Elucidating the origin of *Woodsia scopulina* subsp. *laurentiana* (Woodsiaceae) in the

Great Lakes region, an integrated systematic approach

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Introduction

Pteridophytes (ferns and fern allies) are abundant in the fossil record and previous studies have placed the initial radiation of these spore-bearing vascular plants to the Devonian period, about 430–400 million years ago (Mya), by integrating fossil and molecular sequence data (Taylor & Taylor, 2008; Schneider et al., 2004; Fiz-Palacios et al., 2011; Testo & Sundue, 2016). In the early and mid-Mesozoic (Triassic, Jurassic, and early Cretaceous, roughly 252–140 Mya) ferns composed a dominant element of the global terrestrial flora (Lidgard & Crane, 1988). However, at the end of the Cretaceous period, fossil evidence and historical spore data have shown that fern diversity declined by about half the number of genera (Lupia et al., 1999; Nagalingum et al., 2002). Today there are about 10,500 extant fern species that grow in a wide variety of habitats, spanning emergent aquatic environments to mesic forest floors, the high canopy (as epiphytes), and even desert endemics (PPG 1, 2016).

One striking feature in fern evolution is their unusually high number of chromosomes. In homosporous ferns, a single cell nucleus can hold as many as 1,260 chromosomes (*Ophioglossum reticulatum*, 2*n*; Abraham & Ninan, 1954)—the highest number documented for any organism. High chromosome numbers in the ferns result, at least in part, from whole genome duplication (polyploidy), which results in a cell nucleus with more than two full sets of chromosomes. Polyploidy is common in ferns, making them a useful system for studying the evolutionary impacts of whole genome duplication (DeMaggio et al., 1971).

Polyploidy has been actively scrutinized for more than a century and is recognized in cytogenetic and evolutionary studies of both plants and animals (e.g.,

Winge, 1917; Dobzhansky, 1937; Barker et al., 2016). During the second half of the 20th century, speculation regarding the relative evolutionary importance of polyploidy caused interest in the topic to wane. During this time, the widespread prevalence of diploid species (i.e., species with 2 genome copies per sporophyte cell nucleus, $2n = 2x$) was used by some researchers to pigeon-hole polyploidy as "evolutionary noise" (Wagner, 1970; Stebbins 1971).

Since the late 20th century, however, advances in molecular biology have propelled evolutionary studies, providing new avenues for research on polyploid, non-model systems. A paradigm shift resulted, as researchers re-evaluated the impact of whole genome duplication on plant diversification. Now, it is widely accepted that polyploidy has directly shaped the evolution of all vascular plants (Jiao et al., 2011; Arrigo and Barker, 2012; Li et al., 2015; Wendel, 2015). Even so, despite signatures of polyploidy being detected deep in plant evolutionary history, the long-term fate of polyploid taxa is still debated (Mayrose et al., 2011, 2015; Soltis et al., 2014). Some authors argue that polyploid lineages are mostly evolutionary "dead ends" that are destined to go extinct shortly after forming, at higher rates than diploid taxa (Mayrose et al., 2011). Others contend that polyploids are vastly understudied, and that the estimates used in some studies are a poor representation of the overall diversity of polyploids in nature (Soltis et al., 2014).

Whole genome duplication has had a measurable impact on the evolutionary history of all land plants, and this process continues to play a key role in shaping the diversification of many living species. This is especially true of the ferns (Huang et al., 2020), in which polyploidy often generates species complexes that encompass many

ploidy levels (e.g., $2n = 2x, 3x, 4x$; Grusz et al., 2009). Whole genome duplication, whether it occurred recently or deep in fern evolution, can make it challenging to identify the direct origin(s) of polyploid lineages, e.g., when testing evolutionary hypotheses. In polyploid lineages, determining the biparental (i.e., maternal and paternal) origins of a species is complicated by the presence of multiple copies of each chromosome (≥ 3) within a given cell nucleus. For this reason, it is useful to first examine evolutionary relationships using uniparentally-inherited genomic regions, e.g., the chloroplast genome, when elucidating the parent(s) of polyploid and/or hybrid fern species (Gatsony & Yatskievych 1992; Beck et al., 2010). In ferns, maternal inheritance of the chloroplast genome was originally explored in the '*Asplenium* Triangle', first conceived of by Wagner (1954). Wagner noticed that three out of 11 taxa in the complex appeared to represent morphological extremes, with all other species possessing intermediate morphological features, leading him to conclude that these morphological intermediates represented hybrid species.

Wagner (1954) was the first of many later authors to expose reticulate patterns of hybridization and whole genome duplication in ferns. Since his work on the '*Asplenium* Triangle', a range of evolutionary studies and subsequent taxonomic revisions have directly examined the diverse origins of polyploid fern species. Among these, one well-studied lineage with a history of polyploidy and hybridization is the genus *Woodsia* R. Br. (Woodsiaceae), which hosts a diverse array of diploid ($2n = 2x$), triploid ($2n = 3x$), and tetraploid ($2n = 4x$) species (Windham, 1993a; Fig. 1).

Study system—As a family, Woodsiaceae has fluctuated both in estimates of species number (22–59 spp.) and in the number of recognized genera (ranging from 1–7;

Ching, 1932; Brown, 1964; Shao et al., 2015; Shmakov 2015; Lu et al., 2020). But the most stable classification of the group encompasses a monogeneric family comprising only *Woodsia*. A recent study by Lu et al. (2020) proposed to split the family between two genera, *Woodsia* and *Physematium* Kauf., based on a combination of morphological characteristics and phylogenetic relationships, inferred from the analysis of five maternally-inherited chloroplast regions. Lu et al. (2020) justified their taxonomic conclusions using a variety of morphological characteristics, but, upon closer inspection, many of these synapomorphies break down, especially among species from North America (e.g., presence of bicolored stem scales). It is unclear whether the tenuous morphological synapomorphies presented by Lu et al. (2020) justify the recognition of two distinct genera. Thus, for this study, we apply the most stable classification of one genus [*Woodsia* sensu lato (s. l.)].

Woodsia s. l. is primarily circumboreal, including ~43 species worldwide, including 10 species in North America (Rothfels et al., 2012; PPG, 2016). The highest species richness for the genus is in the Rocky Mountains and the Himalayan Mountains (Brown, 1964), where plants grow on or near rocks, especially in crevices on cliffs. They can also be found growing in mats on top of boulders, or on talus slopes at the base of cliffs, usually on weakly basic substrates (Wherry, 1920; Brown, 1964; Windham, 1993a, 1993b). Within *Woodsia*, whole genome duplication is common (Brown 1964; Windham 1993a). Past studies have identified a variety of polyploid and hybrid species within the genus, and have also speculated on the putative parent species of unstudied taxa (Tryon 1948; Butters 1941; Taylor 1947).

Published phylogenies of *Woodsia* s. l., to date, have mostly included species from Asia (Shao et al., 2015; Shmakov 2015; Lu et al., 2020). When species from North America have been included, they are mostly circumboreal, excluding key American endemics. Indeed, several endemic species of *Woodsia* in North America are in need of further study and would benefit from detailed morphological, molecular, and cytogenetic assessment (Larsson, 2014).

Among *Woodsia* species that are endemic to the Americas, many are polyploids, including the putative polyploid hybrid *Woodsia scopulina* subsp. *laurentiana* Windham. The Laurentian Cliff Fern, *W. scopulina* subsp*. laurentiana*, is of particular interest in the Great Lakes region of North America, where it was first recognized by Windham (1993b) based on its disjunct geographic range compared to *W. scopulina* subsp. *scopulina* D.C. Eaton, a putative diploid ($2n = 2x$) progenitor that is mostly confined to the Pacific Northwest (Fig. 2). Windham also observed that disjunct populations on the Laurentian Shield are distinguishable from *W. scopulina* subsp. *scopulina* (in western North America) and *W. scopulina* subsp. *appalachiana* (T. M. C. Taylor) Windham (in southeastern North America) using spore diameter (≥ 50 μm) and rhizome scale coloration; *W. scopulina* subsp. *laurentiana* has weakly bicolorous rhizome scales with a narrow, usually discontinuous, dark central stripe (Fig. 3). Windham (1993b) hypothesized that populations of *W. scopulina* subsp. *laurentiana* from the Laurentian Shield are primarily tetraploid ($2n = 4x$), possibly resulting from hybridization between *Woodsia scopulina* subps. *scopulina* and *Woodsia scopulina* subsp. *appalachiana*, in the Rocky Mountains and the Appalachian Mountains, respectively (Windham,

1993a). Together, these three subspecies make up the *Woodsia scopulina* complex, the focus of this systematic study.

Geographical disjunctions and the Great Lakes region—Population disjunctions occur when the geographic distribution of a given taxon is discontinuous in space. In the Great Lakes region, some notable range disjunctions have been documented. For example, the western Great Lakes share many climatic features with the Pacific Northwest and both regions sustain similar plant communities (Wagner, 1972); small microclimates along Lake Superior and Lake Huron support arctic alpine plants, e.g., *Castilleja septentrionalis* (Northern Paintbrush), *Pinguicula vulgaris* (Butterwort), and *Euphrasia hudsoniana* (Hudson Bay Eyebright) (Butters & Abbe, 1953; Zlonis & Gross 2018); and, plant species from the Great Lakes region are also found in eastern North America, especially in coastal areas, e.g., *Rhexia virginica* (Meadow Beauty) is found in isolated populations near the Great Lakes and is otherwise found across eastern North America (Reznicek, 1994). A variety of historical factors influence the geographic distribution of modern plant species (Whitehead, 1972). In the Great Lakes region, glaciation has played a pivotal role in shaping the spatial distribution of plant communities today, especially the cycling of Quaternary glacial/interglacial cycles (Weiss & Ferrand, 2007).

During the last glacial maximum (LGM) roughly 21,500 years ago, sea level decreased as a large portion of North America was covered by ice (Clark et al., 2009). The ice moved southward during this time and many species were pushed into more southern locations, permanently altering the regional flora (Stewart & Dalen, 2008). As the ice retreated after the LGM, plant communities migrated northward, occupying newly

exposed terrestrial environments. Fluctuations in temperature and precipitation during the glacial retreat caused dramatic habitat shifts, leading, in some cases, to plant populations that were at one time contiguous becoming geographically separated (Loehle, 2007).

Research objective—This study aims to examine the evolutionary origin and biogeographical history of the Laurentian Cliff Fern, *Woodsia scopulina* subsp. *laurentiana*, in the Great Lakes region, using a combined systematic approach. To this end, I united geospatial, environmental, genetic (maternal), and cytogenetic data to examine this fern from the Great Lakes region. First, I used niche modeling to estimate the current and historical range of *W. scopulina* subsp*. laurentiana* and its putative progenitors (*W. scopulina* subsp. *scopulina* and *W. scopulina* subsp. *appalachiana*)*.* Second, I amplified and sequenced three chloroplast genes (*trnG–trnR* intergenic spacer, *atpA*, and *rbcL*) to build a robust, well-supported phylogenetic hypothesis for the *W. scopulina complex*, giving special consideration to the origin(s) of *W. scopulina* subsp. *laurentiana*. Finally, I integrated my newly-generated molecular sequences (*trnG–trnR*) for the *W. scopulina* complex, from the Great Lakes region and across North America, with previously published fossil and molecular data to estimate the timing of diversification for complex as a whole.

<div align="center">**Methods**</div>

Historical niche modeling

  Study species and occurrence records—Specific localities were extracted from verified specimens borrowed on loan from four North American herbaria (MINN, DUL, MICH, LDKH). Each specimen was closely examined to confirm species identification; I avoided sampling duplicate specimens, considering them to be redundant. In total, three taxa and 134 voucher specimens for members of the *W. scopulina* complex were incorporated into my analyses, including *W. scopulina* subsp. *scopulina* (95 specimens)*, W. scopulina* subsp. *laurentiana* (21 specimens), and *W. scopulina* subsp. *appalachiana* (18 specimens) (Fig. 4).

  Geospatial extent—The current geographical distribution of the *W. scopulina* complex was considered when defining the spatial extent of my niche modeling analyses (Fig. 2). Subspecies within this complex occur across North America, from the east coast to the west coast (Windham, 1993a). Considering that the LGM pushed many species southward from their current ranges, the spatial extent was inclusive of North America, encompassing the current known distributions as well as the expected range shifts brought on by past climates.

  Environmental layers and selection of climate variables—Climatic layers were obtained from the WorldClim 1.4 dataset (Hijmans, et al., 2005), which includes 19 bioclimatic variables: mean annual temperature (bio1), mean diurnal range (bio2), isothermality (bio3), temperature seasonality (bio4), maximum temperature of the warmest month (bio5), minimum temperature of the coldest month (bio6), annual temperature range (bio7), mean temperature of the wettest (bio8) and driest quarter

(bio9), mean temperature of the warmest (bio10) and coldest quarter (bio11), annual precipitation (bio12), precipitation of the wettest (bio13) and driest month (bio14), precipitation seasonality (bio15), precipitation of the wettest (bio16) and driest quarter (bio17), and precipitation of the warmest (bio18) and coldest quarter (bio19). For these variables, data were extracted at all specimen-verified specific localities (1) for current conditions, (2) for the LGM (21,000 years ago), and (3) for the Mid-Holocene (6,000 years ago). All bioclimatic layers were taken at the same spatial resolution of 2.5 arc-seconds. A correlation analysis was then conducted for all layers using ENMTools (version 1.4.4), and highly correlated layers (r > 0.80) were discarded (Warren et al., 2010).

Environmental niche modeling—Following correlation analysis, I created species distribution models within MaxEnt (version 3.4.3) for the three sampled timeframes (current conditions, Mid-Holocene, and the LGM; Phillips et al., 2006). For each model, 25% of the presence data for each taxon were targeted as the 'test data' and MaxEnt was run using default parameter values, with three exceptions: the 'replicated run type' was set to subsample, the replicate number was changed to five, and the maximum iterations was set to 5,000.

Chromosome counts and spore measurements

Spore diameter measurements and ploidy estimation—Measurements of spore diameter are a 'tried and true' indicator of ploidy level in ferns (Barrington et al., 1986). To assess ploidy level and confirm the identification of sampled taxa, spores were measured for all members of the *W. scopulina* complex. Specifically, for each individual

sampled, spores were removed from intact sporangia with a dissecting pin, placed on a slide in glycerol, photographed, and measured. These measurements were then transformed to ploidy level using karyotype-calibrated measurements of spore diameter from several members of the genus.

Chromosome counts—To determine the chromosome number of *W. scopulina* subsp. *laurentiana*, developing fronds with young sporangia (mostly but not completely opaque) were collected in the field and pickled in a 3:1 mix of 95% ethanol and glacial acetic acid, preserved on ice for 24–36 hours. After a minimum of 24 hours, pickled leaf fragments were transferred into 70% ethanol for long-term, cold storage. Pickled tissue was prepared in Hoyer's medium, stained with 50% acetocarmine, and pressed between a slide and coverslip to disperse the stained, condensed chromosomes. Karyotype preparations were then examined using a Nikon Eclipse CiL Phase Microscope with LED Illumination and photographed with a trinocular Nikon Photometrics Dyno CCD camera.

Molecular and phylogenetic analyses

Taxon sampling—In total, 25 freshly-collected and herbarium-dried specimens of *Woodsia* were sampled for phylogenetic analyses, along with one accession of *Cystopteris fragilis* from Genbank (Larsson 21) that served as an outgroup. Specimens representing members of the *W. scopulina* complex were sampled broadly to maximize sampling of their geographic extent: *W. scopulina* subsp. *appalachiana* (2 samples), *W. scopulina* subsp. *scopulina* (3 samples), *W. scopulina* subsp. *laurentiana* (7 samples). Additional species of *Woodsia* from the Great Lakes region were also sampled: *W.*

ilvensis (L.) Brown, *W. cathcartiana* B. L. Rob, *W. oregana* D. C. Eaton, *W. × abbeae*

Butters (Appendix 1).

DNA extraction, amplification, and sequencing—In the field, small fragments of

leaf tissue (~1 cm^2) were removed from freshly-collected leaf material and preserved in

silica desiccant. Whole genomic DNA was extracted from silica-dried and herbarium-

preserved leaf tissue using the cetyl trimethyl-ammonium bromide (CTAB) procedure

(Doyle & Doyle, 1978, as modified by D. Barrington) and using the DNeasy Plant Mini

Kit (Qiagen).

Three chloroplast loci (*atpA*, *rbcL*, and the intergeneric spacer *trnG-trnR*) were

amplified for all 25 accessions using the polymerase chain reaction (PCR) (Table 1). PCR

reactions were conducted on a Veriti 96-well, gradient thermal cycler (Applied

Biosystems) with reactions [comprising ~10–50 ng whole genomic DNA, Phusion High-

Fidelity Master Mix (Thermo Scientific), and forward and reverse PCR primers] that

entailed an initial denaturation step (98°C for 2 min) followed by 35 denaturation,

annealing, and elongation cycles (98°C for 30 sec, 45°C for 30 sec, and 72°C for 1 min),

and a final elongation step (72°C for 10 min). Amplicons were visualized on a 1%

agarose gel. The amplified PCR products were cleaned using ExoSAP-IT PCR Product

Cleanup (Applied Biosystems). Cleaned samples were sent to the Minnesota Genomics

Center for Sanger Sequencing on an Applied Biosystems 3730xl DNA Analyzer.

Sequence alignment and phylogenetic analyses—Sequence chromatograms were

visualized, assembled into contigs, and manually verified using Geneious Prime 2020.2.4

(https://www.geneious.com). Cleaned sequences for each locus were initially aligned

using Clustal Omega 1.2.3 within Geneious, followed by manual curation and exclusion

of ambiguous regions. In total, four alignments were generated for downstream

phylogenetic analyses: three single-locus alignments (one for each chloroplast region

sequences) and a concatenated, three-gene, "all-in" alignment. Each alignment was

analyzed using a maximum likelihood (ML) optimality criterion as implemented in

RaxML 8.2.11., with 1000 bootstrap replicates and a GTR+G model of sequence

evolution. Phylogenies for each single-locus analysis were then compared to identify any

well-supported topological conflicts.

Time-calibration and molecular dating—Next, newly-generated sequence data

(*trnG–trnR*) for the *W. scopulina* complex were combined with previously published

fossil and molecular data spanning the large eupolypods II clade, nested within which is

Woodsiaceae (Larsson 2014, and Rothfels et al., 2012). I estimated the node ages for

Woodsia s. l. using BEAST 2.6.3 (Bouckaert et al., 2019), following the same

methodologies as, and including data from, Rothfels et al. (2012) and Larsson (2014).

Fossil calibrations for our newly-generated *trnG–trnR* phylogeny were based on age

estimates for six nodes deep in the fern phylogeny (Schuettpeltz & Pryer, 2009), not

including members of the Woodsiaceae (or close relatives), for which there is insufficient

fossil evidence available; these node ages are: root of tree = 165.6 Mega annum (Ma),

Dennstaedtiaceae =119.3 Ma, Eupolypods =116.7 Ma, Pteridaceae =110.8 Ma,

Eupolypods II = 103.1 Ma, and Eupolypods I =98.9 Ma. The age parameters were given a

normal distribution with a standard deviation of 10% of the estimated mean ages. The

analysis was performed using a relaxed molecular clock model with the following

settings: birth-death prior, log-normal uncorrelated relaxed clock, and GTR+I+Γ

substitution model. All other priors were set to default values, except the node ages

previously described.

Results

Historical niche modeling

After assessing correlation among environmental variables, all layers except bio1, bio2, bio7, bio8, bio12, bio14, bio15, and elevation were discarded due to high correlation. Before discarding each environmental layer, the ecological impacts tied to the organism was considered, to ensure that no biologically important layers were discarded in error.

For each of the three subspecies comprising the *W. scopulina* complex, the remaining climatic variables were used to build models corresponding to three independent time points: Current, Mid-Holocene, and LGM (Fig. 4). The 'Current' time point model exposes climatic conditions across a geographic range that is mostly overlapping with the present-day distribution reported for each subspecies (Fig. 2). Climatic distributions from the Mid-Holocene time point, approximately 6000 years ago, are, likewise, consistent with current estimates across the focal taxa. Overall, during the LGM, climatic models for each member of the *W. scopulina* complex identify a more southern distribution of associated climatic conditions, relative to Current. suitable habitat for *W. scopulina* subsp. *scopulina* was detected south of its present-day range, including disjunct regions in the western and southeastern United States; for *W. scopulina* subsp. *laurentiana*, suitable habitat at the LGM was detected south of the Great Lakes region, with high probabilities for a range overlapping with the present-day southern United States, and along the Pacific coast of North America; the inferred LGM range of *W. scopulina* subsp. *appalachiana* is south of its current range, with high probabilities in present-day regions of Central America and along the Pacific coast of British Columbia.

Chromosome counts and spore measurements

For this study, karyotype was newly-obtained from one sample of *W. scopulina* subsp. *laurentiana* (Zenzen 46) from northern Minnesota, revealing this subspecies to be a sexual tetraploid ($2n = 4x$) (Fig. 5). Two additional chromosome squashes of *W. scopulina*, provided by Dr. Michael Windham (pers. comm.), were also consulted, including diploid ($2n = 2x = 76$ chromosomes) and tetraploid ($2n = 4x = 152$ chromosomes) accessions. These karyotypes allowed us to directly assess ploidy for individuals sampled, and also provided a valuable proxy for inferring ploidy level in other samples representing these (and closely-related) species, using chromosome-calibrated measurements of spore diameter. For the *W. scopulina* complex, spore diameter was measured for 29 fertile specimens. The average spore size for each taxon is as follows: *W. scopulina* subsp. *scopulina* is 42.6 μm, *W. scopulina* subsp. *laurentiana* is 52.3 μm, and *W. scopulina* subsp. *appalachiana* is 42.0 μm (Table 1).

Molecular and phylogenetic analyses

Three-gene plastid phylogeny—Three chloroplast loci (*atpA*, *trnG-trnR*, and *rbcL*) were newly amplified and sequenced for 18 accessions (Appendix 1). Phylogenetic hypotheses were then generated for four data sets using maximum likelihood bootstrap analyses (MLBS): comparison of topologies among individual loci showed no well-supported conflicts. Maximum likelihood analysis of the concatenated, three-gene alignment revealed a well-supported and (mostly) resolved phylogeny of the *W.*

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scopulina complex and its close relatives from the Great Lakes region (Fig. 6). The first bifurcation in the recovered phylogeny distinguishes a well-supported clade (MLBS 100%) comprising all samples of *W. ilvensis* from a maximally-supported (MLBS 100%) clade containing all other *Woodsia* species sampled split between two well-supported (MLBS 100%) sister clades: one clade consists of *W. oregana* and *W. cathcartiana*, which are, together, sister to *W. × abbeae* (MLBS = 78%), and another clade that includes all samples representing the *W. scopulina* complex, within which *W. scopulina* subsp. *appalachiana* is resolved as sister to a clade comprising all samples of *W. scopulina* subsp. *laurentiana* and *W. scopulina* subsp. *scopulina*, with strong support (MLBS = 89%). Within the '*scopulina-laurentiana*' clade, a diploid ($2n$ = 2x) sample of *W. scopulina* subsp. *scopulina* from Idaho is recovered as sister to all other samples (MLBS = 75%). There is no statistical support to distinguish sequences of *W. scopulina* subsp. *scopulina* from samples of *W. scopulina* subsp. *laurentiana*, with the exception of two sexual diploid samples of *W. scopulina* subsp. *scopulina* from Colorado and British Columbia that form a clade (MLBS = 71%).

Time-calibrated phylogeny

Time-calibrated molecular phylogeny—Newly-sequenced samples and previously published data from Larsson (2014) were combined to infer a fossil-calibrated molecular phylogeny with all of the samples examined in this study (Appendix 2). In Figure 6, I present a subset of this chronogram that includes the *W. scopulina* complex, for which the median estimated age of the split from the sister group [containing other American *Woodsia*: *W. mollis* (Kaulf.) J. Sm., *W. montevidensis* (Spreng.) Hieron, and *W.*

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canescens (Kunze) Mett.)] is 11.37 Ma (± 5–20 Ma, 95% HPD; Fig. 7). The chronogram

in Figure 6 also indicates the geographic region where each taxon can be found.

Discussion

The purpose of this study was to test specific hypotheses proposed by Windham (1993b) regarding the evolutionary and biogeographical history of the Laurentian Cliff Fern, *Woodsia scopulina* subsp. *laurentiana*, in the Great Lakes region. To elucidate the evolutionary and geographic origins of this taxon, I used a combined systematic approach. Specifically, I compiled geospatial, environmental (climatic), genetic (maternal), and cytogenetic data from freshly-collected and herbarium-preserved specimens to ask: (1) How did historical climate impact past [Mid-Holocene and Last Glacial Maximum (LGM)] and current distributions of *W. scopulina* subsp. *laurentiana* and its putative parents? (2) Does karyotype confirm estimates of ploidy in *W. scopulina* subsp. *laurentiana* that were inferred previously from spore diameter measurements? and (3) What species is/are the maternal progenitor(s) of *W. scopulina* subsp. *laurentiana* and when did the *W. scopulina* complex initially radiate?

Historical niche modeling

Subspecies comprising the *W. scopulina* complex are distributed across distinct regions of North America: in the Rocky Mountains (diploid *W. scopulina* subsp. *scopulina* and tetraploid *W. scopulina* subsp. *laurentiana*), the Appalachian Mountains (diploid *W. scopulina* subsp. *appalachiana*), and around the Great Lakes (tetraploid *W. scopulina* subsp. *laurentiana*). In this study, I leveraged verified specimens of all three subspecies to model current and historical environmental niche, using climatic variables as a proxy. The goal of historical niche modelling (conducted in MaxEnt) was to provide a visual representation of how historical climate may have shaped the current distribution

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of focal taxa. MaxEnt was chosen for this study in particular because it performs well, even when few presence records are available (Pearson et al., 2007; Ferrer-Sanchez & Rodriguez-Estrella, 2016). Because two of the subspecies examined in this study are relatively rare (*W. scopulina* subps. *laurentiana* and *W. scopulina* subsp. *appalachiana*), MaxEnt was helpful for inferring probable habitats with our climatic modeling approach. By far, one of the most powerful historical events that has shaped modern plant communities across North America are the glacial/interglacial cycles (Weiss and Ferrand, 2007). In this study, I mapped the distribution of suitable climatic envelopes over the most recent glacial cycle, using data for three time points: the LGM (ca. 20,000 years ago), the Mid-Holocene (ca. 6,000 years ago), and Current.

The general trend observed for members of the *W. scopulina* complex is a northward shift in suitable climatic conditions since the LGM. For *W. scopulina* subsp. *appalachiana*, suitable climatic conditions during the LGM were only inferred within Central America and the Pacific Northwest. The niche model for *W. scopulina* subsp. *laurentiana* at the LGM—when glaciers covered most of its modern-day habitat— identified an abundance of probable areas in the southern United States. Once the glaciers receded, during the Mid-Holocene, the probable habitat of *W. scopulina* subsp. *laurentiana* moved northward, ultimately to where modern populations reside, with the total area of optimal climatic niche narrowing in size.

The true distribution of any taxon is limited by dispersal and other conditions, e.g., microhabitat. Microhabitat is especially important for ferns because they have two independent, free-living stages to their life cycle, the sporophyte ($2n$) and gametophyte (n). The sporophyte is the dominate stage, where meiosis takes place to generate haploid

spores. Haploid spores then develop into gametophytes, upon which the gametes are produced and where reproduction occurs. Gametophytes require water, in part because flagellated sperm swim through water to meet and fertilize the egg, and also because the gametophyte is very thin, sometimes only one cell layer thick (Haufler et al., 2016). Along with dispersal of sperm and avoiding desiccation, *Woodsia* collectors have noted that distinct species can be found growing on specific rock types (Wherry, 1920; Brown 1964).

Chromosome counts and spore measurements

 Spore diameter measurements provide an easy and efficient way to assess whole genome duplication (i.e., polyploidy) in ferns (Barrington et al., 1986), but measurements must be calibrated with a closely-related cytogenetic voucher, i.e., karyotype. Here, I leveraged new (*W. scopulina* subsp. *laurentiana*) and existing (*W. scopulina* subsp. *scopulina*; M. D. Windham, pers. comm.) chromosome counts to assess whether karyotype confirms previous estimates of ploidy for *W. scopulina* subsp. *laurentiana* inferred using spore diameter (Windham, 1993b). *Woodsia* can be difficult material to work with when preparing a chromosome squash, as they have relatively few sporangia that are each covered with a filamentous indusium. Our newly-presented chromosome count for *W. scopulina* subsp. *laurentiana* (Fig. 5) reveals that material procured from Cook County, MN has ~ 152 chromosomes, and is, thus, a tetraploid ($2n = 4x$). This new count is the first to confirm Windham's hypothesis, based on spore diameter, that populations of *W. scopulina* in the Great Lakes region are tetraploid. This information, combined with the two squashes that were generously provided by Dr. Michael Windham

allowed us to estimate ploidy level for all samples in our phylogenetic analyses, using spore diameter as a proxy. The published spore diameter ranges for members of the *W. scopulina* complex are: *W. scopulina* subsp. *scopulina*, 42–50 μm; *W. scopulina* subsp. *laurentiana*, 50–57 μm, and *W. scopulina* subsp. *appalachiana*, 39–46 μm (Windham, 1993a), which are consistent with our newly-calibrated estimates for these three taxa (Table 1).

Three-gene chloroplast phylogeny

The best maximum likelihood topology inferred from our phylogenetic analysis of the three-gene (*atpA*, *rbcL*, *trnG-trnR*) dataset revealed an early split between *W. scopulina* subsp. *appalachiana* and the other two members of the *W. scopulina* complex (Fig. 6). Furthermore, all specimens of *W. scopulina* subsp. *scopulina* and *W. scopulina* subsp. *laurentiana* sampled form a monophyletic clade, with strong support (MLBS 89%; Fig. 6). This reveals that *W. scopulina* subsp. *scopulina* is the chloroplast donor and, thus, maternal progenitor of tetraploid *W. scopulina* subsp. *laurentiana* on the Laurentian Shield. Our best maximum likelihood phylogeny is consistent with a single origin for *W. scopulina* subsp. *laurentiana.*

One unexpected outcome of including related species of *Woodsia* from the Great Lakes region was to reveal the maternal parent of the putative hybrid *W. × abbeae* sampled in our analysis. A prior study by Wagner (1987) proposed that the parent species of *W. × abbeae* co-occur in the Great Lakes region, a diploid *W. ilvensis* and a tetraploid *W. cathcartiana*. Wagner based this conclusion on his examination of morphological features, as well as chromosome numbers, for *W. scopulina*, *W. ilvensis*, and *W.*

cathcartiana. Previous authors, however, hypothesized *W. × abbeae* to be a cross between *W. scopulina* and *W. ilvensis*, based on morphological traits (Tryon, 1948; Brown 1964). All four of Wagner's focal taxa were included in our phylogenetic analyses, revealing that *W. × abbeae* is sister to a clade containing *W. oregana* and *W. cathcartiana* (Fig. 6), not nested within the *W. scopulina* complex (Fig. 6), consistent with Wagner's hypothesis. Nuclear data is required to confirm the other DNA contributor to *W. × abbeae*, and fully support Wagner's full conclusion of parentage.

Time-calibrated phylogeny

Previous molecular dating of *Woodsia* s. l. did not include the three taxa comprising the *W. scopulina* complex (Larsson, 2014). Samples generated in this study, therefore, improve our understanding of the evolutionary history and timing of diversification for species in the focal clade. My fossil-calibrated molecular phylogeny reveals an estimated age of 11.37 Mya (± 5–20 Ma, 95% HPD) for the divergence of the *W. scopulina* complex from other North American taxa. However, given the lack of fossil calibrations within the focal genus (or even the Woodsiaceae), I recommend that these age estimates be interpreted with caution.

Future directions

In this study, I united multiple systematic approaches to explore the evolutionary origin(s) and biogeographic provenance of *W. scopulina* subsp. *laurentiana*, a rare, endemic species of Cliff Fern in the Great Lakes region of North America. Notably, throughout the course of conducting this study, we revisited many specimen-verified,

historical localities for *W. scopulina* subsp. *laurentiana* (including sites mentioned in the original taxonomic description), but populations were rarely encountered and were presumed to be locally extirpated. Instead, in its place, we frequently found robust populations of the geographically widespread and morphologically variable species *W. ilvensis*, possibly indicating that the latter is undergoing a range expansion. For this reason, historical specimens were paramount for gathering morphological and environmental data on this species.

In the future, sequencing of biparentally-inherited nuclear regions should be used to determine the paternal genome donor(s) of *W. scopulina* subsp. *laurentiana* in the Great Lakes region. To accomplish this, one obstacle that must be overcome is dealing with multiple gene copies in this confirmed tetraploid. Multiple methods have been used in recent years, with varying success, to evaluate biparental inheritance in polyploid ferns. Early studies used isozyme data as a trusted (albeit potentially harmful) technique for identifying autopolyploid and allopolyploid lineages (Roose & Gottlieb, 1976; Werth et al., 1985; Haufler et al., 1990; Pryer & Haufler, 1993). The use of isozymes declined with the increasing popularity of DNA-sequencing, and then molecular cloning became a favored, but expensive and labor-intensive approach for examining the nucleotide sequence of duplicated nuclear regions in polyploids (e.g., Grusz et al., 2009; Dufresne et al., 2014). With the advent of next-generation sequencing came a variety of new, cost-effective methods for sequencing biparentally-inherited loci in polyploid systems, e.g., leveraging long-read sequencing platforms to generate data relatively quickly and cheaply (Rothfels et al., 2017), though with varying degrees of success (data not shown). More recently, probe-based approaches have been introduced that are reliable for testing

the inheritance of nuclear genes in polyploids like *W. scopulina* subsp. *laurentiana*, such as the GoFlag probe set (Breinholdt et al., 2021), which has shown increasing promise for the phasing of duplicated alleles among low-copy nuclear loci in polyploid ferns.
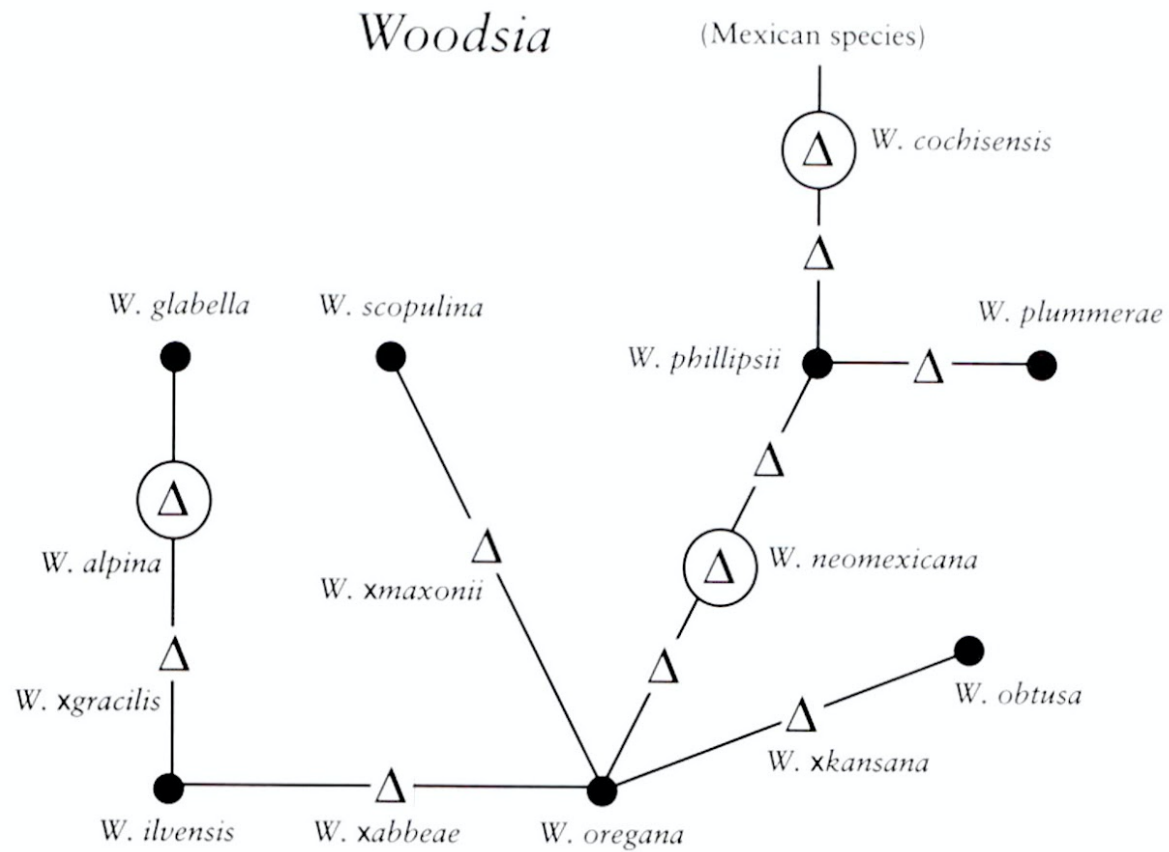
Relationships in *Woodsia*. Solid circles represent parental taxa; circled triangles represent allotetra-ploids; and triangles represent sterile hybrids.

Figure 1: Relationships in *Woodsia*. From *Woodsia*: Flora of North America, Volume 2 (Windham, 1993a).
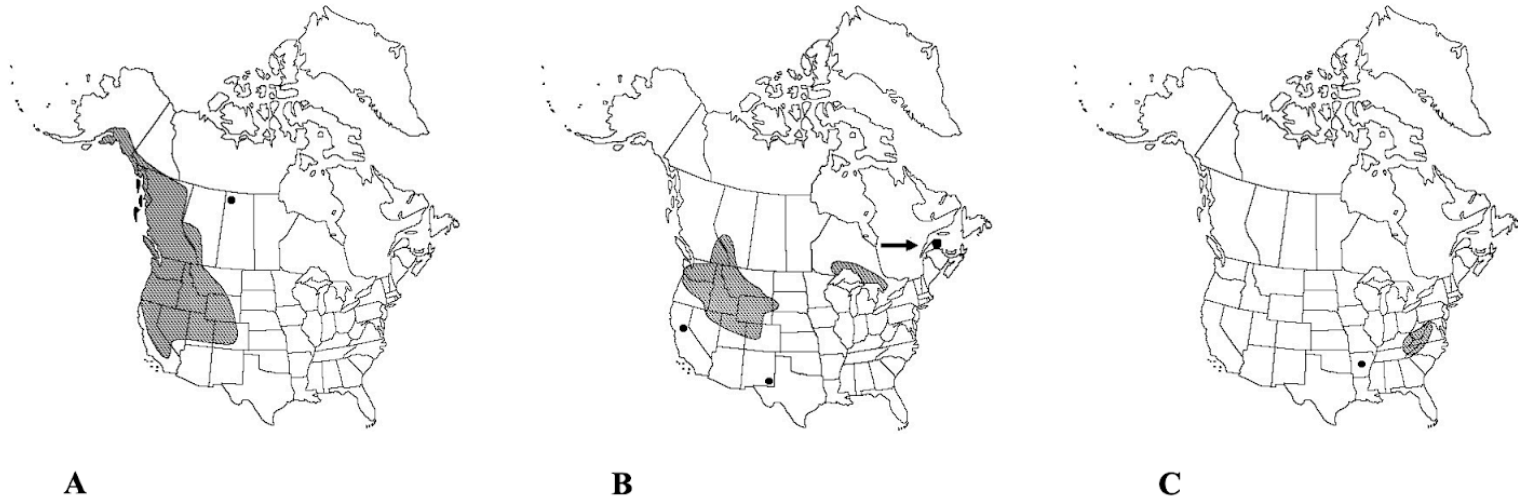
Figure 2: A) Distribution map of *Woodsia scopulina* subsp. *scopuliana*. **B)** Distribution of *Woodsia scopulina* subsp. *laurentiana*. **C)** Distribution of *Woodsia scopulina* subsp. *appalachiana.* Species distribution maps from *Woodsia*: Flora of North America, Volume 2 (Windham, 1993a).
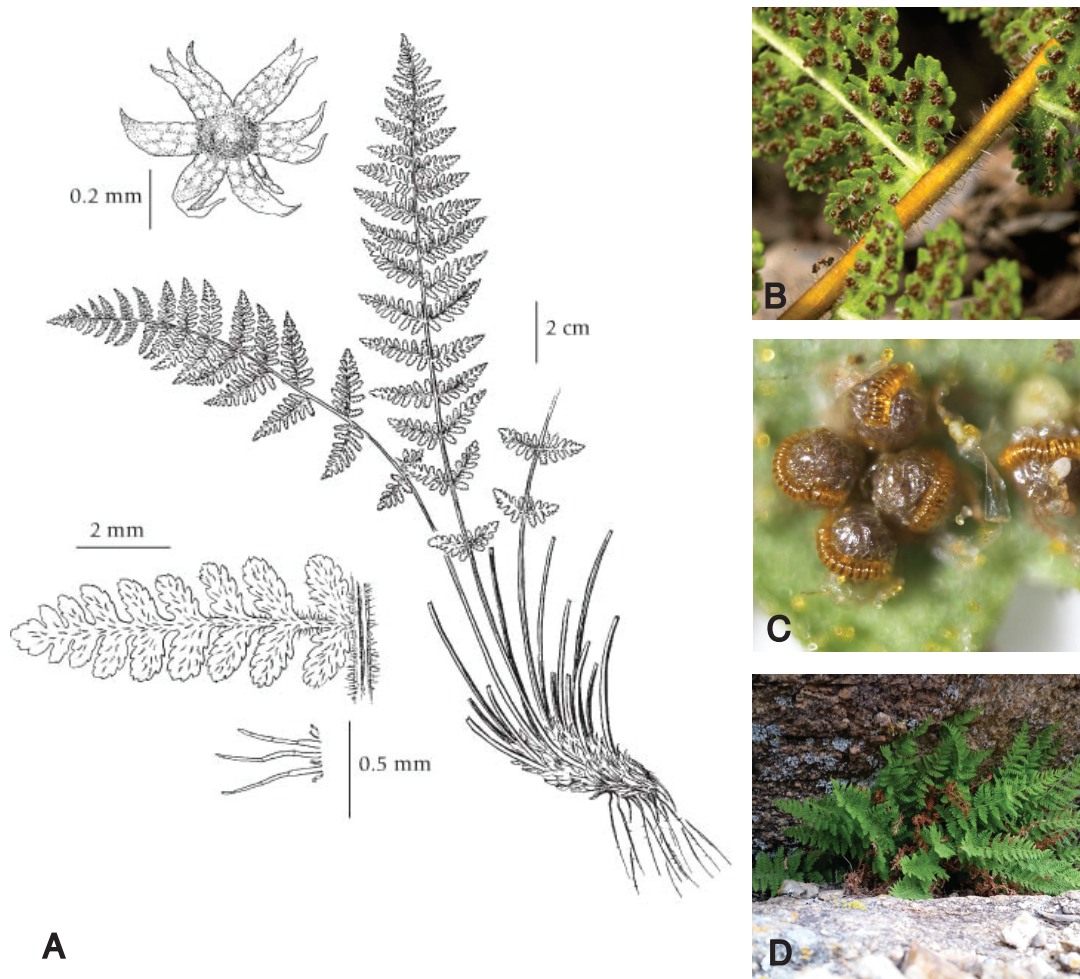
Figure 3: Characteristics of *Woodsia scopulina*. **A)** Overview of characteristics. From *Woodsia*: Flora of North America, Volume 2 (Windham, 1993a). **B)** Stipe of *Woodsia scopulina*. **C)** Sporangia of *Woodsia scopulina*. **D)** Habit of *Woodsia scopulina*.
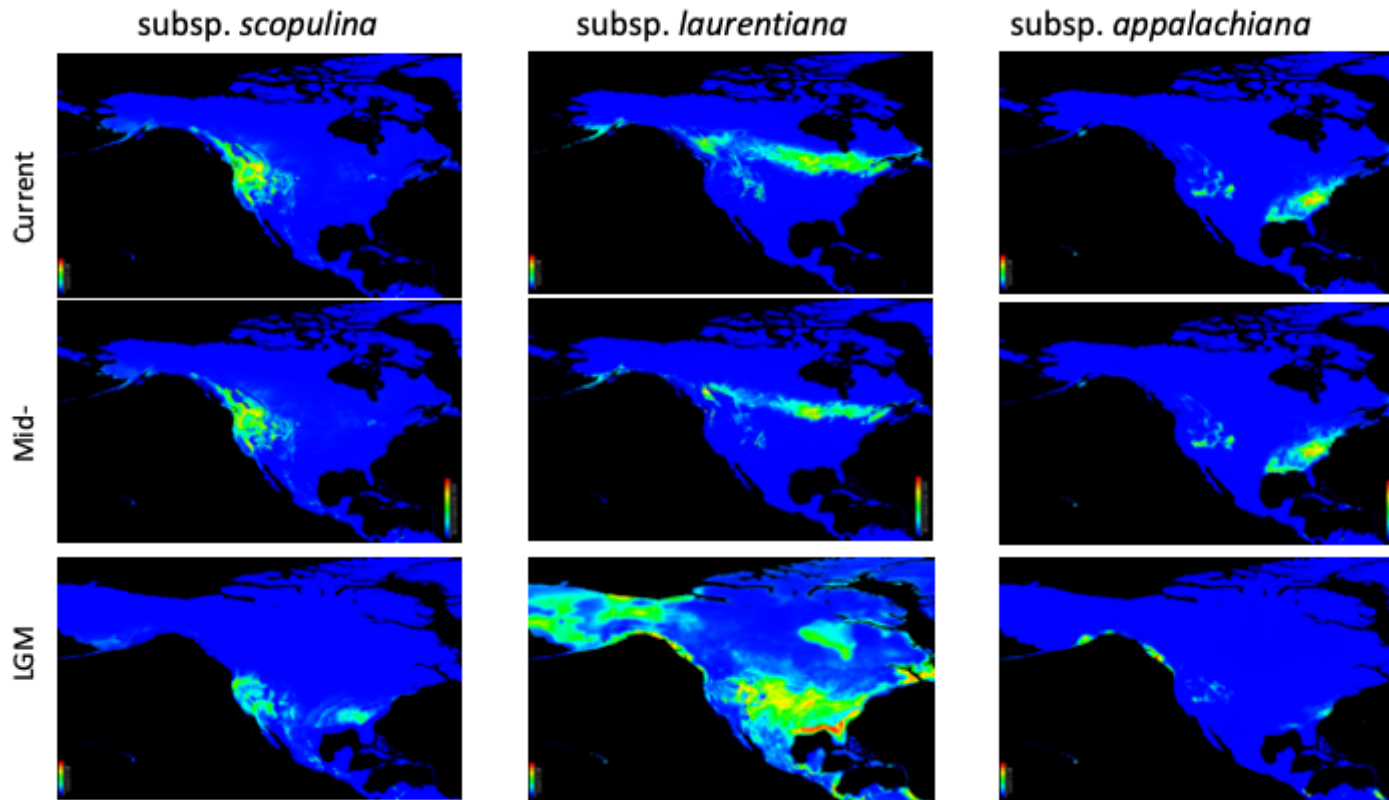
Figure 4: MaxEnt distribution maps for subspecies of the *W. scopulina* complex for three time points. Columns, from left to right: *W. scopulina* subsp. *scopulina* (n = 95), *W. scopulina* subsp. *laurentiana* (n = 21), and *W. scopulina* subsp. *scopulina* (n = 18). Bottom row: Last Glacial Maximum (LGM). Center row: Mid-Holocene (Mid). Top row: Present-day (Current). Scale ranges from 0–1, with 1 (red) indicating the most likely habitats, inferred from global climatic variables.
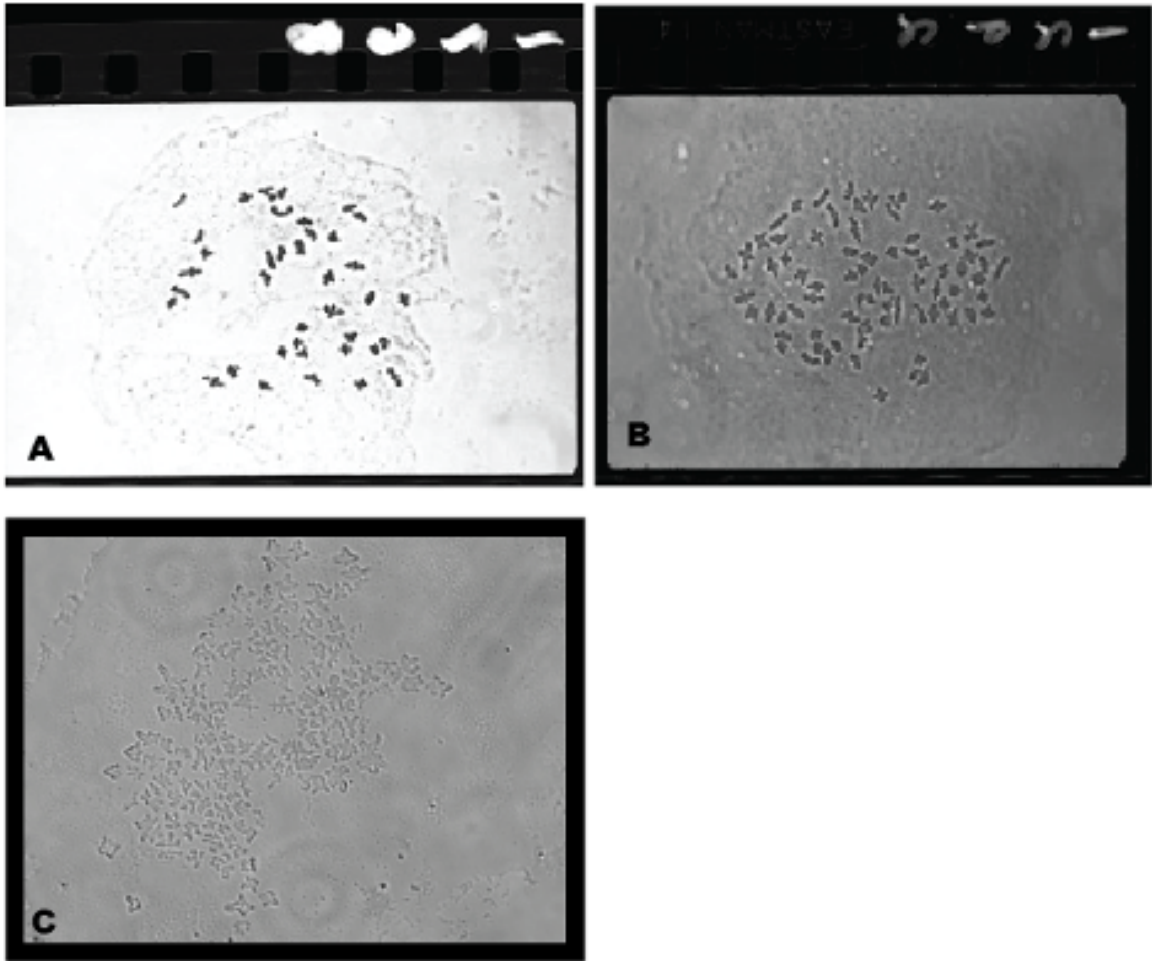
Figure 5: A) Karyotype of *Woodsia scopulina* subsp. *scopulina* ($2n = 2x = 76$) from Taylor River, Gunnison Co., CO. Provided by Dr. Michael Windham. **B)** Karyotype of *Woodsia scopulina* subsp. *laurentiana* ($2n = 4x = 152$) from Kootenai, Lincoln County, MT. Provided by Dr. Michael Windham. **C)** Newly-generated karyotype of *Woodsia scopulina* subsp. *laurentiana* ($2n = 4x \cong 152$) from Mt. Rose, Lake County, MN, USA.

Table 1: Spore diameter (μm) for members of the *W. scopulina* complex.

Taxon	Average diameter [s.d]	Observed limits	Sample size	Ploidy
W. scopulina subsp. *scopulina*	42.6 [1.6]	41.0–45.3	n = 8	2x
W. scopulina subsp. *laurentiana*	52.3 [2.3]	47.7–56.0	n = 16	4x
W. scopulina subsp. *appalachiana*	42.0 [1.9]	39.5–44.0	n = 5	2x

Table 2: Primers used in DNA amplification and sequencing.

DNA Region	Primer	5′–3′ Primer Sequence	Usage*	Primer Source
trnG–trnR	trnGIF	GCGGGTATAGTTTAGTGGtAA	A, S	Nagalingum et al. 2007
trnG–trnR	trnG353F	TTGCTTMTAYGACTCGGTG	S	Nagalingum et al. 2007
trnG–trnR	trnG63R	GCGGGAATCGAACCCGCATCA	S	Nagalingum et al.2007
trnG–trnR	trnR22R	CTATCCATTAGACGATGGACG	A, S	Metzgar et al. 2007
atpA	ESATPA412F	GARCARGTTCGACAGCAAGT	A, S	Schuettpelz et al. 2006
atpA	ESTRNR46F	GTATAGGTTCRARTCCTATTGGACG	A, S	Schuettpelz et al. 2006
atpA	ESATPA557R	ATTGTATCTGTAGCTACTGC	S	Schuettpelz et al. 2006
atpA	ESATPA856F	CGAGAAGCATATCCGGGAGATG	S	Schuettpelz et al. 2006
atpA	ESATPA877R	CATCTCCCGGATATGCTTCTCG	S	Schuettpelz et al. 2006
atpA	ESATPA535F	ACAGCAGTAGCTACAGATAC	S	Schuettpelz et al. 2006
rbcL	ESRBCLF1	ATGTCACCACAAACGGAGACTAAAGC	A, S	Schuettpelz & Pryer 2007
rbcL	ESRBCL1361R	TCAGGACTCCACTTACTAGCTTCACG	A, S	Schuettpelz & Pryer 2007
rbcL	ESRBCL628F	CCATTYATGCGTTGGAGAGATCG	S	Schuettpelz & Pryer 2007
rbcL	ESRBCL654R	GAARCGATCTCTCCAACGCAT	S	Schuettpelz & Pryer 2007

*A=PCR amplification, S=DNA sequencing reaction
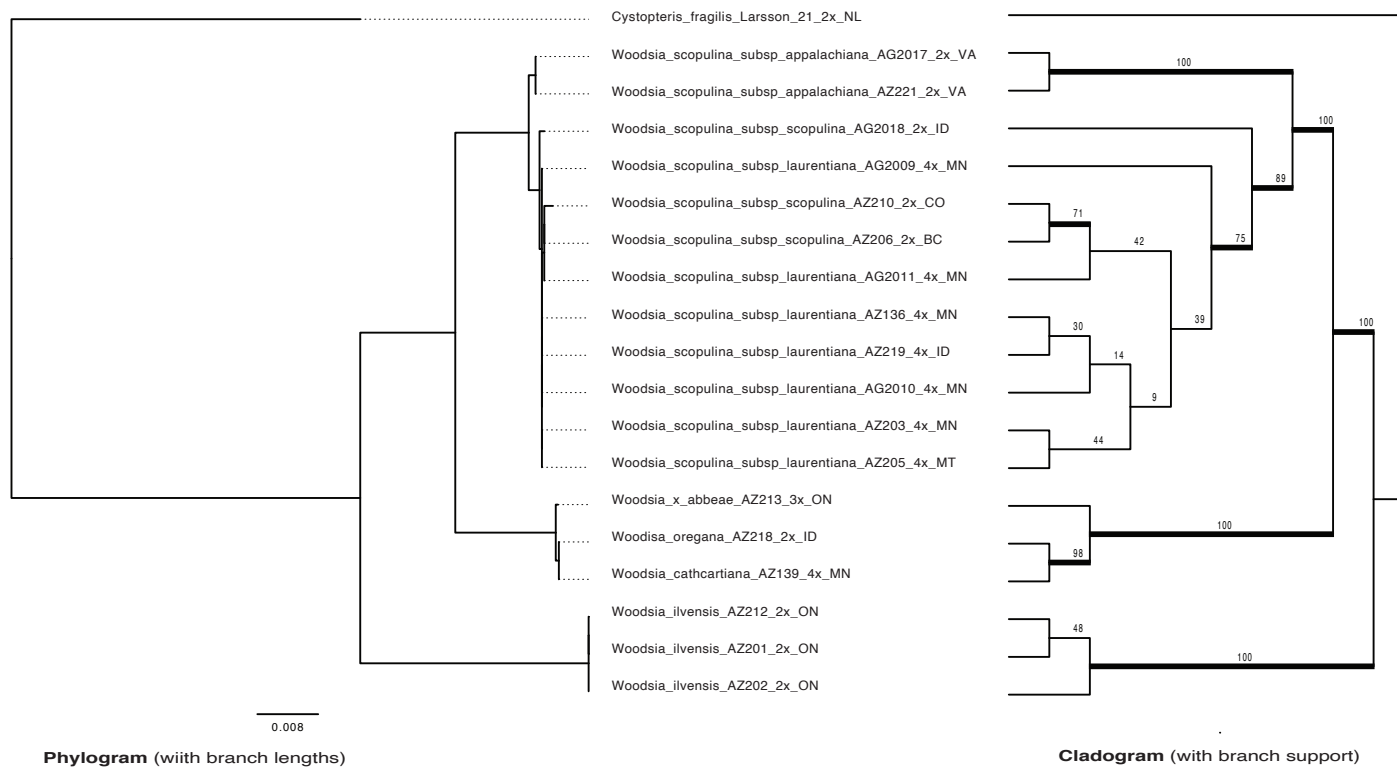
Figure 6: Maximum likelihood best tree for the *Woodsia scopulina* complex (and close relatives) based on the three-gene, concatenated dataset (*rbcL*, *atpA*, and *trnG-trnR*). Presented as a phylogram (left) with branch lengths proportional to substitutions per site, and as a cladogram (right) for presentation of the maximum likelihood bootstrap (MLBS) estimates. Branches with MLBS ≥ 70% are indicated in bold.
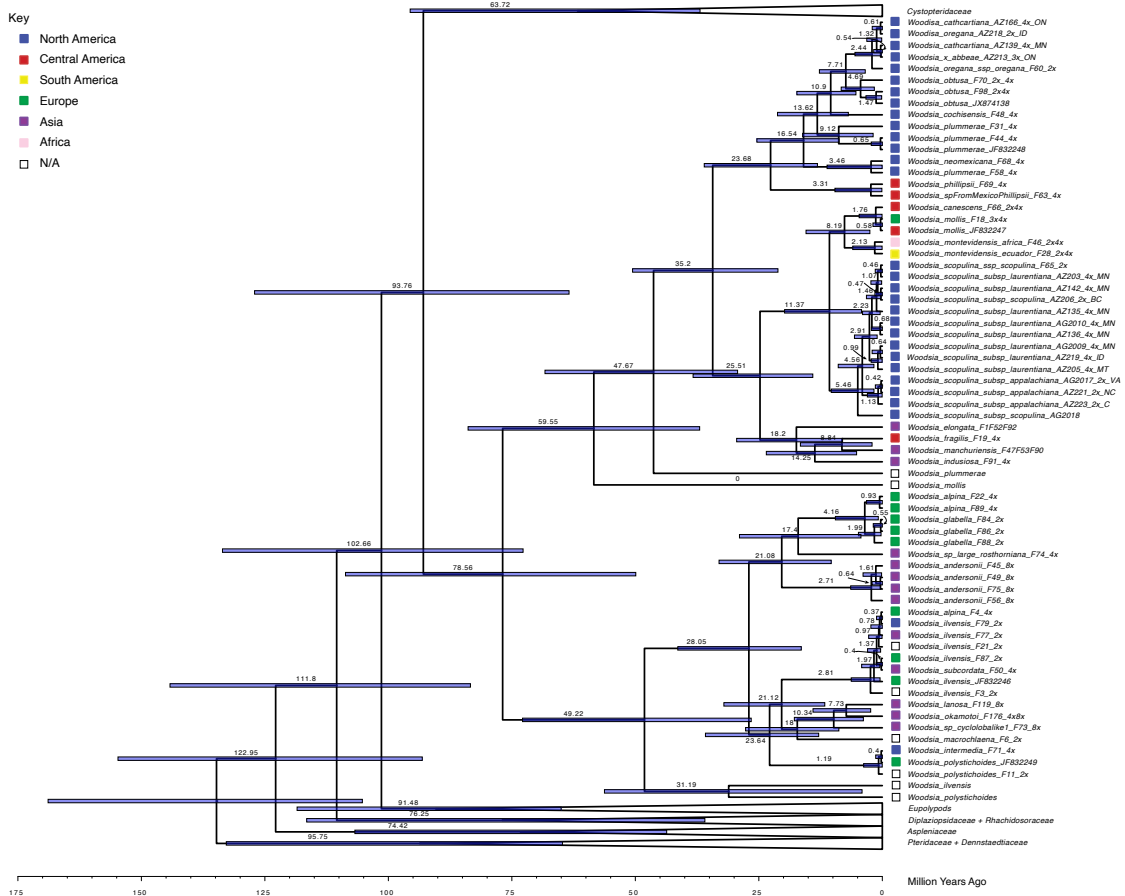
Figure 7: Time-calibrated BEAST phylogeny. Median divergence times, with blue bars representing 95% HPD intervals. Geographic distribution indicated by the color-coded icon next to each sample name. Chronogram derived from combined analysis of samples newly-sequenced here and *trnG–trnR* sequences published previously by Larsson (2014).

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Appendices

Appendix 1: Individuals sampled for DNA sequencing and phylogenetic analyses.

Sample ID	Taxon	Country, State	Ploidy (2*n*)	Collector and #	*rbcL*	*atpA*	*trnG-trnR*
AG2009	*W. scopulina* subsp. *laurentiana*	USA, MN	4x	Grusz 233	x	x	x
AG2010	*W. scopulina* subsp. *laurentiana*	USA, MN	4x	Grusz 234	x	x	x
AG2011	*W. scopulina* subsp. *laurentiana*	USA, MN	4x	Grusz 235	x	x	x
AG2017	*W. scopulina* subsp. *appalachiana*	USA, VA	2x	Windham & Pryer sn	x	x	x
AG2018	*W. scopulina* subsp. *scopulina*	USA, ID	2x	Rothfels 4187	x	x	x
AZ135	*W. scopulina* subsp. *laurentiana*	USA, MN	4x	Gerdes & Converse 741			x
AZ136	*W. scopulina* subsp. *laurentiana*	USA, MN	4x	Gerdes 5786	x	x	x
AZ139	*W. cathcartiana*	USA, MN	4x	Lynden B. Gerdes & Joe W. Walewski 6265	x	x	x
AZ142	*W. scopulina* subsp. *laurentiana*	USA, MN	4x	K. A. Lederle, sn			x
AZ166	*W. cathcartiana*	CAN, ON	4x	P. A. Scott 2824			x
AZ201	*W. ilvensis*	CAN, ON	2x	Zenzen 08	x	x	x
AZ202	*W. ilvensis*	CAN, ON	2x	Zenzen 26	x	x	x
AZ203	*W. scopulina* subsp. *laurentiana*	USA, MN	4x	Zenzen 46	x	x	x

AZ205	*W. scopulina* subsp. *laurentiana*	USA, MT	4x	Sigel 2011-36	x	x	x
AZ206	*W. scopulina* subsp. *scopulina*	CAN, BC	2x	Rothfels 4079.1	x	x	x
AZ210	*W. scopulina* subsp. *scopulina*	USA, CO	2x	S. F. Smith 123	x	x	x
AZ212	*W. ilvensis*	CAN, ON	2x	Zenzen 77	x	x	x
AZ213	*W.* x *abbeae*	CAN, ON	3x	Zenzen 132	x	x	x
AZ218	*W. oregana*	USA, ID	2x	Rothfels 4198	x	x	x
AZ219	*W. scopulina* subsp. *laurentiana*	USA, ID	4x	Rothfels 4194	x	x	x
AZ221	*W. scopulina* subsp. *appalachiana*	USA, VA	2x	Windham 4074	x	x	x
AZ223	*W. scopulina* subsp. *appalachiana*	USA, NC	2x	Windham 4440			x

Appendix 2: All-inclusive BEAST chronogram of *Woodsia*. Median divergence times

provided, with blue bars representing 95% highest posterior density (HPD) intervals.

Chronogram includes newly-generated samples and previously-published sequences

(Larsson, 2014).