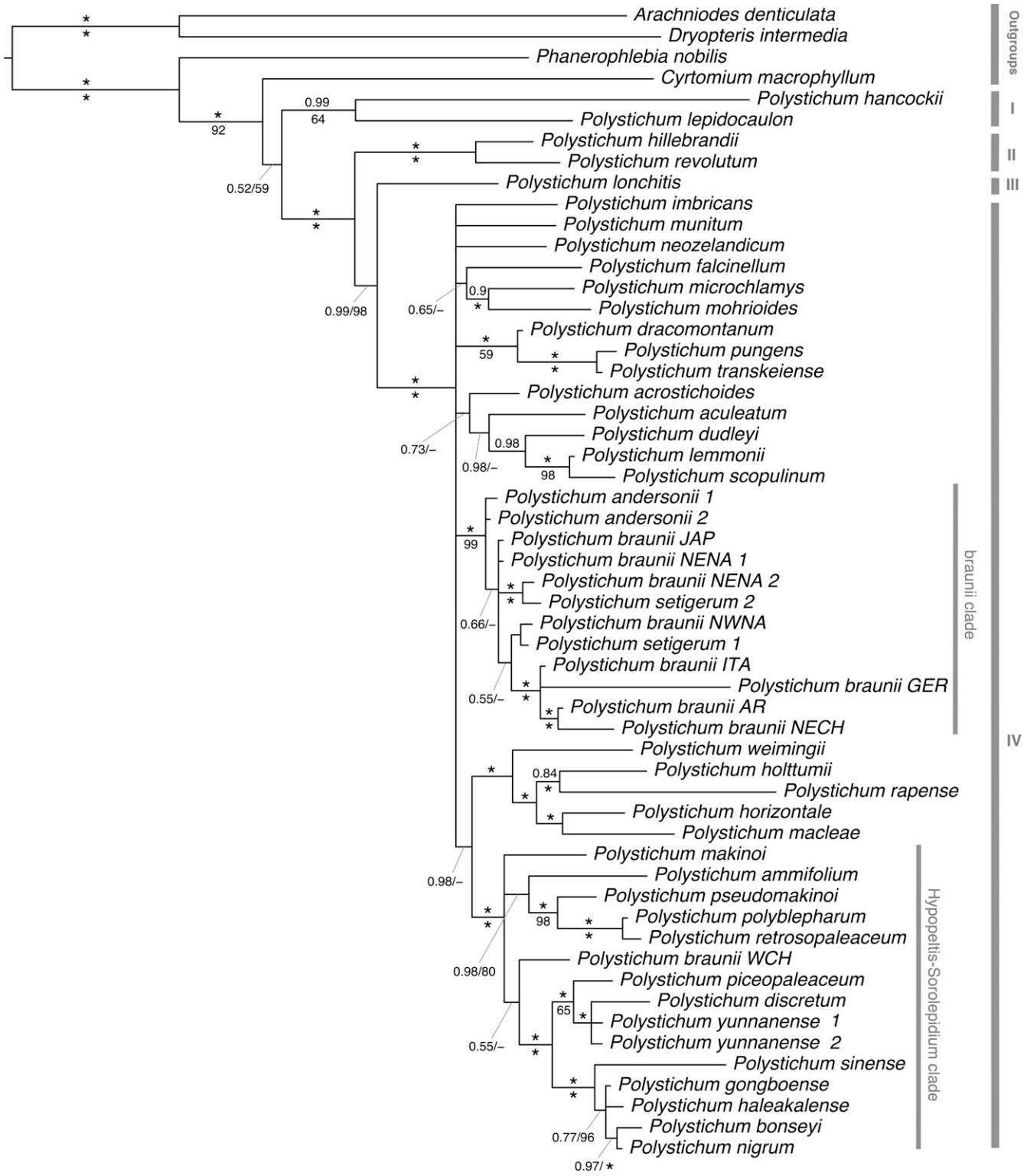

FIG. 2. Phylogram based on combined *rbcL*, *rps4-trnS*, and *trnL-F* data. Bayesian posterior probabilities (above branches) and maximum parsimony bootstrap percentages (below branches) given where support values exceed 0.5 or 50% respectively. Asterisk indicates 1.0 PP or 100% BS. Roman numerals indicate clades as reported in Driscoll and Barrington (2007). AR = Altai, Russia. GER = Germany. ITA = Italy. JAP = Japan. NECH = northeastern China. NENA = northeastern North America. NWNA = northwestern North America. WCH = western China.

Divergence-time Analysis—The maximum clade credibility chronogram obtained from the divergence-time analysis (Fig. 3) had a similar topology to that of the uncalibrated phylogeny (Fig. 2). Our divergence-time estimates for the two haplotypes retrieved from *Polystichum braunii* are both Late Miocene. The single accession constituting the western China/Sino-Himalayan (WCH) lineage is the product of a divergence event 7.7 mya, whereas the widespread braunii-clade lineage is the product of a divergence event 10.0 mya and has a crown age is 3.1 MYA. Patterns within the

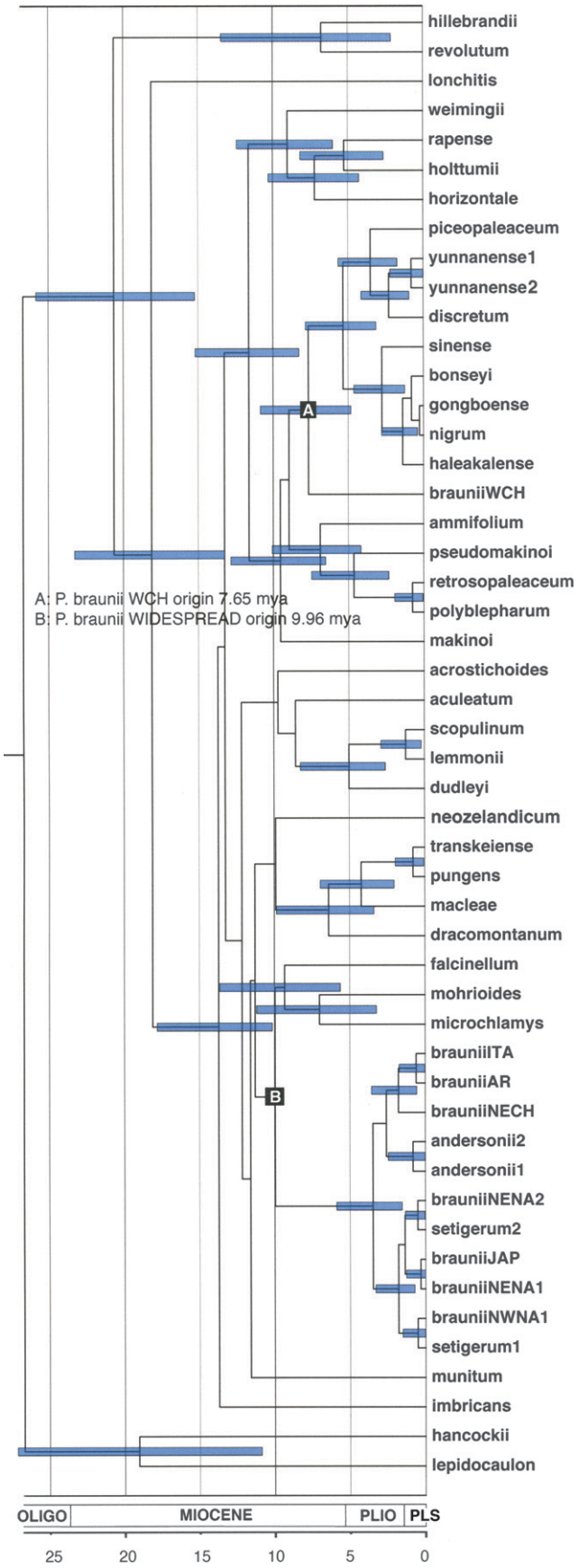

FIG. 3. Maximum clade credibility (MCC) chronogram with the divergence time estimates from the BEAST analysis. Numbers to right of nodes indicate mean node-age estimates. Mean age and 95% HPD are indicated for each node by the grey bars. Plio = Pliocene, Pls = Pleistocene.

braunii-clade lineage were incongruent with those in the Bayesian analysis but without support, consistent with the low divergence of the haplotypes within the lineage.

Nuclear Phylogeny—The combined data set of the two nuclear markers for accessions of *Polystichum braunii* comprised 1065 characters, 275 of which were variable, and 66 (6.5%) of which were parsimony informative (for details of length and variation of each marker, see Table 1). Bayesian and MP analyses of the nuclear DNA sequences retrieved consensus trees with congruent topologies. Considering the nuclear data for just *P. braunii*, two distinct clusters, which we call variants 1 and 2, were recovered from both the separate (not shown) and combined analyses of *gapCp* and *pgiC* (1.0 PP, 100% BS; Fig. 4).

The pattern of two *P. braunii* clusters persisted in the 2-nuclear-marker phylogeny including direct sequences of the remaining accessions (Fig. 5). The variant-1 sequences of

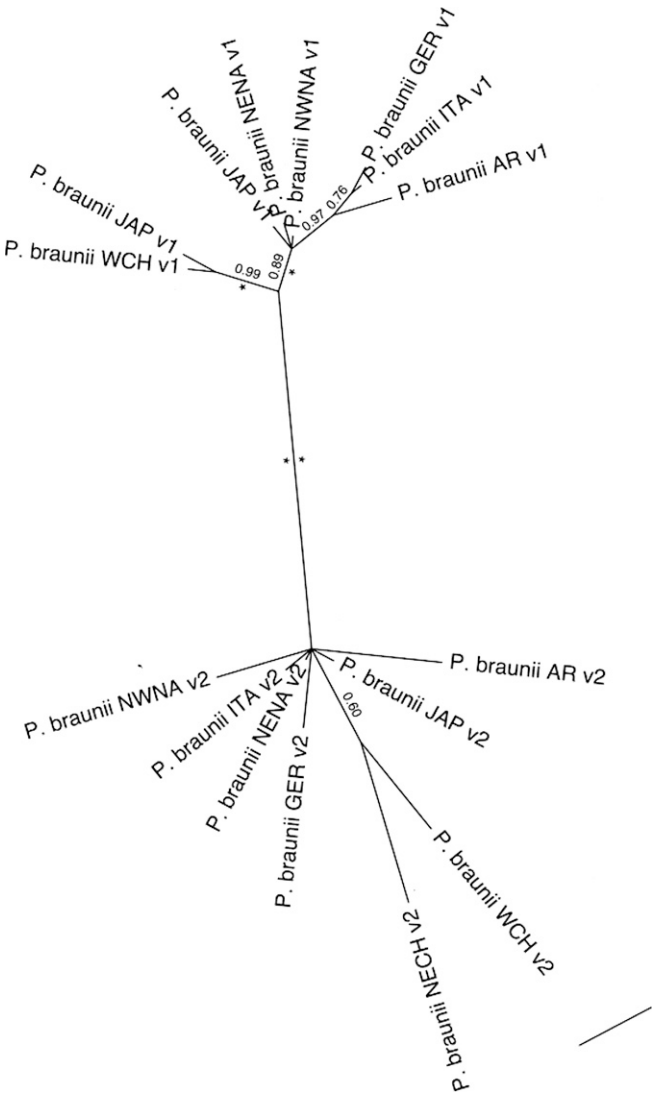

FIG. 4. Unrooted network based on concatenated *gapCp* and *pgiC* data for *Polystichum braunii*. Bayesian posterior probabilities (above branches) and maximum parsimony bootstrap percentages (below branches) given where support values exceed 0.5 or 50% respectively. Asterisk indicates 1.0 PP or 100% BS. Scale bar represents 0.005 expected substitutions per site. AR = Altai, Russia. GER = Germany. ITA = Italy. JAP = Japan. NECH = northeastern China. NENA = northeastern North America. NWNA = northwestern North America. WCH = western China.

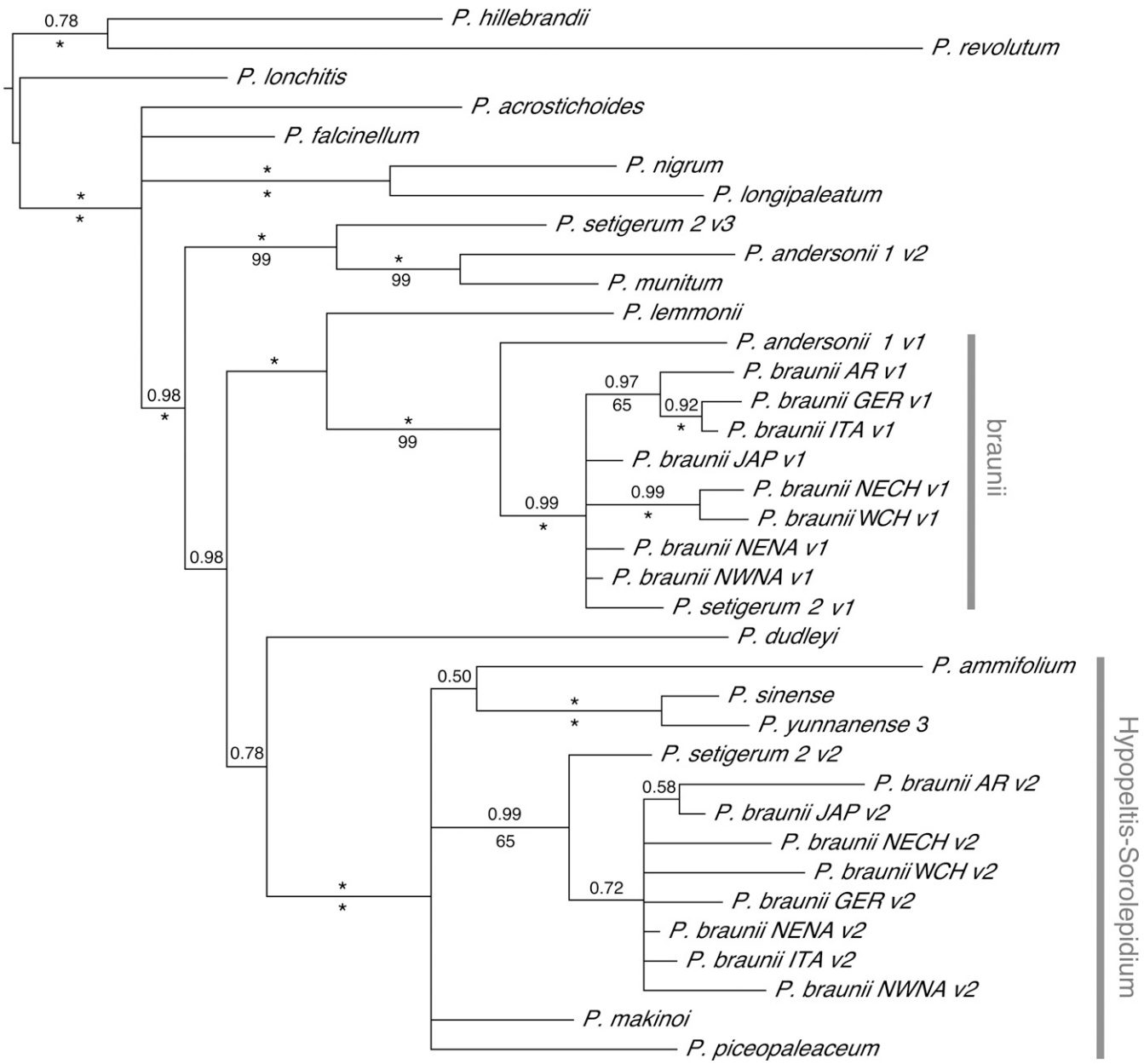

FIG. 5. Phylogram based on combined *gapCp* and *pgiC* data. Variants are distinguished with v# suffixes to the accession names. Bayesian posterior probabilities (above branches) and maximum parsimony bootstrap percentages (below branches) given where support values exceed 0.5 or 50% respectively. Asterisk indicates 1.0 PP or 100% BS. AR = Altai, Russia. GER = Germany. ITA = Italy. JAP = Japan. NECH = northeastern China. NENA = northeastern North America. NWNA = northwestern North America. WCH = western China.

P. braunii resolved in a clade, the North American clade, with sequences from three North American taxa (1.0 PP, 99% BS): *P. andersonii*, *P. setigerum*, and *P. lemmonii*. The *P. braunii* variant-2 sequences resolved in a single clade (0.99 PP, 65% BS) that also included a variant isolated from *P. setigerum*, within a clade of Asian species from sections *Hypopeltis* and *Sorolepidium* (1.0 PP, 100% BS), the Asian clade. All the *P. braunii* accessions, including the one from Western China, had variants that resolved in both clades.

Variants isolated from *Polystichum andersonii* and *P. setigerum*, the two polyploid species with which *P. braunii* co-occurs in northwestern North America, reveal relationships in the analysis using nuclear markers. The hexaploid *P. setigerum* yielded three nuclear variants. *Polystichum setigerum* variant 1 clustered with *P. braunii* in the North American clade (0.99 PP, 100% BS; Fig. 5). Variant 2 clustered with *P. braunii* in the Asian clade (0.99 PP, 65% BS), with *P. braunii* variant 2 as its nearest neighbor (0.72 PP). Variant 3 was closely associated with the diploid *P. munitum* and a variant isolated from *P. andersonii* (1.0 PP, 99% BS), but without any variants of *P. braunii*. The tetraploid *P. andersonii* yielded two nuclear variants, one sister to the North American clade (1.0 PP, 99% BS) and the other in the clade with *P. munitum* and *P. setigerum* (1.0 PP, 99% BS).

Inferring Climate Parameters for Polystichum braunii—We retrieved a mean annual temperature for the *P. braunii* records in the GBIF database of 5.3°C±1.8°. For the same records, the annual temperature range of their localities was 22.7°C±5.6°.

Discussion

***Genetic Profile of Polystichum braunii*—** The chloroplast phylogeny resolved *Polystichum braunii* in two places (Fig. 2). This pattern could be the result of two polyploid origin events involving the same two species, but with a different progenitor donating the chloroplast in each case. Alternatively, the pattern could represent more than one combination of progenitor species passing under the name *Polystichum braunii*.

The unrooted network of combined nuclear sequences recovered from *Polystichum braunii* presented two clusters (Fig. 4). We infer that the two clusters recovered in the analysis of the nuclear data (variants 1 and 2) represent the two component genomes of allotetraploid *P. braunii*. Each of the *P. braunii* specimens yielded two variants; each cluster included sequences from every specimen. The nuclear data thus indicate that there is only one pair of progenitors; we conclude that the data from the chloroplast evidence a minimum of two origins for *P. braunii* from these two progenitors.

Only one variant of *gapCp* was recovered from *Polystichum braunii* specimens from Italy and Germany, though they had both variants for *pgiC*. A total of 17 *gapCp* clones were sequenced for the European samples; all had identity to variant 1. The implied change in nucleotide sequence in the primer sites of *gapCp* we take to be a synapomorphy for the European accessions, as both copies were retrieved in the remainder of the species' range. This synapomorphy suggests that the most recent event in the history of *P. braunii* was its arrival in Europe, the source of its nomenclatural type.

***The Relationships of the Progenitors of Polystichum braunii*—** An allopolyploid origin for *Polystichum braunii* from two remotely related progenitors in clade IV of Driscoll and Barrington (2007) is implied by the patterns retrieved in both the chloroplast and nuclear results. One progenitor, represented by nuclear variant 1, is sister to but morphologically and genetically divergent from the North American *P. lemmonii* in the nuclear phylogeny (Fig. 5). As we sampled all North American diploid species, we conclude that nuclear variant 1 was contributed by a North American species that is unknown and likely extinct. This progenitor is present in the chloroplast phylogeny, but without near allies, as the maternal progenitor of *Polystichum braunii* from everywhere in the world except western China (Fig. 2). The second progenitor, represented by nuclear variant 2, has its closest allies among the sampled species of sections *Hypopeltis* and *Sorolepidium* (H. Christ) Tagawa (sensu Zhang and Barrington 2013), centered in eastern Asia, in the nuclear phylogeny (Fig. 5). This progenitor is present in the chloroplast tree as the maternal progenitor of *P. braunii* from western China (Fig. 2). Neither the nuclear nor chloroplast sequences of this second progenitor resolved in close relationship to any of the sampled Asian species. We conclude that the second nuclear variant's origin lies in a species with East Asian relationships, but that this progenitor is at large and perhaps extinct. Thus, it appears that one of the progenitors of *P. braunii* is allied to species with East Asian affinities and the other to a species with North American affinities.

Our analysis of divergence dates based on cpDNA data yielded insights into the history of the two progenitors of *Polystichum braunii*. Within the constraints of our sample, the earliest dates of origin for the two progenitors (stem lineage origin) are Late Miocene (i.e. 7.7 [for braunii WCH] – 10.0 [for the braunii clade] mya), and the latest date for the origin of the tetraploid itself is 3.5 mya (i.e. the crown age of the braunii-clade cpDNA haplotype). Some context for these dates comes from within our dataset. We have included the tetraploid *P. scopulinum* and *P. lemmonii*, one of its progenitors (the other is *P. imbricans*, Soltis et al. 1991). The divergence of their genomes at 1.4 mya suggests a date of origin in the Pleistocene for this tetraploid with two extant, identified progenitors. Sigel et al. (2014) retrieved similar results in tracing the history of the North American diploid *Polypodium* species with a BEAST analysis of cpDNA markers. The divergence history that yielded the diploid progenitors (both extant) of the well-known northeastern North American allopolyploid *Polypodium virginianum* L. is entirely Pleistocene, i.e. less than 1.5 mya long. Sessa et al. (2012a) provide additional context for our analysis of divergence dates, as they retrieved Pleistocene (1–2 mya) cpDNA haplotype divergence dates for the three North American *Dryopteris* polyploids *D. clintoniana, D. cristata, and D. carthusiana*, all of which share the presumptively extinct *"Dryopteris semicristata"* genome. *Polystichum braunii* does appear to have a relatively ancient origin among Northern-hemisphere polyploid ferns subjected to dating analysis.

Polystichum braunii, a widely distributed allotetraploid with genomes originating in the Late Miocene and likely extinct progenitors, fits the Stebbins historical model (Stebbins 1940) for polyploids. The isolation of the lineages that include *P. braunii*'s progenitors on different continents also suggests that a long time has passed since they were close enough to interbreed. The distant relationship between the progenitors' clades relates well to the anomalously low degree of pairing of its homoeologous genomes.

Where were the encounters that yielded *Polystichum braunii*? At the boundary between Asia and North America lies Beringia, above water and forested in the Mid- to Late Miocene, (Wolfe 1994). Was the climate appropriate for the origin of *P. braunii* in Beringia at that time? Forests characteristic of regions with the mean annual temperature and annual temperature-range parameters we retrieved are documented in both Alaska and Siberia in the Mid- to Late Miocene, at latitudes from 60° to 64° North (Wolfe 1994, Fig. 2). We suggest that the encounters of *P. braunii*'s progenitors took place in these Beringian forests.

The remarkable genetic uniformity of *Polystichum braunii* across its circumboreal range revealed in both chloroplast and nuclear sequences suggests that the species, though relatively ancient, has expanded to occupy its current broad range in the recent past. The opening of broad-ranging appropriate habitat in the wake of the last glacial retreat likely provided the opportunity for *P. braunii*, a highly dispersible species likely capable of selfing, to expand rapidly into its current interrupted circumboreal range.

***Relationships with Other Pacific Northwestern Polyploids*—** Our analysis also allows us to test established hypotheses for the origin of two polyploids endemic to northwest North America, tetraploid *Polystichum andersonii* and hexaploid *P. setigerum*. In the chloroplast phylogeny, the two accessions of *P. andersonii* resolved in the braunii clade with *P. setigerum* and the *P. braunii* accessions outside of Western China, but no other species of *Polystichum*. Thus, the three either share a diploid progenitor or they have a genome from species in the same lineage. Given that the three polyploids resolve in a single clade, they may have a progenitor species in common. However, since *P. andersonii* lies outside the subclade comprising the *P. braunii* and *P. setigerum* accessions, it may have a genome from a different but closely allied species.

Now considering the nuclear tree, as expected we retrieved three variants from hexaploid *Polystichum setigerum*, one lying with each of the two variants found in *P. braunii* and one lying sister to *P. munitum*, providing support for D. H. Wagner's (1979) proposal that *P. setigerum* arose from a hybrid between *P. munitum* and *P. braunii*. The *P. andersonii* variants resolved in two places, in concert with D. H. Wagner's (1979) hypothesis that *P. andersonii* is an allotetraploid. One *P. andersonii* variant lies in the exclusive clade with *P. munitum* and *P. setigerum*, in agreement with D. H. Wagner's (1979) proposal that *P. munitum* is one progenitor of both. The single remaining *P. andersonii* sequence is sister to the North American lineage.

What is the origin of the *P. andersonii* genome that resolves with *P. braunii* and *P. setigerum*? The simplest explanation is that *Polystichum andersonii* and *P. braunii* share a diploid progenitor, but they differ by *P. andersonii* bearing subterminal rachis bulbils absent in *P. braunii* and *P. munitum*. We suggest that a single North American *Polystichum* lineage comprised a bulbil-bearing progenitor of *P. andersonii* and a non-bulbil-bearing progenitor that gave rise to *P. braunii*. *Polystichum kwakiutlii* D. H. Wagner may be the bulbil-bearing progenitor of *P. andersonii*, as proposed by Wagner (1979, 1990).

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Appendix 1: Sources of material for DNA sequencing. Ploidy reported for the species when available; voucher location included in parentheses, abbreviations follow Thiers [continuously updated]; short accession names used in trees and Genbank are provided in brackets for taxa with more than one accession; GenBank accession numbers for the

five markers in order are *rbcL*, *rps4-trnS*, *trnLF*, *gapCp*, *pgiC*; V#s are cloned versions within nuclear markers.

Arachniodes denticulata (Sw.) Ching: COSTA RICA, Pr. Cartago, *E. Sigel & M. McHenry s.n.* (VT); KX768031, KX768068, KX768066, -, -. ***Cyrtomium macrophyllum*** (Makino) Tagawa: CHINA, Yunnan Pr., Kunming, *S. G. Lu B15* (PYU); AB575111, EU106600, EU106596, -, -. ***Dryopteris intermedia*** (Benth.) C. Chr.: U.S.A., Vermont, *D. P. Little 345* (VT); AF537229, KX768069, KX768067, -, -. ***Phanerophlebia nobilis*** (Schlecht. & Cham.) C. Presl var. ***nobilis***: MEXICO, Volcán Iztaccihuatl, *G. Yatskievych et al. 85-211* (IND); AF537231, EU031782, EF177269, -, -. ***Polystichum acrostichoides*** (Michx.) Schott, (diploid): U.S.A.,Vermont, *S.A. Jorgensen 57* (VT); KT602429, KX768071, JX476097, KX866661, KX786207. ***Polystichum aculeatum*** (L.)Roth (tetraploid): U.S.A. Hort., New York Botanical Garden, *J. T. Mickel s.n.* (VT); KX768041, KX768072, KX768064, -, -. ***Polystichum ammifolium*** (Poir.)C.Chr.: RÉUNION, Cilaos, T. A. Ranker 1537 (VT); AF537237, KF020391, EF177287, KX866660, KX786209. ***Polystichum andersonii*** Hopkins (tetraploid): CANADA, British Columbia, *S. A. Jorgensen 70* (VT) [andersonii 1]; KX768032, KX768073, JX476099, V1 KX866640 V2 KX866647, V1 KX786238 V2 KX786239. U.S.A, Alaska, *M. Stensvold 8413* (VT) [andersonii 2]; KX768033, KX768074, JX476098, -, -. ***Polystichum bonseyi*** W. H. Wagner & R. W. Hobdy (hexaploid): U.S.A., Hawaii, Maui, *H. Driscoll 320* (VT); KX768043, KX768075, KX768063, -, -. ***Polystichum braunii*** (Spenner) Fée (tetraploid): CHINA, Heilongjiang Pr., L. *Zhang s.n.* (VT) [braunii NECH]; KX768049, KX768080, JX476105, V1 KX866653 V2 KX866650, V1 KX786216 V2 KX786217. Sichuan Pr., *D. S. Barrington 2219* (VT) [braunii WCH]; KX768042, KX768084, JX476106, V1 KX866646 V2 KX866657, V1 KX786222 V2 KX786223. GERMANY, Hort., Munich Botanical Garden, *J. Lockwood s.n.* (VT) [braunii GER]; KX768058, KX768079, KX768061, V1 KX866642 V2 -, V1 KX786210 V2 KX786211. ITALY, Valsesia, *R. L. Viane 5833* (VT) [braunii ITA]; KX768036, KX768077, JX476107, V1 KX866643 V2 -, V1 KX786212 V2 KX786213. JAPAN, Hokkaido, *C. Takeda A* (VT) [braunii JAP]; KX768034, KX768078, JX476104, V1 KX866644 V2 KX866649, V1 KX786214 V2 KX786215. RUSSIA, Altai Mountains, *A. I. Shmakov s.n.* (VT) [braunii AR]; KX768048, KX768076, KX768062, V1 KX866641 V2 KX866648, V1 KX786237 V2 KX786235. U.S.A., Vermont, *S. A. Jorgensen 37* (VT) [braunii NENA 1]; KX768037, KX768081, JX476100, V1 KX866645 V2 KX866651, V1 KX786218 V2 KX786219. CANADA, Québec Pr., *S. A. Jorgensen 58* (VT) [braunii NENA 2]; KX768040, KX768082, JX476101, -, -. U.S.A., Alaska, *E. Anderson 751* (VT) [braunii NWNA]; KX768035, KX768083, JX476103, V1 KX866654 V2 KX866656, V1 KX786220 V2 KX786221. ***Polystichum discretum*** (D. Don) J. Sm. (diploid): CHINA, Kunming, *S. G. Lu B41* (PYU); KX768056, DQ151864, DQ150401, -, -. ***Polystichum dracomontanum*** Schelpe & N. C. Anthony: SOUTH AFRICA, *J. P. Roux 2715* (VT); AF537240, KX768085, EF177290, -, -. ***Polystichum dudleyi*** Maxon (diploid): U.S.A. California: *W.A. Born s.n.* (VT); AF537241, KX768086, JX476108, KX866663, KX786224. ***Polystichum falcinellum*** (Sw.) C. Presl (octoploid): PORTUGAL, Madeira, *R. L. Viane 8981* (VT); KX768055, KX768087, JX476109, KX866664, -. ***Polystichum gongboense*** Ching & S.K.Wu: CHINA, Yunnan, *S. G. Lu K43* (PYU); KX768044, DQ151867, DQ150404, -, -. ***Polystichum haleakalense*** Brack. (tetraploid): U.S.A., Hawaii, *H. Driscoll 323* (VT); KX768046, KX768088, JX476110, -, -. ***Polystichum hancockii*** (Hance)Diels (diploid): TAIWAN, Nantou Co., *T. A. Ranker 2086* (COLO); -, KX768070, JX476111, -, -. ***Polystichum hillebrandii*** Carruth., (diploid): U.S.A., Hawaii, *H.E. Driscoll 312* (VT); -, -, -, -, KX786225. U.S.A., Hawaii, *H.E. Driscoll 315* (VT); EF177323, KX768089, EF177279, KX866667, -. ***Polystichum holttumii*** C.Chr.: BORNEO, Sabah, *T. Ranker 2163* (VT); KX768059, KX768090, JX476112, -, -. ***Polystichum horizontale*** C. Presl: CHINA, Kunming, *S. G. Lu PK5* (PYU); KX768050, KX768091, JX476113, -, -. ***Polystichum imbricans*** (D. C. Eaton) D. H. Wagner (diploid): U.S.A., Oregon, *D. H. Wagner 9112* (VT); AF537262, KX768092, EF177313, -, -. ***Polystichum lemmonii*** Underw. (diploid): U.S.A., Washington, *P. Zika 18982* (VT); EF177324, KX768093, EF177280, KX866665, KX786226. ***Polystichum lepidocaulon*** (Hook.) J. Sm. (diploid): CHINA, Zhejiang, *S. G. Lu Q12* (PYU); AF537224, DQ151855, DQ150392, -, -. ***Polystichum lonchitis*** L. (diploid): U.S.A., Alaska, *D. P. Little 344* (VT); AF537247, KF020393, KX768065, -, -. SWITZERLAND, Hort., Botanical Garden, Zurich, *J. Scheller 01* (VT); -, -, -, KX866669, KX786227. ***Polystichum macleae*** (Baker) Diels (tetraploid): SOUTH AFRICA, Sabie, *J. P. Roux 2561* (VT); AF537249, KX768094, EF177294, -, -. ***Polystichum makinoi*** (Tagawa) Tagawa (tetraploid): CHINA, Sichuan Pr., *D. S. Barrington 2185* (VT); KX768057, KX768095, JX476114, KX866666, KX786228. ***Polystichum microchlamys*** (Christ) Matsum. (tetraploid): USA, Alaska, Attu Island, *S.S. Talbot et al. 018-X-1* (ALA); KX856980, KX856982, KX856981, -, -. ***Polystichum mohrioides*** (Bory) C. Presl: CHILE, Magallanes, *B. Connolly 2* (VT); AF537250, KX768096, JX476115, -, -. ***Polystichum munitum*** (Kaulf.) Presl (diploid): U.S.A., Washington, *P. Zika 18932* (VT); EF177343, KX768097, EF177315, KX866659, KX786229. ***Polystichum nigrum*** Ching & H. S. Kung: China, Yunnan Pr., *D. S. Barrington 2243* (VT); KX768045, KX768098, JX476116, KX866668, KX786230. ***Polystichum neozelandicum*** Fée ssp. ***zerophyllum*** (Colenso) Perrie (octoploid): NEW ZEALAND, South Island, *H. White s.n.,* (VT) [neozelandicum]; AF208394, KX768099, JX476120, -, -. ***Polystichum piceopaleaceum*** Tagawa (tetraploid): CHINA, Yunnan, *S. G. Lu K50* (PYU); -, DQ151874, -, -, -. TAIWAN, Nantou Co., *T. Ranker 2030* (COLO); EF177338, -, EF177308, -, -. CHINA, Sichuan Pr., *D. S. Barrington 2223* (VT); -, -, -, KX866670, -. ***Polystichum polyblepharum*** (Röm. ex Kunze) C. Presl (tetraploid): CHINA, Zhejiang Pr., *S. G. Lu P7* (PYU); KX768051, DQ202468, DQ202436, -, -. ***Polystichum pseudomakinoi*** Tagawa (tetraploid): CHINA, Jiang Xi Pr., *D. S. Barrington 2083* (VT); KX768053, KX768100, JX476117, -, -. ***Polystichum pungens*** (Kaulf.) C. Presl (octoploid): SOUTH AFRICA, Western Cape Pr., *J. P. Roux 2370* (VT); AF537253, KX768101, EF177295, -, -. ***Polystichum rapense*** E. Brown: RAPA, *K. Wood 9355* (VT); KC896009, KX768102, JX476118, -, -. ***Polystichum retrosopaleaceum*** (Kodama) Tagawa: CHINA, Zhejiang, *S.G. Lu P21* (PYU); KX768052, DQ202470, DQ202438, -, -. ***Polystichum revolutum*** P. S. Wang (diploid): CHINA, Sichuan Pr., *D. S. Barrington 2206* (VT); KX768060, KX768103, JX476119, KX866672, KX786231. ***Polystichum scopulinum*** (D. C. Eaton) Maxon (tetraploid): CANADA, Québec Pr., *P. Zika 18579* (VT); KX768047, KX768104, JX476121, -, -. ***Polystichum setigerum*** C. Presl (hexaploid): CANADA, British Columbia, *S. A. Jorgensen 79* (VT) [setigerum 2]; KX768039, KX768105, JX476123, V1 KX866655 V2 KX866658 V3 KX866652, V1 KX786232 V2 KX786233 V3 KX786234. U.S.A., Alaska, *M. Stensvold 8414* (VT) [setigerum 1]; KX768038, -, JX476122, -, -. ***Polystichum sinense*** (Christ) Christ (tetraploid?): CHINA, Sichuan Pr., *D. Boufford 27808* (VT); EF177334, -, EF177304, KX866662, -. Sichuan Pr., *D. Boufford 27542* (VT); -, KX768106, -, -, KX786235. ***Polystichum transkeiense*** N. Jacobsen (tetraploid): SOUTH AFRICA, KwaZulu-Natal Pr., *J. P. Roux 2493* (VT); AF537257, KX768107, EF177297, -, -. ***Polystichum weimingii*** Li Bing Zhang and H. He: CHINA. Yunnan Pr., *D. S. Barrington 2257* (VT); KX768054, KX768108, JX476124, -, -. ***Polystichum yunnanense*** Christ (tetraploid): CHINA, Yunnan Pr., *D. S. Barrington 2087* (VT) [yunnanense 1]; EF177333, KX768109, EF177303, -, -. *S. G. Lu 28* (PYU) [yunnanense 2] AY545504, DQ151869, DQ150406, -, -. Yunnan: *R. L. Viane 9369* (VT) [yunnanense 3]; -, -, -, KX866671, KX786208.