

# Evolutionary history of the *Asplenium scolopendrium* complex (Aspleniaceae), a relictual fern with a northern pan-temperate disjunct distribution

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*Asplenium scolopendrium* is distributed in northern temperate forests with many global biogeographic disjunctions. The species complex of *A. scolopendrium* has been generated by spatial segregation coupled with divergent evolution. We elucidated the biogeographic history of the *A. scolopendrium* complex by exploring its origin, dispersal and evolution, thus providing insights into the evolutionary history of the Tertiary floras with northern pan-temperate disjunct distributions. The results revealed that all infraspecific taxa descended from a widely distributed common ancestor in the Northern Hemisphere. This pan-temperate ancestral population formed by unidirectional westward dispersal from European origins primarily during the Early Eocene when the Earth's climate was much warmer than today. The splitting of European, American and East Asian lineages occurred during the Early Miocene due to geo-climatic vicariations. Polyploidy events in the American ancestral populations created additional reproductive barriers. The star-shaped haplotypes in each continent indicated that local disjunctions also led to derived genotypes with potential to diverge into different taxa. This intracontinental lineage splitting is likely related to latitudinal range shift and habitat fragmentation caused by glacial cycles and climate change during the Pleistocene. The evolutionary history of the *A. scolopendrium* complex supported the Boreotropical hypothesis exhibiting range expansion during the Early Eocene Climatic Optimum.

**ADDITIONAL KEYWORDS:** Arcto-Tertiary Geoflora – Boreotropical hypothesis – Early Eocene Climatic Optimum – geo-climatic vicariance – hart's tongue fern – species complex – unidirectional dispersal

## INTRODUCTION

Disjunct distributions of Northern Hemisphere plants are common and reported for a variety of plant taxa (Raven, 1972; Barrington, 1993; Wen, 2001; Deng *et al.*, 2015). The disjunct distribution between eastern Asia and eastern North America, known as the North Pacific pattern, has long been recognized across many related or apparently identical species (Kato & Iwatsuki, 1983; Wen, 2001; Wen *et al.*, 2016). There exists a similar disjunction in the amphi-Atlantic species that inhabit eastern North American and western Europe, a pattern that is apparently ancient and includes evidence from several extant and fossil taxa (Tiffney, 1985). Ferns frequently have global biogeographic disjunctions at lower taxonomic ranks, such as between closely related

species or even varieties (Li, 1952). As one of the most widespread fern groups, the genus *Asplenium* contains several lineages with global disjunct distributions, including *Asplenium septentrionale*, *Asplenium ruta-muraria*, *Asplenium trichomanes* and *Asplenium viride* (Schneider *et al.*, 2004). Although some taxa are thought to have experienced multiple latitudinal shifts between temperate and tropical regions, the aforementioned ferns typically occur in northern temperate forests (Wolfe, 1975; Tiffney & Manchester, 2001; Davis *et al.*, 2002; Schneider *et al.*, 2004).

As many floras and many taxa exhibit similar patterns of disjunct distribution, it is likely that global environmental change has played a significant role in the discontinuity at the community level. The most widely accepted explanation for these modern disjunctions is that they represent remnants of the

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ancestral forest that once extensively covered the Northern Hemisphere during the Palaeogene and Neogene periods (previously known as the Tertiary) (Gardner & Ettinghausen, 1879; Wolfe, 1975). It is presumed that multiple geological and climatic changes, including Quaternary glacial cycles, have caused the range contraction and fragmentation of northern pan-temperate forests. However, there are two different hypotheses regarding the origin of the separate northern temperate floras (Baskin & Baskin, 2016). The Arcto-Tertiary Geoflora concept assumes that modern temperate floras are remnants of the Arcto-Tertiary forest that developed in high latitudes during the Late Cretaceous and Early Tertiary. This forest is thought to have maintained a fairly homogenous floristic composition which gradually expanded southward to mid-latitudes due to a decrease in global temperature and an increase in seasonality. Alternatively, the Boreotropical hypothesis proposes that the modern temperate flora originated from multiple tropical or subtropical floras that expanded northward during a period of equable climate of the Early Eocene (Wolfe, 1975, 1977; Tiffney, 1985). As climate fluctuated between warm and cool, including the Late Oligocene and Miocene, cool-adapted taxa were selected from the Boreotropical flora.

Biogeographic processes that shape species distributions have focused on the two different mechanisms of dispersal and vicariance (Lomolino *et al.*, 2017). Dispersal is the expansion of the distributional area of a taxon. As it operates on different spatial and temporal scales, various forms have been identified, including jump dispersal (dispersal by individuals to very distant areas within a generation, usually followed by a founder effect), diffusion (gradual spread of a population), and secular migration (slow and progressive range expansion over many generations). Alternatively, vicariance is the appearance of a barrier that allows division of the distribution of an ancestral population, after which the fragmented populations may evolve in isolation. Vicariance can occur due to large-scale geological changes such as continental drift, mountain uplift and sea level rise (Keast, 1971; Woodroffe & Grindrod, 1991). Climatic change can also lead to shifts or contractions in species ranges along with differential regional extirpation. The interplay of climate change and other geographic barriers (referred to as geo-climatic barriers) further exacerbates geographic isolation by weakening dispersal. Species have their own biogeographic history involving a variety of forces such as speciation, dispersal, expansion, fragmentation, contraction and/or extinction (Kato, 1993). Therefore, the processes underlying the distribution of a species should be described as a series of events triggered by global

geophysical changes and culminating in a particular phylogenetic history (Barrington, 1993).

Fern biogeography is typically challenging because vicariance patterns can often be conflated with long-distance dispersal (Wolf *et al.*, 2001). Cumulative biogeographic histories of multiple reticulate evolution events, incomplete lineage sorting and differing local extinction patterns can also mask vicariance patterns (Barrington, 1993). Furthermore, an accidental or deliberate dispersal, vicariance or range contraction due to anthropogenic activity must be considered as causes of biogeographic disjunction in some ferns. Nevertheless, advances in molecular approaches have facilitated much more intensive investigations of species' dynamic spatial and temporal distributions (Riddle *et al.*, 2008). For example, studies of maternally inherited chloroplast DNA variations reveal a network of haplotypes, which allows analysis of historical processes that shaped the geographical distribution of a given lineage (Iwasaki *et al.*, 2012; Njuguna *et al.*, 2013). With the help of fossil calibration, a molecular clock can contribute to estimating divergence time, which plays an important role in historical biogeography when integrated with geological, palaeoclimatic and other data (Donoghue & Moore, 2003; Renner, 2005; Qiu *et al.*, 2011). Modelling approaches also allow inference on ancestral population ranges by testing the various modes of biogeographic processes (Matzke, 2013).

*Asplenium scolopendrium* L., commonly known as the hart's tongue fern, has drawn considerable scientific interest due to its unusual distribution and abundance patterns (Cinquemani *et al.*, 1988; Testo & Watkins, 2013; Fernando *et al.*, 2015). As a temperate evergreen fern, *A. scolopendrium* is primarily distributed in the Northern Hemisphere with minor exceptions including recently introduced populations in New Zealand (Brownsey & Perrie, 2018). Latitudinally, it has an intercontinental disjunct distribution across widely separated regions. Three main population clusters occur in Europe, eastern North America and East Asia. Accordingly, these three major lineages are largely isolated by several major geographic barriers: the Atlantic Ocean between Europe and America; the Ural Mountains and the Eurasian steppe between Europe and East Asia; and the Pacific Ocean and the Bering Strait between North America and East Asia. Moreover, these biogeographical disjunctions appear related to polyploidy events in tandem with geographical segregation as the European lineage is diploid while the other lineages are tetraploids. It exhibits a clear north to south gradient in population abundance. This pattern is most apparent in North America where northern populations located along the Niagara Escarpment are relatively abundant, while those found further south in areas including Alabama and Tennessee are sparser (COSEWIC, 2016; USFWS, 2020). It is abundant throughout western Europe,

particularly in the British Isles (Löve, 1954); however, its abundance tends to decrease towards southern and eastern Europe. Mainland East Asian populations such as those in the Russian Far East, north-eastern China and the Korean Peninsula are generally rare [Lee & Lee, 1997; Lin & Viane, 2013; Shiyong *et al.*, 2017; Global Biodiversity Information Facility (GBIF), <https://www.gbif.org/>] but they are relatively abundant on many islands including the Japanese Archipelago. Nevertheless, the degree of divergence varies by taxa in association with their evolutionary history, and ongoing speciation will likely generate additional cryptic species due to further ecological fragmentation. To date, five taxa have generally been recognized as members of the *A. scolopendrium* complex: two European taxa (*A. scolopendrium* subsp. *scolopendrium* and subsp. *antri-jovis*), two American taxa (vars *americanum* and *lindenii*) and one East Asian taxon (*Asplenium komarovii*) (Tropicos, <https://www.tropicos.org/>; GBIF; World Plants, <https://www.worldplants.de/>).

This study seeks to explain the biogeographic history of the *A. scolopendrium* complex using molecular biogeographic approaches. We hypothesize that the diploid European hart's-tongue fern evolved earlier than the tetraploid American and East Asian lineages based on the diploid first hypothesis (Beck *et al.*, 2010). Given that large populations typically produce sufficient propagules to cross oceanic barriers (Barrington, 1993), the directional dispersal from abundant European populations to North America is probable, particularly during periods of more favourable climate, presumably around the Early Eocene Climatic Optimum (EECO). The pattern and degree of lineage splits in *A. scolopendrium* are consistent with major geological and climatic changes. To test this hypothesis, we first investigate the geographical distribution and relationships among chloroplast DNA haplotypes to identify their origin and dispersal patterns. We then estimate the divergence time of each haplotype and its ancestral range to recognize the specific periods of active dispersal and vicariance events by incorporating known geological and palaeoclimatic conditions. Lastly, we further infer the effects of Pleistocene glaciation, modern climate change, polyploidization and secondary contacts caused by anthropogenic activities on diversification using species distribution modelling, cytogeography and population genetic structure. The results of this study elucidate the origin, dispersal and evolution of the *A. scolopendrium* complex and provide insights into the evolutionary history of the Tertiary floras with northern pan-temperate disjunct distributions. In future research, these findings may serve as a basis for resolving present taxonomic uncertainties and developing effective conservation plans.

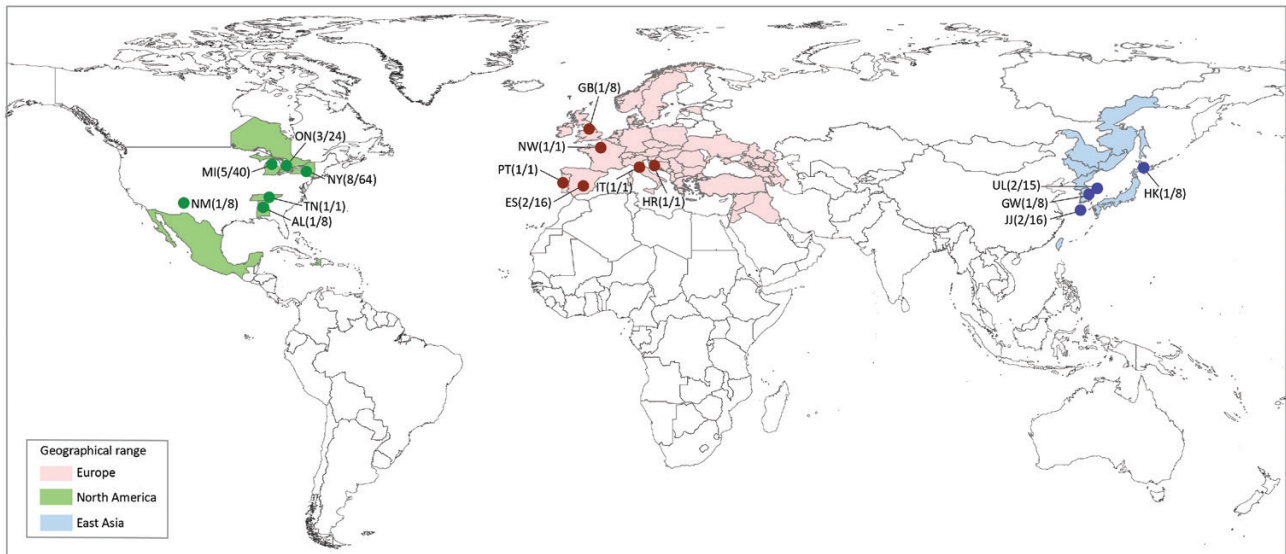
## MATERIAL AND METHODS

### SAMPLE COLLECTION

Fresh and herbarium leaf samples of the *A. scolopendrium* complex were collected from the three main population clusters of Europe, North America and East Asia (Fig. 1; Supporting Information, Table S1). A total of 218 individuals were sampled from 29 populations, and the three chloroplast DNA sequences used in prior studies were downloaded from GenBank (Supporting Information, Table S1). In addition, the cpDNA sequences (*rbcL* and *trnL-trnF*) of nine *Phyllitis* sister species and an outgroup (*Asplenium nidus*) were also obtained from GenBank for interspecific phylogenetic analysis (Supporting Information, Table S2). The number of samples per population varied from one to eight, depending on population size and accessibility. Official permits for sample collection in legally protected areas of North America were obtained from the U.S. Fish and Wildlife Service (FWS), the New York State Office of Parks, Recreation and Historic Preservation (OPRHP), Hiawatha National Forest and the Michigan Nature Association. Voucher numbers are provided in the Supporting Information (Table S1), but vouchers could not be deposited for the sampled North American populations due to their rarity and conservation status.

### DNA EXTRACTION AND SEQUENCING

Total genomic DNA were extracted from fresh (~100 mg) or dried (~25 mg) leaf samples using the DNeasy Plant Mini Kit according to the manufacturer's instructions (Qiagen, Carlsbad, California, USA). Polymerase chain reactions (PCR) were performed using universal primers for three plastid markers—a genic region (*rbcL*) and two intergenic spacers (*atpB-rbcL* and *trnL-trnF*)—and four pairs of simple sequence repeat (SSR) markers for nuclear DNA (AS4, AT5, AS7 and AT10) (de Groot *et al.*, 2011). PCR was conducted in a final volume of 20 µL containing 0.5 µL genomic DNA, 0.5 µL of each primer (10 µM), 0.5 µL of dimethylsulfoxide (DMSO, 5%), AccuPower PCR premix (Bioneer, Korea) and 18 µL of double distilled water (ddH<sub>2</sub>O). The PCR conditions consisted of an initial denaturation at 95 °C for 5 min followed by 35 cycles with denaturation at 94 °C for 45 s, annealing at different temperatures for each primer for 40 s, extension at 72 °C for 1 min, and then final extension at 72 °C for 10 min (Supporting Information, Table S3). All PCR products were confirmed by electrophoresis on 1% agarose gels. The confirmed products were sequenced at Massachusetts General Hospital Center for Computational and Integrative Biology (MGH CCIB) DNA Core (Massachusetts, USA) and Psomagen Inc. (Maryland, USA). The DNA sequences



**Figure 1.** Species ranges, sample locations, and the number of populations and individuals of sampled from each population. The colours on the map indicate the species range of each lineage. (/): (No. of populations/No. of individuals). GB, United Kingdom; PT, Portugal; ES, Spain; NW, north-western Europe; IT, Italy; HR, Croatia; ON, Ontario (Canada); NY, New York; MI, Michigan; TN, Tennessee; AL, Alabama; NM, New Mexico (USA); GW, Gangwon Province; UL, Ulleung Island; JJ, Jeju Island (Korea); HK, Hokkaido (Japan).

were aligned using Geneious v.10.2.2. (Kearse *et al.*, 2012). Allele size and genotype peaks were scored using GENEMAPPER v.5.0 (Applied Biosystems).

#### HAPLOTYPE NETWORK ANALYSIS AND MOLECULAR DATING

Sequences from the three cpDNA regions (*atpB-rbcL*, *rbcL* and *trnL-trnF*) were concatenated and used for the haplotype network and phylogenetic analysis. A cpDNA haplotype network was constructed using TCS v.1.21 (Clement *et al.*, 2000). Each indel was considered a single-step mutation event. Gaps were coded and messy polyA and polyC regions were deleted. We estimated the divergence times of each haplotype using BEAST v.2.6.2 (Drummond & Rambaut, 2007). We set the GTR+G nucleotide substitution model and the uncorrelated lognormal clock model as the best models for molecular evolution (ModelFinder, Kalyaanamoorthy *et al.*, 2017) and molecular clock, respectively. The clock model was set to a mean of 1.0. The birth-death process of the tree prior had a starting value of 1.0 for speciation and 0.5 for extinction rates (uniform prior from 0 to 10 for speciation rate and 0 to 1 for relative extinction rate). We used two secondary references to calibrate trees: the stem age of *A. nidus* was set to 99.12 Mya, and the crown age of *Phyllitis* (*Asplenium sagittatum* and *A. scolopendrium*) and *Ceterach* clades was set to 56.15 Mya, with both given a mean of 5.0 and standard deviation of 1.0 using lognormal priors (Testo & Sundue, 2016). We performed

MCMC analyses for 40 million generations, sampling every 1000 generations. The posterior distribution of all statistics was examined using Tracer v.1.6.0 and convergence was determined if the effective sample size (ESS) values were above 200. We discarded the first 10% of trees and then generated a maximum clade credibility tree using TreeAnnotator v.1.7.1. The age of each node and 95% highest posterior density (HPD) intervals were displayed using FigTree v.1.4.4.

#### ANCESTRAL RANGE ESTIMATION

The probabilities of ancestral geographical ranges were estimated using the R package *BioGeoBEARS* v.1.1.2 (Matzke, 2013). We utilized the same phylogenetic tree used for molecular dating and the maximum number of areas per node was specified to three continents: Europe, North America and Asia. The extant distributions of each taxon were identified using the occurrence data from GBIF. Given that the *A. scolopendrium* complex has an intercontinental distribution, we considered jump dispersal as an important process in its species range evolution. The result of model testing based on AIC criteria and likelihood ratios also supported that a dispersal-extinction-cladogenesis model with a long-distance dispersal or founder-event speciation parameter (DEC+J) provided significantly better estimates than alternatives (Supporting Information, Table S4). However, prior research has criticized model comparisons between DEC and DEC+J models (Ree & Sanmartín, 2018).

## POPULATION GENETIC STRUCTURE

Population genetic structure was analysed to determine possible genetic admixtures caused by secondary contact. The genetic structures of each population were estimated based on the four nuclear SSR data (AS4, AT5, AS7 and AT10). Given that the *A. scolopendrium* complex contains multiple cytotypes, nSSR data were transformed into a binary matrix displaying the presence or absence of each allele to reduce artifacts arising from multiple cytotypes (Lo *et al.*, 2009; López-Vinyallonga *et al.*, 2015). Bayesian model-based clustering algorithms were implemented using STRUCTURE v.2.3.4 to assign individuals to one of K genetically distinct clusters (Pritchard *et al.*, 2000). An admixture model was set to estimate the number of population clusters (K), ranging from 1 to 15. Each operational run was performed with  $10^5$  burn-ins and  $10^5$  Markov Chain Monte Carlo (MCMC) iterations with ten runs per K value (Evanno *et al.*, 2005). We identified the best K using STRUCTURE HARVESTER v.0.6.94 (Earl & vonHoldt, 2012), and visualized a population genetic structure using CLUMPAK (Kopelman *et al.*, 2015).

## SPECIES DISTRIBUTION MODELLING

The georeferenced occurrence data were obtained from GBIF (GBIF Occurrence Download <https://doi.org/10.15468/dl.kjja72>, accessed 13 August 2021). We filtered for only preserved specimens and cleaned the data using the R package *CoordinateCleaner* v.2.0-20 (Zizka *et al.*, 2019) by removing duplicates and flagged records with coordinate errors, such as country centroids and capitals, zero or equal coordinates, and locations of biodiversity institutions. To reduce geographical sampling bias and model overfitting, the spatially autocorrelated occurrence points were rarefied with a resolution of 10 km using the R package *humboldt* v.1.0.0.420121 (Brown & Carnaval, 2019). Outliers and taxonomically misclassified records were manually identified based on herbarium specimens and metadata from GBIF, leaving 1347 records for use in the species distribution modelling. Excluding BIO2, 3, 5, 6 and 7 to ensure data compatibility between geological periods (as these were unavailable for the Pliocene: M2), we retrieved 14 bioclimatic layers at a 2.5-minute spatial resolution from PaleoClim using data from the Pliocene (*c.* 3300 Kya), the Last Glacial Maximum (*c.* 21 Kya), the Mid-Holocene (*c.* 8.3–4.2 Kya) and the Present (1979–2013) (Dolan *et al.*, 2015; Fordham *et al.*, 2017; Karger *et al.*, 2017; Brown *et al.*, 2018). Current and past potential species distributions were predicted based on the maximum entropy (MaxEnt) model using the R package *dismo* v.1.1-4 (Hijmans *et al.*, 2021). Eighty percent of occurrence

data were randomly selected to train the model, while the other 20% were used to test the model. Ten thousand random points were also generated as background values to provide absence data. Model performance was assessed based on AUC (Area Under the receiver-operator Curve).

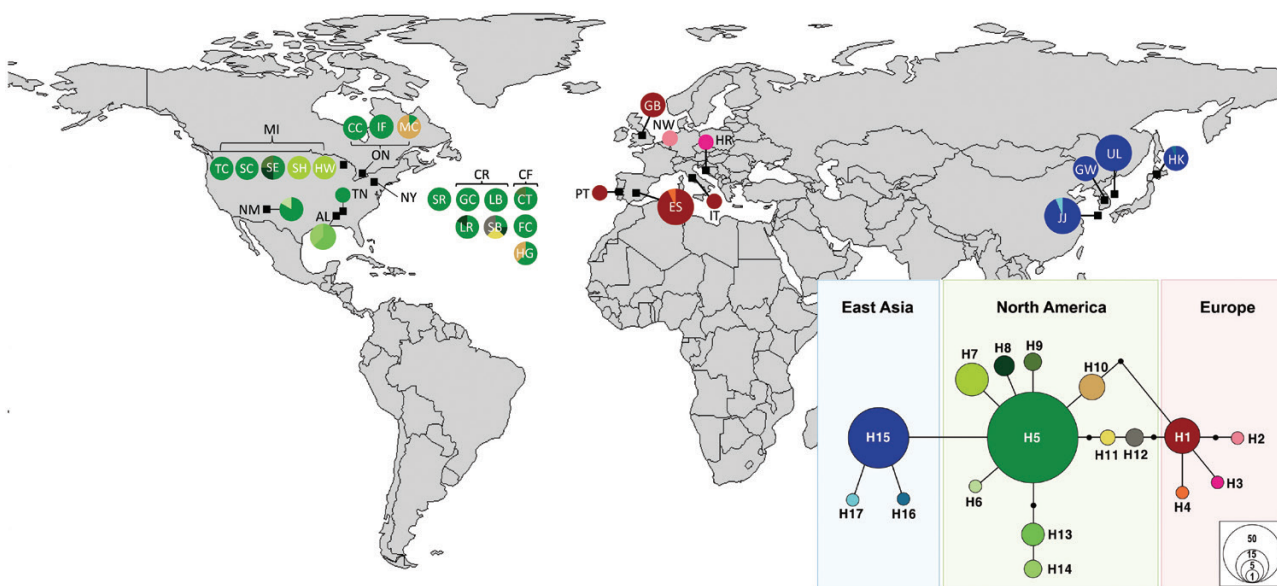
## RESULTS

## HAPLOTYPE DISTRIBUTION AND NETWORK

A total of 17 geographically segregated plastid haplotypes were identified (Fig. 2). Four and three haplotypes were respectively found in Europe (H1–H4) and East Asia (H15–H17), while ten haplotypes (H5–H14) were identified in North America. There were no shared haplotypes between the three regions. The H1, H5 and H15 haplotypes were notably dominant in Europe, North America and East Asia, respectively. In the haplotype network, European haplotypes were closely related to those of North America. In particular, the H10 and H12 haplotypes of North America were linked to the European H1 haplotype with intervening missing haplotypes. Like a stepping stone, haplotype H10 of North America linked the two regionally dominant haplotypes of H1 and H5. Despite the contiguous Eurasian continent geographically linking the European and Asian populations, East Asian haplotypes were separated by at least four mutational steps from European haplotypes. On the other hand, the Asian dominant haplotype of H15 was directly linked with the North American dominant haplotype of H5.

Samples from North America generally formed a star-shaped haplotype network. The relatively low frequency haplotypes from the H6 to H14 haplotypes were derived from the dominant haplotype of H5. The dominant H5 haplotype was largely shared by several populations, including Ontario, New York, Michigan (SC, SE and TC), Tennessee and New Mexico. In contrast, the derived haplotypes H6, H7, H13 and H14 were distributed locally: H6 in New Mexico, H7 in Michigan (SH and HW), and H13 and H14 in Alabama. H10 was found in MC (Ontario) and CF (HG, New York) and H11 and H12 were only found in CR (SB, New York). H10 and H12 haplotypes were closely related to the European dominant haplotype of H1.

Fewer haplotypes were identified from samples in East Asia. Most populations contained the H15 haplotype which was closely related to the major American H5 haplotype. Haplotypes H16 and H17 were derived from H15 and were found in island regions such as H16 in HK (a northern Japanese population) and H17 in JJ (a southern Korean population).



**Figure 2.** Geographical distribution of the cpDNA haplotypes and their relationships. Haplotypes are represented by coloured circles according to sample localities. Haplotype frequency in each population is expressed as a pie chart. Lines represent one mutational step and black dots represent missing haplotypes. A 95% probability haplotype network is shown in the bottom right box with circle size proportional to the number of sequences sharing a haplotype. GB, United Kingdom; PT, Portugal; ES, Spain; NW, north-western Europe; IT, Italy; HR, Croatia; ON, Ontario (Canada); NY, New York; MI, Michigan; TN, Tennessee; AL, Alabama; NM, New Mexico (USA); GW, Gangwon Province; UL, Ulleung Island; JJ, Jeju Island (Korea); HK, Hokkaido (Japan); TC, Taylor Creek; SC, Sugar Camp; SE, Southeast of East Lake; SH, Scherer; HW, Hiawatha; CC, Clark's Corner; IF, Inglis Falls; MC, Mono Cliffs; SR, Split Rock Unique Area; CR, Clark Reservation State Park; CF, Chittenango Falls State Park; GC, Grand Canyon; LB, Lower Basin; LR, Long Ravine; SB, Sentinel Basin; CT, Chip's Trail; FC, Falls Colony; HG, Horseshoe Gorge.

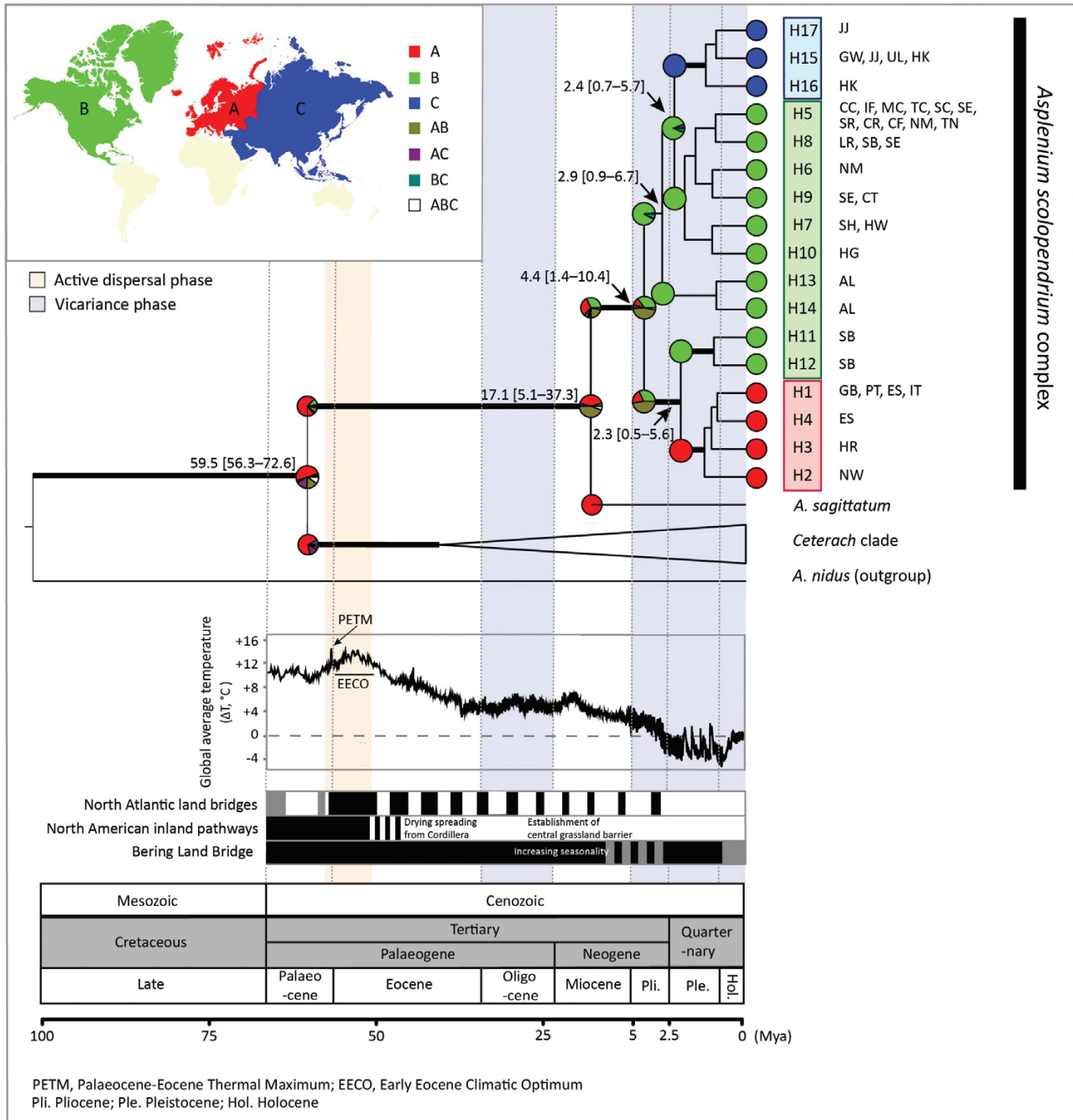
#### PHYLOGENETIC RELATIONSHIPS AND ANCESTRAL POPULATION RANGES

The haplotypes of the *A. scolopendrium* complex identified in this study formed a monophyletic group (Fig. 3). Ancestral range estimation revealed that the common ancestor of *A. scolopendrium* and its sister taxon *A. sagittatum* expanded its ranges from Europe to all continents in the Northern Hemisphere, particularly with the opening of the North Atlantic and Bering land bridges around the EECO. The split between *A. scolopendrium* and *A. sagittatum* was estimated to have occurred between the Late Eocene and Late Miocene (17.1 Mya, 95% HPD, 5.1–37.3 Mya). After the lineage split between the species, the *A. sagittatum* lineage became mostly confined to Europe, while the *A. scolopendrium* lineage maintained its broad range of distribution. A major split within the *A. scolopendrium* complex was likely to have occurred during the Late Miocene and Pleistocene. The split between European and other haplotypes was estimated to have occurred approximately 4.4 Mya (95% HPD, 1.4–10.4 Mya), which was followed by the haplotype splits within North America (eastern US and other haplotypes) during the Pliocene at 2.9 Mya (95% HPD, 0.9–6.7 Mya). The split between North American and East Asian haplotypes

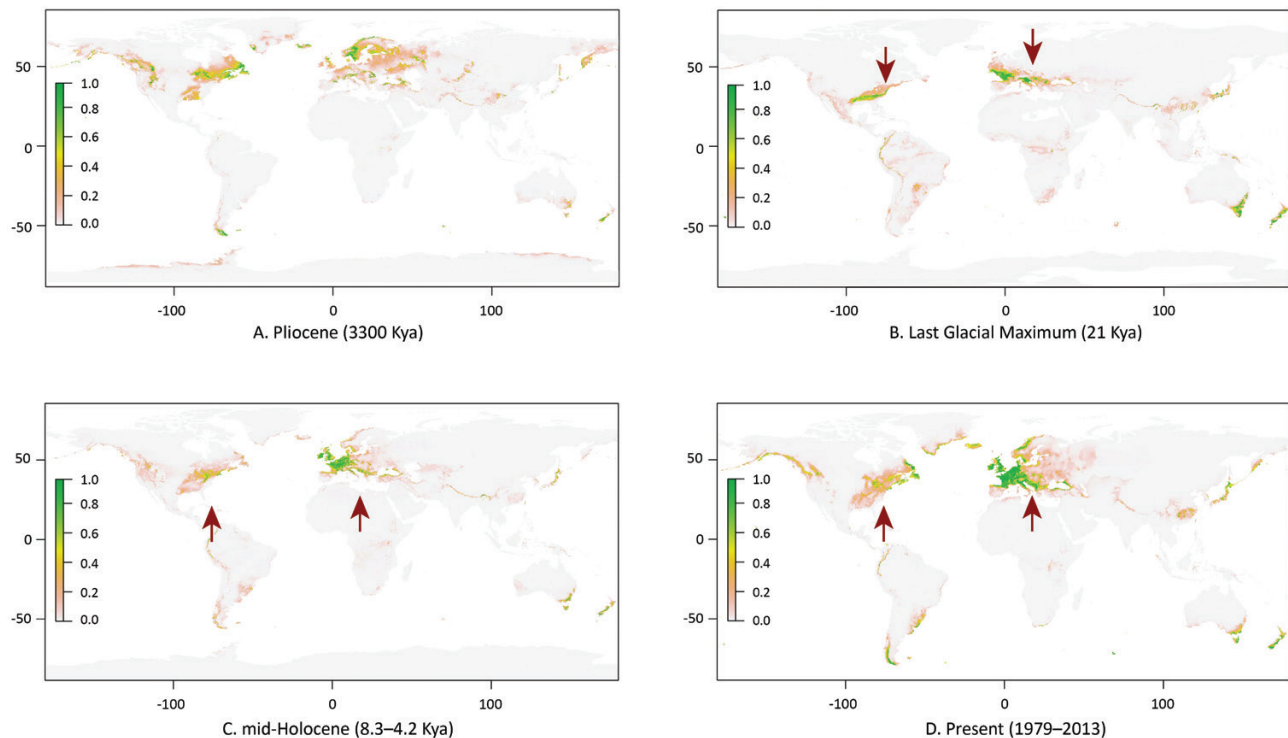
occurred at approximately 2.4 Mya (95% HPD, 0.7–5.7 Mya). The ancestral range of *A. scolopendrium* became segregated into Europe, North America and East Asia, indicating that these divisions were associated with geoclimatic vicariations driven by multiple glacial cycles during the Pleistocene.

#### SPECIES DISTRIBUTION MODELLING

The AUC value of the MaxEnt model was 0.935 (Supporting Information, Fig. S1). Latitudinal range shifts were identified from species distribution modelling (Fig. 4). The species ranges (or suitable habitats) of *A. scolopendrium* on all Northern Hemisphere continents extended to mid-latitudes during the Pliocene (Fig. 4A). The ranges contracted farther south, particularly as glaciers advanced from the north (Fig. 4B). These distributional changes were particularly pronounced in Europe and North America where the impact of glaciation was greater and may have induced range collapses. However, the ranges returned northward over the past 21 000 years since the Last Glacial Maximum (Figs 4C, D). Due to increasing aridity, multiple habitat fragmentations may have accompanied additional vicariations within each biogeographic region.



**Figure 3.** Phylogenetic relationships of cpDNA haplotypes and ancestral range estimation. The three major biogeographical regions (Europe, North America and Asia) are colour coded. Pie graphs at each node denote the geographical regions inferred to have been occupied by ancestral taxa. Numbers above selected internodes indicate the median divergence time estimates. Numbers in brackets indicate the 95% HPD intervals. Global average temperature is relative to the global mean for 1960–1990 (Burke *et al.*, 2018). References: North Atlantic land bridges (Tiffney & Manchester, 2001; Brikiatis, 2014); North American inland pathways (Tiffney & Manchester, 2001); Bering Land Bridge (Tiffney & Manchester, 2001). Branches with bootstrap support above 50% and posterior probabilities of greater than 0.5 are shown in bold in the tree. The timescale in the most recent 5 Mya was increased in size to provide greater clarity. Note that the haplotypes H11 and H12 were considered artificially introduced to North America. See Figure 2 or Table S2 for keys to all location abbreviations.



**Figure 4.** Species distribution modelling of *A. scolopendrium* complex. Note that habitat suitability is predicted based on climate conditions only; thus, some suitable regions, particularly in the Southern Hemisphere may not be occupied by the *A. scolopendrium* complex due to dispersal limitations. A, Pliocene. B, Last Glacial Maximum. C, Mid-Holocene. D, Present. Arrows indicate the regions with notable change in habitat suitability.

#### PLOIDY BARRIER AND POPULATION GENETIC STRUCTURE

Given that European populations are diploid, whereas Asian and American populations are tetraploid (Wagner, 1955; Emmott, 1964; Tryon, 1972), ploidy barriers were likely formed between Europe and North America, as well as Europe and Asia (Fig. 5A). The optimal number of population genetic clusters was determined to be 4 ( $K = 4$ ; Supporting Information, Fig. S2). Accordingly, the population genetic structures were separated at the continental scale: Europe (orange), North America (light blue) and Asia (dark purple) (Fig. 5B). The SB (NY) population likely included individuals that were introduced from European sources, whereas IF (ON), GW and UL (KR) were considered genetically admixed populations.

#### RECONSTRUCTION OF EVOLUTIONARY HISTORY

The integration of the results discussed above resulted in the reconstruction of the origin and divergent history of the *A. scolopendrium* complex in three major biogeographic phases (Fig. 6). The Early Tertiary (until the Late Eocene but mostly prior to Palaeocene-Eocene Thermal Maximum [PETM]) was considered an active dispersal phase as the species range of *A. scolopendrium*

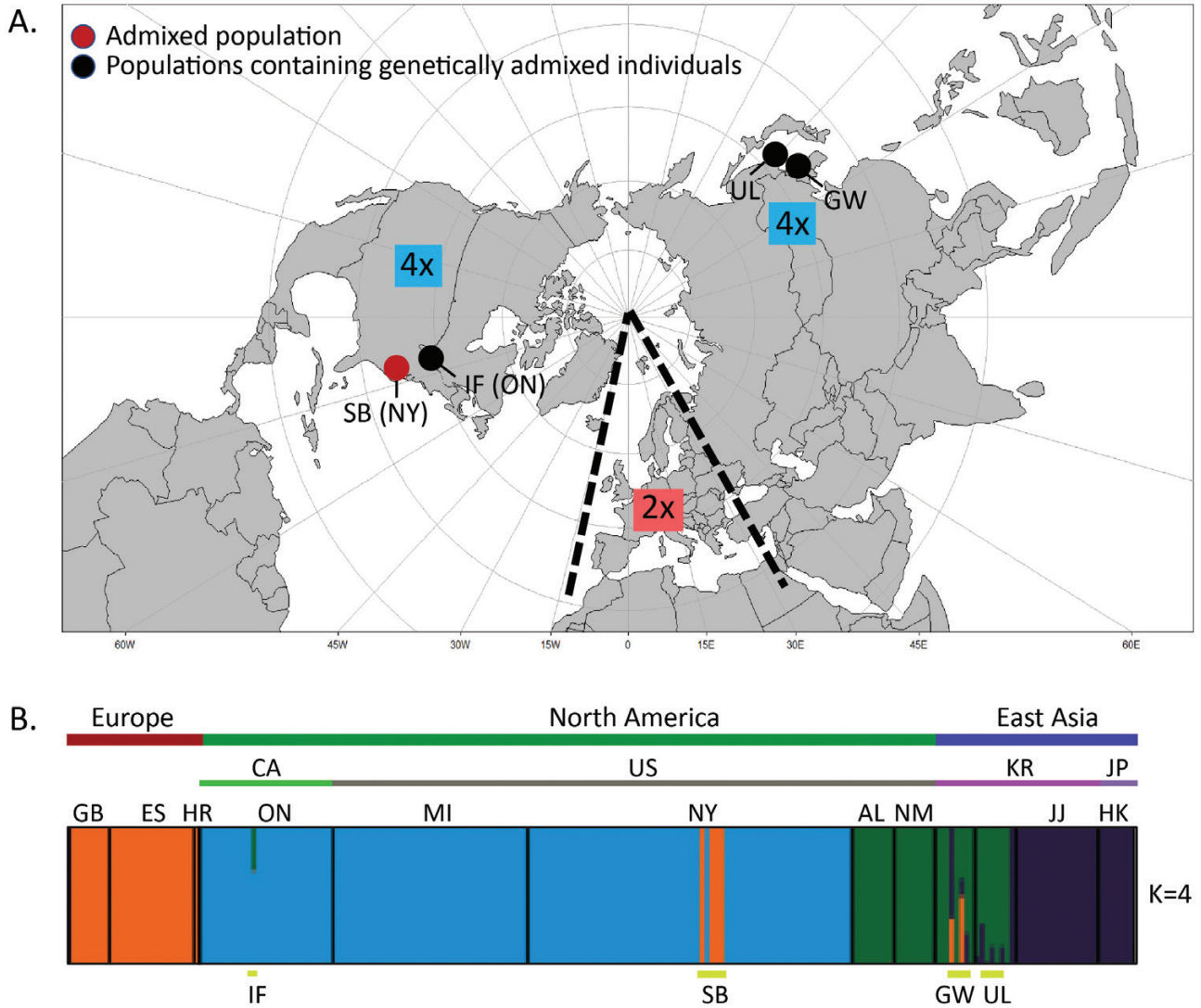
expanded through sequential dispersal from Europe to North America and then from North America to East Asia. A fairly homogenous pan-temperate ancestral population formed during this phase (Fig. 6A). In particular, the tetraploid founder populations, which arose through major polyploidy events in North America, then expanded slowly and progressively over many generations across East Asia. During the subsequent two phases of the vicariance (phase I: during the Oligocene; and phase II: after the Pliocene), oceanic, mountain, ecological and cytological barriers impeded gene flow resulting in genetic differentiation between populations (Fig. 6B). Moreover, the interplay of climate change and other geographic barriers exacerbated population fragmentation, further increasing isolation between populations. Subsequently, the emergence of distinct lineages of *A. scolopendrium* on each continent has been promoted.

#### DISCUSSION

##### FORMATION OF THE PAN-TEMPERATE ANCESTRAL POPULATION

The monophyly of the three major lineages of European, North American and East Asian hart's tongue ferns suggests that they descended from a

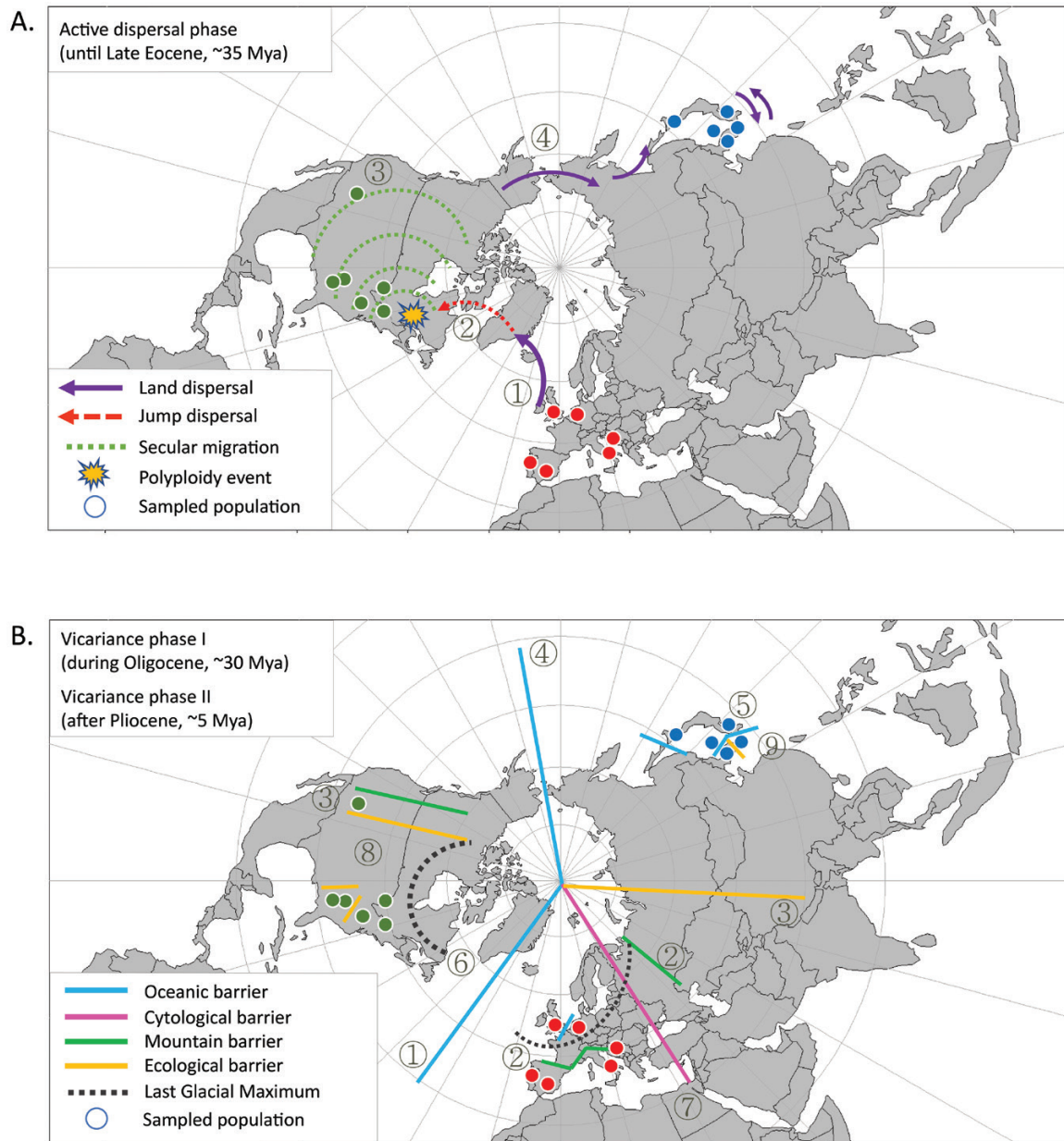




**Figure 5.** Cytogeography and population genetic structure. A, cytotypes of three major lineages. B, population genetic structure and genetic admixture patterns based on nuclear DNA. See Figure 2 or Table S2 for information on all location abbreviations.

common ancestor that once had a northern pan-temperate distribution (Fig. 3). Along with cytotype variation, the result of the haplotype network analysis supports the hypothesis that *A. scolopendrium* originated in Europe and spread westward, as the haplotypes were sequentially linked from diploid European, tetraploid American and then East Asian lineages. Given that each continent contained several satellite haplotypes that were derived directly from a dominant haplotype, it implies that these major intercontinental dispersal events occurred before the modern lineage diversification. Molecular dating and ancestral range estimation, integrated with geological data, suggest that the unidirectional dispersal events occurred predominantly during the Early Eocene (~55 Mya), even before the lineage split with *A. sagittatum*.

Active dispersals of ancestral *A. scolopendrium* populations likely occurred during the Early Eocene when principal continental units in northern temperate latitudes were grouped much more closely together around an enclosed Arctic basin. Land bridges likely played an essential role in the intercontinental dispersals. The North Atlantic land bridges (NALB) that connected the British Isles to southern Greenland (Thulean Bridge) and Greenland to Baffin Island (i.e. island stepping stones in the Davis Straits) existed briefly in the Late Palaeocene and Early Eocene (McKenna, 1983; Brikiatis, 2014). It appears that dispersal through this route occurred much more readily because the British Isles were connected to mainland Europe, and Greenland was free of glaciers during the period. The links between



**Figure 6.** Reconstruction of the evolutionary history of the *A. scolopendrium* complex. A, active dispersal phase during the Palaeocene-Eocene Thermal Maximum. B, two phases of major vicariance events. Numbers indicate the estimated sequence of major biogeographic events (several events may have concurrently occurred). Ecological barriers refer to unfavourable biotic or abiotic factors between two ecosystems. Note that climate change reinforces the difficulty of dispersal across existing physical barriers, further increasing isolation between populations.

Greenland and northern Europe (De Geer Bridge), and Greenland and the Queen Elizabeth Islands, are estimated to have formed before the Thulean Bridge (~63–71 Mya), likely providing an alternative northern route in the early stages of dispersal. Despite the great dispersal potential of ferns, long-distance dispersal directly from Europe to North America was unlikely due to the westerlies blowing

from the south-west across the Atlantic Ocean (Quan *et al.*, 2014).

Cytogeographic patterns suggest that a major polyploidy event occurred in the founder populations in North America. This event was likely associated with jump dispersals through island stepping stones in the Davis Straits, as haplotypes found near this route (Mono Cliffs, Ontario, Canada) were phylogenetically

more related to European populations. Given that the founder populations were likely small, they would have been more dependent on self-fertilization. Under this scenario, a small number of tetraploids may have arisen by autopolyploidization and subsequently dominated new North American populations. Despite possible artificial hybridization between *A. scolopendrium* and its closely related sisters (Emmott, 1964), its tetraploid populations likely emerged from autopolyploidization given morphological similarity to their diploid progenitor maintaining simple fronds. Polyploid-related traits such as gene redundancy, particularly during the gametophyte stage, might also have been advantageous in the novel environment of North America (Ranney, 2006). Moreover, minority cytotype exclusion, a selective disadvantage due to frequency-dependent effects, may have resulted in a homogeneous cytotype in the early North American populations if tetraploids became more numerous very early in colonization. This cytotype exclusion effect may also have contributed to the formation of a reproductive barrier between European and North American populations, thus preventing both further colonization by European populations and reverse dispersal from America to Europe.

The early American founder populations expanded across central and western North America, presumably during the Early Eocene. The range expansion began with the retreat of the epicontinental seaway (the Western Interior Seaway) which opened physical pathways to spread westward. However, local extinctions of central North American populations would have quickly followed as the rising Rocky Mountains cast an increasingly long rain shadow to their east, changing the area to a continental climate zone (Leopold & MacGinitie, 1972). These processes formed a strong phytogeographic barrier that separated the western and eastern North American-European floristic provinces (Muller, 1970). Therefore, the extinction of the central North American population would have led to vicariance between the western and eastern populations, which later became a founder for subsequent westward range expansion into East Asia. The existence of the ancestral lineage of the genus *Asplenium* in the fossil record in central North America may indirectly support its diffusional dispersal toward the western USA and subsequent dispersal to East Asia (<https://paleobiodb.org>).

The Bering Bridge between Alaska and Siberia may have served as a dispersal route for American populations to East Asia during the Eocene exposures (Brikiatis, 2014). The Bering Bridge has been above water for various substantial periods of geological time, forming a broad highway for intercontinental dispersal (Neil, 1969). However, Beringia was likely a filter rather than a corridor during the Late Palaeocene

and Early Eocene because it was located at a higher latitude than today (Brikiatis, 2014). Although the Eocene floras near the bridge were dominated by deciduous angiosperm taxa, subtropical flora is also presumed to have exchanged, particularly during the EECO (Tiffney & Manchester, 2001). The Bering Land Bridge likely existed as late as the Late Miocene epoch and flora exchanges frequently occurred in both directions (Kurtén, 1966; Neil, 1969; Novodvorskaja & Janovskaja, 1977; Wen *et al.*, 2016). Thus, the dispersal of the American ancestral population to East Asia may have occurred over a longer period than that of Europe to North America, and bidirectional exchanges may have increased the similarity between the two taxa (i.e. incomplete lineage sorting).

Despite contiguous land connecting Europe and Asia, less genetic admixture or hybridization occurred between European and East Asian populations. Dispersal from Europe to East Asia may have been prevented by the Turgai Straits that existed near the present-day Caspian Sea until the end of the Eocene (Kurtén, 1966). Although this oceanic barrier disappeared as the Turgai Straits closed in the Oligocene, physical barriers such as the Ural Mountains and ecological barriers such as the Gobi Deserts and Eurasian Steppe still impeded European populations' dispersal to East Asia. Likewise, ecological barriers in the Eurasian continent may have limited the far western expansion of East Asian populations to Europe.

#### ORIGIN OF *A. SCOLOPENDRIUM* AND MAJOR LINEAGE SPLITS

*Asplenium scolopendrium* was estimated to have evolved approximately 20 Mya (Early Miocene) by splitting with *A. sagittatum*. The phylogenetic relationship of two taxa was consistent with other large-scale phylogenetic studies on genus *Asplenium* (Lin & Viane, 2013; Xu *et al.*, 2020). As both species are diploid and their ranges rarely overlap, they likely underwent allopatric speciation within Europe. The European hart's tongue fern is currently found mostly in central and northern Europe, whereas *A. sagittatum* has a pan-Mediterranean distribution along the coast of southern Europe (Mifsud *et al.*, 2016). Mountain ranges, such as the Pyrenees (formed ~80–20 Mya), the Alps and the Carpathian Mountains (~65 Mya), enclose the areas currently inhabited by *A. sagittatum* and appear to act as barriers to gene flow between the two species. Given that the major orogenesis of these aforementioned mountain ranges preceded the speciation, climate change during the Late Oligocene and Early Miocene may have exacerbated the impermeability of these already formed geological barriers. Subsequently, populations separated by mountain barriers were likely confined to the

Mediterranean region and speciated into *A. sagittatum* under different environmental conditions and low gene flow. Emmott (1964) produced artificial hybrids between *A. scolopendrium* and *A. sagittatum*, which showed some degree of genetic affinity and suggested that they had partially diverged from a common ancestor by geographic isolation.

Phylogenetic divisions among the *A. scolopendrium* complex were estimated to have occurred quite contemporaneously since the Late Pliocene or Pleistocene. The ancestral populations in each continent could have initially maintained fairly homogenous haplotypes with continuous gene flow. Subsequent vicariations were likely initiated by multiple glacial cycles during the Pleistocene (Zachos *et al.*, 2008; Nagalingum *et al.*, 2011). In addition, population fragmentations may have resulted in multiple haplotypes derived from the geographically confined larger ones. This haplotype pattern indicates that current gene flow has been sufficiently low, and random genetic drift promoted genetic divergence of populations that were in historical contact.

#### *Range shifts of American hart's tongue fern*

American ancestral populations experienced multiple geographic range expansions and contractions during the Early Pleistocene. The star-shaped haplotype network indicated that they have a peripatric divergence as apomorphic traits are likely formed from isolated peripheral populations. With respect to eastern USA populations, glaciation may have shifted their ranges southward to at least Mexico and the Caribbean (Maxon, 1900). During the multiple range shifts of eastern North American populations, refugia appear to have played a role in supporting populations under harsh and changing conditions but also facilitated population differentiation.

Rockhouses are one example of potential refugia during the Pleistocene glaciation (Farrar, 1998; Testo & Watkins, 2011). Although rockhouses take on many forms, they conventionally refer to semicircular recesses extending under cliff overhangs (Farrar, 1998). It is therefore plausible that southern populations such as those in Mexico and the USA (New Mexico, Tennessee and Alabama) are pre-Pleistocene remnants that became isolated during the Late Pliocene or Early Pleistocene. As their refugia provided suitable environments for their survival, they could successfully overwinter during periods of glaciation. However, it is possible that their confinement to refugia has separated them from the dominant populations that have returned northward after the Last Glacial Maximum (~21 000 years ago), resulting in the current star-shaped haplotype network with genetic differentiation. Another example of refugia is nunataks, which are ice-free islands (outcrops) formed in ridges of a

mountain protruding out of a glacier. The high haplotype diversity near the Great Lakes may have resulted from the multiple outcrops of the Niagara Escarpment that existed during the Wisconsin Glaciation, facilitating the accumulation of mutations in many distinct and isolated populations. Furthermore, the lake effect of this region provides different types of refugia from the current dry climate conditions of North America. Thus, multiple local factors, in addition to geographic proximity to the European origin and colonization routes, contribute to the Great Lake populations forming the centre of American hart's tongue fern populations.

#### *The emergence of East Asian hart's tongue fern*

The common ancestral population of Asia and North America seemed to have been separated by geoclimatic vicariance because the estimated phylogenetic divergence time was congruent with the intermittent disappearance of the Bering Bridge. However, the recent haplotype split between North American and East Asian populations implied that these two lineages have not completely sorted. Despite the large sample size, the East Asian populations had the lowest haplotype richness, and a single major haplotype was shared by most of the populations. These results supported the idea that the East Asian hart's tongue fern is the youngest among the three lineages.

Considering that the volcanic Jeju and Ulleung islands in South Korea formed after sea level rise during the Holocene, the extension of populations to several islands within East Asia would have occurred through long-distance spore dispersal. However, dispersal to continental islands such as the Japanese Archipelago appear to have a more complex evolutionary history. East Asian ancestral populations may have dispersed through land bridges in the Late Pleistocene, such as those in the Tsushima Strait that linked southern Korea and western Japan, and those in the northern East Sea (also known as Sea of Japan) that connected the Russian Far East, Sakhalin and Hokkaido. The Kuril Islands may also have served as stepping stones for dispersal. Moreover, land bridges within the Japanese Archipelago such as Hokkaido-Honshu may have further facilitated dispersal throughout Japan. More data are required to understand these biogeographic relationships at a higher resolution. Nevertheless, unique haplotypes were identified both in the northern Japanese island of Hokkaido and southern Jeju Island suggesting that *in situ* evolution occurred on those isolated islands, thus showing potential for speciation.

#### FORMATION OF THE *A. SCOLOPENDRIUM* COMPLEX

Although dominant haplotypes were found in each major continent, derived haplotypes in remote

populations resulted in star-shaped haplotype networks, which implies that those populations have undergone regional genetic differentiation. Climate change may have driven recent habitat fragmentation as the result of species distribution modelling predicting not only a large-scale northward population shift but also range collapse of potential habitats since the Last Glacial Maximum. As a result, increased fragmentation in association with decreased gene flow between populations may have hastened genetic differentiation at the population level. Populations in southern regions such as *A. scolopendrium* var. *lindenii* in Mexico and *A. scolopendrium* subsp. *antrijovis* in Crete are examples of this pattern. These palaeo-endemic remnant populations likely survived in only a small part of their once-expansive former range, which has ultimately increased both local genetic differentiation and extinction risk.

Anthropogenic impacts would be another driver for population fragmentation. Currently, hart's tongue fern populations are threatened throughout most of their range due to trampling, logging, and development within and near their habitats (USFWS, 2020). Vegetational changes may also play a role in population decline. Cinquemani Kuehn and Leopold (1993) stated that hart's tongue ferns were absent beneath evergreen woody species like *Tsuga canadensis* because of the vegetational changes that have led to light and soil moisture changes. Records showed that invasive species inevitably accompany climate change, such as the European pale swallow wort (*Cynanchum rossicum*) displacing American populations where they co-occur (USFWS, 2020). These patterns were also observed by the authors in Gangwon Province, Korea, where populations were historically abundant but have gradually been replaced by a variety of angiosperm taxa. Intensive land use in association with global warming has the potential to affect population size and fragmentation of hart's tongue ferns as is the case for the scarce Tokyo populations (A. E. Ebihara, pers. comm.). These interwoven relationships between range collapse and human-induced landscape or vegetational change, as well as ecological and anthropogenic effects on the persistence and evolution of hart's tongue ferns, should be further investigated.

#### SECONDARY CONTACTS

The regions of IF (Ontario), GW and UL (KR), located near the putative ancestral dispersal route, showed genetically admixed populations based on nuclear DNA, indicating possible historical secondary contacts. However, the SB (NY) population may include individuals that were likely introduced from multiple sources, increasing the likelihood of secondary contacts

by humans. Given that the SB population had several distinct cpDNA haplotypes that were not shared, even with geographically close neighbouring populations, it is unlikely that individuals with these haplotypes could selectively reach the SB population through random natural dispersal. It is also unlikely that the SB population could exceptionally generate multiple haplotypes that were not directly derived from the dominant haplotypes in North America and Europe. Translocation history in the Clark Reservation State Park (Benedict, 1927; Wherry, 1936; Faust, 1960) and anecdotal evidence regarding the prevalence of spore sowing by private individuals earlier in the 20<sup>th</sup> century (The Nature Conservancy, 1990; Finger Lakes Native Plant Society, 2009) also supported a possibility of human-mediated introduction. Further studies should aim to identify the origins of admixed individuals, which may include DNA forensic tracking targeting unsampled regions (e.g. cultivars in commercial stocks, other natural populations in Canada and Europe, and historical herbarium specimens).

#### INSIGHTS INTO THE ORIGIN AND EVOLUTION OF NORTHERN PAN-TEMPERATE DISJUNCT FLORA

The biogeographic history of the *A. scolopendrium* complex supports the Boreotropical flora hypothesis rather than the Arcto-Tertiary Geoflora concept in several aspects. First, the common ancestor of *A. scolopendrium* and its sister taxon *A. sagittatum* originated in Europe where the palaeoclimate was subtropical during the Late Cretaceous and Early Palaeocene. Most of the sister taxa of *A. scolopendrium*, the members of the *Ceterach* clade, remain confined to Europe. Second, the common ancestral populations had pan-temperate distributions during the Early Eocene when the Earth's climate was favourable to subtropical flora exchanges, as postulated by the Boreotropical flora hypothesis. In particular, the range expansion occurred during periods of the Early Eocene when North American inland pathways were temporarily open. If the ancestral population originated from the Arcto-Tertiary Geoflora, its range expansion would have occurred when the global climate cooled and became more seasonable, presumably before or after the EECO. Third, the species range of *A. scolopendrium* has expanded northward since the Last Glacial Maximum, similarly to its range expansion during the Early Eocene in response to global warming. Lastly, coexistence with tropical ferns such as *Didymoglossum*, *Vandenboschia* and *Vittaria* exemplifies that the historical range of *A. scolopendrium* extended far enough south during the Early Eocene to overlap with those of tropical ferns (Farrar, 1978).

The biogeographic processes found in the *A. scolopendrium* complex enrich the discussion of the

evolution of floristic disjunctions by demonstrating direct intercontinental relationships across western Europe, eastern North America and East Asia at lower taxonomic ranks (i.e. between infraspecific taxa). Its evolutionary history involved multiple biogeographic processes including jump dispersal, range expansion and subsequent geo-climatic vicariances. In particular, the westward dispersal from an European origin was opposite to patterns found in ferns with Asian origin such as *Diplazium* (Wei *et al.*, 2015) as well as *Blechnidium*, *Struthiopteris* and *Spicantopsis* from the Blechnaceae group (Molino *et al.*, 2019). This suggests that major directions of dispersal during the Cenozoic were rather diverse and determined by several spatial and temporal factors regarding a taxon's origin, availability of niches and dispersal routes, and ecological constraints for successful establishment. Although biotic exchanges from Eurasia to North America are known to have been higher throughout the Cenozoic, this asymmetric dispersal could be biased due to the greater phylogenetic diversity of these taxa in Eurasia and variable rates and directions of exchange between different geological times (Jiang *et al.*, 2019). Various modes and times of floristic exchange in the Northern Hemisphere have also been reported across many other taxonomic groups (Xiang & Soltis, 2001; Banasiak *et al.*, 2013; Xiang *et al.*, 2015; Wen *et al.*, 2016). Therefore, the evolution of the northern pan-temperate disjunct flora cannot be generalized by typical evolutionary histories but must instead be individually explained as the consequences of cumulative biogeographic processes operating at different time scales.

#### LIMITATIONS OF THIS STUDY

Inferences of evolutionary history will depend critically on an appropriate sampling strategy that incorporates complete taxon sampling throughout the geographic distribution (Templeton *et al.*, 1993). In that sense, this study may be limited by the exclusion of *A. scolopendrium* var. *lindenii* and *A. scolopendrium* subsp. *antri-jovis* due to their rarity. Unfortunately, these taxa are under serious threat and many of their populations may become extirpated before they are examined. Despite the high abundance and wide distribution of European and Japanese populations, the sampling density for these lineages might be less than those of other lineages. Moreover, future studies should include newly established lineages such as in New Zealand, which will provide additional insights regarding the introduction and evolutionary adaptation in novel biogeographic regions.

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#### DATA AVAILABILITY

The data underlying this article can be accessed with OM675058–OM675276 (*atpB-rbcL*), OM675277–OM675495 (*rbcL*) and OM675496–OM675714 (*trnL-trnF*) in GenBank.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Table S1.** Information on plant materials and voucher numbers.

**Table S2.** Taxa and GenBank accession numbers used in the divergence time analysis.

**Table S3.** Marker information and PCR conditions.

**Table S4.** Biogeographic model comparison implemented in BioGeoBEARS.

**Figure S1.** Receiver operating characteristic (ROC) curve.

**Figure S2.** Delta K values from K = 1 to K = 15, indicating that the highest peak was observed at Delta K = 4.