RESEARCH ARTICLE

Herbariomics and the peculiar case of *Alisma wahlenbergii* (Alismataceae)

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DOI https://doi.org/10.1002/tax.70097

Abstract Species identification and delimitation in aquatic plants can be challenging due to their often simple, plastic, and convergent morphology. This has led to varying taxonomic opinions in many genera, including *Alisma*. One debated case is the narrowly endemic *A. wahlenbergii*, which occurs submerged in the Baltic Sea region. Depending on the author, it is either accepted at some rank or synonymized with the widespread amphibious *A. gramineum*. Our study aimed to resolve the status and biogeographic history of this peculiar taxon using herbariomics and environmental niche modelling. Our results indicate that *A. wahlenbergii* and *A. gramineum* are best treated as conspecific. Furthermore, the findings suggest that what is considered *A. wahlenbergii* likely represents two independent colonization events of *A. gramineum* to the Baltic Sea. Environmental niche models projected onto paleoclimate scenarios suggest that the current main population of *A. wahlenbergii* in Bothnian Bay was established approximately 8.3–4.2 thousand years ago from the southeast via range expansion through Karelia. The population in the Gulf of Finland is genetically distinct. These results challenge the current view of a single, rare, and endemic taxon of submerged *Alisma* in the northern Baltic Sea. Instead, it appears that there are two relictual populations of *A. gramineum* of different origins surviving underwater from a deteriorated climate. Similarly, paleoclimatic projections highlight the significant role of Beringia in explaining the current distribution of *A. gramineum* in China and western North America. The eastern North American populations seem to have a different and more recent origin.

Keywords aquatic plants; environmental niche modelling; phylogenetics; taxonomy

INTRODUCTION

Alisma L. is a genus of aquatic or semi-aquatic herbs in the monocot family Alismataceae. The genus is widely distributed, mainly in the Northern Hemisphere, and is currently considered to consist of approximately 10 species (Ito & Tanaka, 2023). However, the species concept and, consequently, the number of accepted species have varied among authors due to the relatively simple morphology and high phenotypic plasticity in the genus (Björkqvist, 1967, 1968).

The phylogenetic relationships in the genus have been studied by Jacobson & Hedrén (2007), Ito & Tanaka (2023), and most recently Lan & al. (2024). Jacobson & Hedrén (2007) sequenced the nuclear ITS region and the plastid *trnL* region and conducted random amplified polymorphic DNA (RAPD) analysis. However, their study was limited by low sequence variability and the small number of markers studied. Later, Ito & Tanaka (2023) sampled more markers (nuclear ITS and *phyA*; plastid *matK*, *ndhF*, *psbA-trnH* and *rbcL*) and specifically tested for multi-locus species delimitation using STACEY (Jones, 2017). In the most recent study, Lan & al. (2024) sequenced entire plastomes from most of the commonly accepted species and sampled multiple populations from some species. Despite the variable taxon sampling, polyploid origins of some taxa, and limited sequence variation in the genus, the results of these studies are quite consistent.

One consistent group is formed by *Alisma gramineum* Lej. and *A. wahlenbergii* (Holmb.) Juz., which are particularly well adapted to submerged life within the genus (Lan & al., 2024). However, they also present taxonomic challenges. *Alisma gramineum* is widely distributed from Europe and northern Africa through Asia to North America and is morphologically extremely variable depending on growing conditions. While often growing submerged, it also produces emersed growth forms (Björkqvist, 1968). Several infraspecific taxa have been recognised accounting for various ecological growth forms, but these are generally no longer taxonomically accepted (e.g., Tournay & Lawalrée, 1949).

Alisma wahlenbergii, on the other hand, is endemic to the northern Baltic Sea and some nearby lakes in Sweden and Russia (Björkqvist, 1968). The main populations occur on the Finnish side of Bothnian Bay, but the species is also

Article history: Received: 21 Jan 2025 | returned for (first) revision: 30 Apr 2025 | (last) revision received: 1 Aug 2025 | accepted: 12 Oct 2025
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present in the Gulf of Finland. The Swedish populations in the Lake Mälar region have been reported to sometimes produce emersed or terrestrial leaves, but this has never been observed in other populations, and the taxon is considered extremely adapted to submerged conditions (Björkqvist, 1968). Juzepczuk (1934) was the first to accept *A. wahlenbergii* at the species level, albeit with some hesitation, but was followed by Björkqvist (1968) and, for example, *Flora Europaea* (Tutin & al., 1980). In contrast, many other authors have not accepted the species status but have treated *A. wahlenbergii* as an infraspecific taxon under *A. gramineum* at various ranks (e.g., Holmberg, 1922; Raymond & Kucyniak, 1948; Lousley, 1957), or as a synonym without any taxonomic status (e.g., Haynes & Hellquist, 2020). Previous molecular studies did not clarify this question. Ito & Tanaka (2023) did not sample *A. wahlenbergii*, whereas Jacobson & Hedrén (2007) and Lan & al. (2024) sampled *A. wahlenbergii* and *A. gramineum*, but in both studies, each taxon was represented by only one specimen. This was enough to show the very close relationship between them but did not provide a proper test of taxonomic hypotheses.

Björkqvist (1968) believed *Alisma wahlenbergii* to have evolved from *A. gramineum* as an isolated ecotype and suggested that the Swedish freshwater populations may represent a separate stage of evolution compared to the obligately submerged populations in the brackish Baltic Sea. This implies a phylogenetic scenario in which *A. gramineum* would be paraphyletic with respect to *A. wahlenbergii*. Similarly, Samuelsson (1934) concluded that *A. wahlenbergii* is a recent ecotype of *A. gramineum* after abandoning his earlier hypothesis of its relictual origin (Samuelsson, 1922, 1932).

Hence, the taxonomic status and evolutionary history of *Alisma wahlenbergii* is of great interest, not only because it may represent recent post-glacial speciation but also due to its conservation status. In Sweden and Russia, the species is found in only a few locations, and because the population on the Finnish coast of the Gulf of Finland was thought to have disappeared, the species is globally assessed as Vulnerable (VU; Lansdown, 2011). In Sweden, the species was assessed as Endangered (EN) before 2015, when it was reassessed as VU, and currently, it is assessed as Near Threatened (NT) (SLU Artdatabanken, 2024a). In Finland, the species was assessed as VU in 2000, as EN in 2010, and as VU again in the latest assessment (Rassi & al., 2010; Hyvärinen & al., 2019). However, after the latest Finnish evaluation, the population thought to be lost in the Gulf of Finland has been found again in the old location. On the other hand, *A. gramineum* has never been found in Finland, is considered regionally extinct (RE) in Sweden (SLU Artdatabanken, 2024b), and Data Deficient (DD) globally, even though it is red-listed by most of the countries where it occurs (Lansdown, 2014).

Here, we aim to reappraise the taxonomic status of *Alisma wahlenbergii*, its phylogenetic relationship with the closely related *A. gramineum* and shed light on its biogeographical history in the Baltic Sea region. To address these objectives, we analysed herbarium specimens and fresh collections sampled across the distribution range of both taxa. We delineate genetic population units and reconstruct a time-calibrated phylogeny, which we then correlate with shifts in potential species distribution over the last glacial cycle, as inferred from environmental niche modelling.

■ MATERIALS AND METHODS

Taxon sampling. — We sampled 27 herbarium specimens collected between 1901 and 2023 from herbaria TUR, TUR-A, H and HIB. Additionally, we collected fresh material and sequenced 2 specimens of *Alisma wahlenbergii* from the recently rediscovered Pyhtää population in the Gulf of Finland. Out of the sampled specimens, 6 failed to produce usable sequence reads. Hence, we obtained data for 23 samples, including 8 specimens representing *A. wahlenbergii*, 11 specimens of *A. gramineum*, and 4 outgroup specimens (Appendix 1). The sampling of *A. wahlenbergii* covered the Finnish and Russian populations in the Gulf of Finland, the Swedish population, and the main population from Bothnian Bay. The sampled populations of *A. gramineum* spanned the entire range of the species from Europe to North Africa, China, and North America.

DNA extraction, sequencing, and sequence assembly. — Total genomic DNA from all samples was extracted using a modified 2× CTAB method (Doyle & Doyle, 1987). The quantity and quality of each sample were assessed via 1% agarose gel electrophoresis and a Qubit fluorometer (Invitrogen, Carlsbad, California, U.S.A.). High-quality DNA was sheared by sonication, while severely degraded DNA was used directly for library construction without shearing. Paired-end libraries with an average insert size of 350 bp were prepared using the MGIEasy FS DNA Library Prep Set (MGI Tech., Shenzhen, China) according to the manufacturer's instructions. Following PCR amplification and purification, library quality was evaluated using the Agilent 5400 system and quantified by qPCR. Qualified libraries were then pooled, circularized, and amplified to generate DNA nanoballs. Paired-end libraries with an average insert size of 350 bp were prepared for 150 bp reads and sequenced on the DNBSEQ-T7 platform (MGI Tech.). Approximately 3 Gb of data per sample was generated and subsequently filtered using Fastp v.0.20 with default parameters (Chen & al., 2018).

We used GetOrganelle v.1.6.2e (Jin & al., 2020) to de novo assemble plastome (ptDNA), mitogenome (mtDNA), and nuclear ribosomal genes (rDNA) and their spacer regions with the parameters "-R 50 -t 10 -k 21,45,65,85,105". The assembled genomes were verified and manually adjusted by remapping the reads in Geneious Prime v.2021.1.1 (Biomatters, https://www.geneious.com/). The mitogenomes were annotated using GeSeq (Tillich & al., 2017) with an existing annotated mitogenome of *Butomus umbellatus* (NC_021399) as a reference, and manually adjusting the annotations in Geneious. The plastomes were annotated in Geneious according to published *Alisma* plastomes. Similarly, the gene boundaries in rDNA were detected and annotated in Geneious.

Molecular data. — Preliminary analyses revealed that while ptDNA grouped one outgroup sample (*Cîrțu & al. s.n.*, TUR) with *Alisma plantago-aquatica* L., rDNA and mtDNA sequences grouped it together with *A. lanceolatum* With. Morphologically, the specimen is indistinguishable from *A. lanceolatum*, but due to the mixed and apparently hybrid phylogenetic signal, the specimen was excluded from further analyses. Hence, 22 samples were used in the subsequent analyses.

The three assembled genomes/regions were aligned separately with MAFFT v.7.453 (Katoh & Standley, 2013). Visual inspection of the alignments revealed a 7 bp long inversion within *rpoC2* gene, and a 16 bp long inversion in the *ccsA-ndhD* intergenic spacer region, both in the plastome. These two inversions introduced more sequence variation than was otherwise present in the whole plastome, and consequently, they were excluded from the final alignments to avoid arbitrary results. The rDNA region was split into seven partitions (5′ETS, 18S, ITS1, 5.8S, ITS2, 26S, 3′ETS) because of the highly divergent evolutionary dynamics of the genes and their spacer regions (Poczai & Hyvönen, 2010), whereas ptDNA and mtDNA were not further partitioned. These nine datasets were then submitted to ModelTest-NG v.0.1.6 (Darriba & al., 2020) to search for appropriate evolutionary models under Bayesian information criteria (BIC; Schwarz, 1978). The final data matrices and the resulting trees are available in TreeBASE (31976).

Identification of population units. — First, we used an unsupervised network clustering method, NetView P (Steinig & al., 2016), to assign individuals to populations based on their genetic distances. A range of 1–20 *k*'s was assessed for the *k*-nearest-neighbour search in R package netviewr v.2.1.0 (Steinig & al., 2016) using Euclidean genetic distances. The optimal value of *k* was determined based on the shape of the curve of the mutual *k*-nearest neighbours' graph based on the Infomap and Fast-Greedy algorithms.

Next, we identified genetic population structure (Sukumaran & Knowles, 2017) by determining the population identities of sampled individuals using a "multipopulation coalescent" model in the program BPP v.4.8.0 (Flouri & al., 2018). We conducted a joint Bayesian species delimitation and species tree estimation (A11-analysis) applying the same data partitioning scheme and substitution models previously used in ModelTest-NG. For organellar genomes, we specified inheritance scalars 1/4 that of rDNA, following Hey & Nielsen (2004). The mutation rate was allowed to vary between the loci, for theta and tau parameters gamma shaped priors were set (2 2000), otherwise default parameters were applied. Each sampled individual was treated as a population *a priori*, and the "species" resulting from the delimitation analysis were treated as populations in the following starBEAST analysis. The population delimitation scheme with the highest posterior probability (PP) grouped *Alisma wahlenbergii* from Sweden (the type locality) in the same population as *A. wahlenbergii* from Bothnian Bay, whereas *A. wahlenbergii* samples from the Gulf of Finland were grouped in another population together with some samples of *A. gramineum*. As we were interested in testing the taxonomic status of *A. wahlenbergii*, the population delimitation scheme with the second-highest PP, in which the Finnish and Russian *A. wahlenbergii* from the Gulf of Finland were grouped in a population of their own, was used for downstream analysis (see Results and Discussion below). In this scheme, there were a total of three populations of *A. gramineum* (Pop. 1–3) and two populations of *A. wahlenbergii* (Pop. 4–5).

Phylogenetic analyses. — IQ-TREE v.2.2.2 (Minh & al., 2020) was used to perform a maximum likelihood analysis for concatenated multi-locus dataset. Substitution models selected in the previous step (JC69: 18S, 5.8S, 3′ETS; TN93: 26S; HKY: 5′ETS, ITS1, ITS2; HKY + Γ: ptDNA, mtDNA) were assigned to data partitions, and ultrafast bootstrap approximation (Hoang & al., 2018) with 1000 replicates was run to estimate nodal support (BS). To obtain a reasonable prior for time calibration, we downloaded the Alismataceae ptDNA dataset of Li & al. (2022) and replaced the *Alisma* samples of that study with one of our *A. gramineum* (Alisma530), one *A. lanceolatum* (TUR350538), and our *A. plantago-aquatica* ptDNA sequences. These data were then time-calibrated in IQ-TREE using least square dating (To & al., 2016), estimating the confidence intervals by resampling branch lengths 100 times, and applying the same calibration points used by Li & al. (2022) in their dating analysis. The resulting time-calibrated tree file was processed with treeio v.1.3.13 (Wang & al., 2020) for interpretation.

We then ran a dated multi-species coalescence analysis using starBEAST3 (Douglas & al., 2022) implemented in BEAST v.2.7.6 (Bouckaert & al., 2014). This analysis required the assignment of individuals to populations. Since our sample included individuals from here and there across a wide range, we divided the sampled individuals into genetically defined populations according to our BPP analysis above. Ploidy level was set at 2.0 for all the nuclear partitions and at 0.5 for the uniparentally inherited organellar genomes (Hey & Nielsen, 2004). Site, clock, and tree priors were unlinked. A strict clock and a Calibrated Yule model (Heled & Drummond, 2012) were implemented. These simple models were considered most appropriate for the population-level dataset with very limited molecular variation. In the time-calibrated IQ-TREE analysis, the split between *Alisma lanceolatum* and the remaining *Alisma* was estimated at 4.87 Ma, with the confidence interval ranging from 3.13 to 6.92 Ma. This was used as a root age calibration point for the species tree in starBEAST3 by setting a normal distribution with a mean of 4.87 and a standard deviation (sigma) 1.0 to approximate the 95% prior distribution within the estimated time interval. As the calibrated IQ-TREE analysis was based on ptDNA data, we used the previously estimated rate of 3×10^{-3} substitutions per site per million years for ptDNA evolution (Wolfe & al., 1987) as a mean for the log-normal clock rate prior to the species tree. The original rate estimate of 1×10^{-3} to 3×10^{-3} was calculated based on selected plastid genes (Wolfe & al., 1987) and we used the highest end of this range because our complete ptDNA genomes include also the non-coding introns and intergenic spacers. Correspondingly, a log-normal clock rate prior with a mean of 1 was set for the ptDNA gene tree. Clock rate priors for other gene trees were set relative to ptDNA rate according to their published rates, i.e., a mean of 0.3 for mtDNA (Zwonitzer & al., 2024), 1.4 for ITS and ETS regions (Kay & al., 2006), 0.05 for 18S and 5.8S genes (Savard & al., 1994), and 0.09 for the 26S gene (Kuzoff & al., 1998). Sigma was set to 1.25 in each case. Because various priors may together produce an unwarranted joint prior in combination (Heled & Drummond, 2012), we verified that the joint priors were reasonable by running a chain of 4×10^9 generations sampling from the prior. We then ran four chains of 4×10^8 generations, sampling every 10,000 generations. LogCombiner v.2.7.6 (http://www.beast2.org) was used to combine the resulting log and tree files after discarding 10% as burn-in, and effective sample sizes (ESS) were assessed in the program Tracer v.1.7 (Rambaut & al., 2018). The posterior trees were visualized in DensiTree v.2.7.7 (Bouckaert, 2010) and summarized with TreeAnnotator v.2.7.7 to find the maximum credibility trees (Heled & Bouckaert, 2013).

Species delimitation. — Species boundaries were tested with the species delimitation program Delineate v.1.2.3 (Sukumaran & al., 2021), replicating the analysis of over 100 species trees randomly sampled from the starBEAST posterior. We assigned a status of known identification to the *Alisma gramineum* populations (Pop. 1–3) and a status of unknown identification to *A. wahlenbergii* populations (Pop. 4–5). Two competing species delimitation hypotheses were then tested using the "constrained" mode: (H0) assuming that *A. wahlenbergii* and *A. gramineum* are conspecific (Pop. 1–5 are conspecific), or (H1) assuming that populations of *A. wahlenbergii* from Sweden, Bothnian Bay and the Gulf of Finland form a species distinct from *A. gramineum* (Pop. 4–5 form an exclusive new species). The marginal probabilities (MPs) of these competing hypotheses were then summarized to test the molecular support of the alternative species-level taxonomies explicitly.

Morphological analysis. — For a morphological analysis, we measured the following 18 characters from the individuals sampled for molecular analyses (excluding the two Chinese samples that we were unable to access for measurement): (1) number of leaves per rosette, (2) submerged blade width / blade length, (3) emerged blade width / blade length, (4) blade length / petiole length, (5) inflorescence length, (6) inflorescence length / leaf length, (7) peduncle length / inflorescence length, (8) number of whorls in inflorescence, (9) number of flowers in lowermost whorl, (10) bract length in lowermost whorl, (11) pedicel length, (12) sepal length, (13) anther length, (14) anther length / filament length, (15) style length / ovary length, (16) achene length, (17) achene length / achene width, (18) distance from the base of fruit to fruit beak / fruit length. Measurements were rescaled, and a principal component analysis (PCA) was performed in R (R Core Team, 2018).

Environmental niche modelling. — We aimed to explore whether environmental niche modelling (ENM) can help elucidate the origins and history of *Alisma wahlenbergii*. For this purpose, we first downloaded the occurrences of *A. gramineum* from GBIF (2024a,b). Duplicate records were removed, and occurrences in mainland western-central Europe were thinned by randomly keeping 20% of the records from this region to avoid overly European-biased models. Records were further thinned in R by keeping just one record per raster grid cell (10 arc-minute scale) of the climatic layers. The remaining occurrence points were plotted on a map, and dubious records (falling in the sea or outside the recorded distribution of the species or representing what is here treated as *A. wahlenbergii*) were manually removed. In the end, 1262 occurrence records were kept. We did not use the occurrences of *A. wahlenbergii* for niche modelling because the taxon only occurs underwater and is thus buffered against many climatic variations, and we were interested in testing whether the niche model of "pure" *A. gramineum* could predict the occurrence of *A. wahlenbergii* in the Baltic Sea region.

To build the current ENM, we used the CHELSA bioclimatic variables on a 10 arc-minute scale (Karger & al., 2017, 2018). The model calibrated on the current climate was then projected onto paleoclimatic scenarios (Brown & al., 2018) covering the Holocene (Fordham & al., 2017) until the last interglacial period ca. 130,000 years ago (Otto-Bliesner & al., 2006). ENMTools v.1.1.0 (Warren & al., 2021) was used to find correlated variables, and Maxent v.3.4.4 (Phillips & Dudík, 2008; Phillips & al., 2024) was used to build the model after dividing the data into training (75% of occurrence points) and validation (25% of occurrence points) sets. After an initial run using all the 19 bioclimatic variables and applying a jackknife test, a set of eight important and least correlated variables were selected for final modelling (BIO1 Annual mean temperature, BIO4 Temperature seasonality, BIO10 Mean temperature of warmest quarter, BIO11 Mean temperature of coldest quarter, BIO12 Annual precipitation, BIO14 Precipitation of driest month, BIO15 Precipitation seasonality, BIO16 Precipitation of wettest quarter). The final model was then projected on paleo-environmental scenarios using 10 bootstrap replications, with clamping and extrapolation turned off (Owens & al., 2013). Potential distributions were then plotted using the "maximum test sensitivity plus specificity" threshold. These thresholded distribution predictions were then averaged over all 10 bootstrap replicates with a custom written R script for presenting the results. ChatGPT (OpenAI, 2024) was used to assist in writing the R codes used in this study.

■ RESULTS

IQ-TREE analysis. — The IQ-TREE analysis using concatenated data resolved *Alisma wahlenbergii* samples into two fully supported but distinct clades nested deeply within *A. gramineum* (Fig. 1). Together, *A. gramineum* and *A. wahlenbergii* formed a fully supported monophyletic lineage. A sample of *A. gramineum* (lzzdb0025) from eastern China formed a clade with a sample from the western U.S.A. (BS = 93), and together they were resolved as sister to the remaining of the *A. gramineum–A. wahlenbergii* clade (BS = 70). *Alisma wahlenbergii* samples originating from the Gulf of Finland grouped with European (Netherlands, Estonia, Hungary, Poland) samples of *A. gramineum* (BS = 100), whereas the samples from Bothnian Bay formed a sister clade to the Swedish *A. wahlenbergii* (BS = 100). Together, they were associated (BS = 66) with a clade composed of samples from western China and eastern Canada (BS = 94).

NetView network clustering analysis. — In the NetView network clustering analysis, the *k*-nearest-neighbours' graph started to plateau from *k* = 5 onwards (Fig. 2A). From *k* = 5, 6, …,18, all *Alisma gramineum* and *A. wahlenbergii* populations formed a cluster together with a varying degree of connectivity, two *A. lanceolatum* populations formed another cluster, and the single *A. plantago-aquatica* was separated. This three-cluster interpretation seems most reasonable also in light of the phylogenetic analyses (Fig. 2B).

Bayesian population delimitation. — The Bayesian population delimitation based on coalescence analysis in BPP supported seven to nine populations in the full dataset. The outgroup species (*Alisma lanceolatum*, *A. plantago-aquatica*) were consistently supported as two separate populations, resulting in five to seven distinct populations within the *A. gramineum–A. wahlenbergii* clade. The best model (PP = 0.031200) supported a total of seven populations, with *A. wahlenbergii* from the Gulf of Finland assigned to the same population as *A. gramineum* from the Netherlands (C.452789) and Egypt (TUR129479). To test the taxonomic status of *A. wahlenbergii*, we selected the second-best BPP model (PP = 0.030680) for further analysis. This model also supported seven populations in total, but *A. wahlenbergii* from the Gulf of Finland (including Finnish and Russian plants) were grouped in their own population (Pop. 5), and *A. wahlenbergii* from Bothnian Bay were assigned to another population with the Swedish sample (Pop. 4). The plant from the western U.S.A. was placed in the same population as the plant from East China (Pop. 1). The Ukrainian plant was assigned to a population of its own (Pop. 3), and the remaining samples from Europe, Africa, eastern North America, and West China were all placed in one population (Pop. 2). The third-best BPP model had a markedly lower probability (PP = 0.018820) but grouped *A. wahlenbergii* the same way as the second-best model.

starBEAST analysis. — ESS values were >500 for most of the estimated parameters, but apparently, due to very limited sequence variation, the TreeDistanceUPGMA.t and TreeDistanceNJ.t parameters remained between 78 and 178 for some of the gene trees. We do not believe this compromises our results, as ESS values were >3000 for the parameters we were mostly interested in, such as posterior, likelihood, root age, and all tree heights. Root age posterior matched the prior and therefore the reliability of the time calibration can be considered fully dependent on the accuracy of the IQ-TREE calibration used to create the age prior. The split between *Alisma*

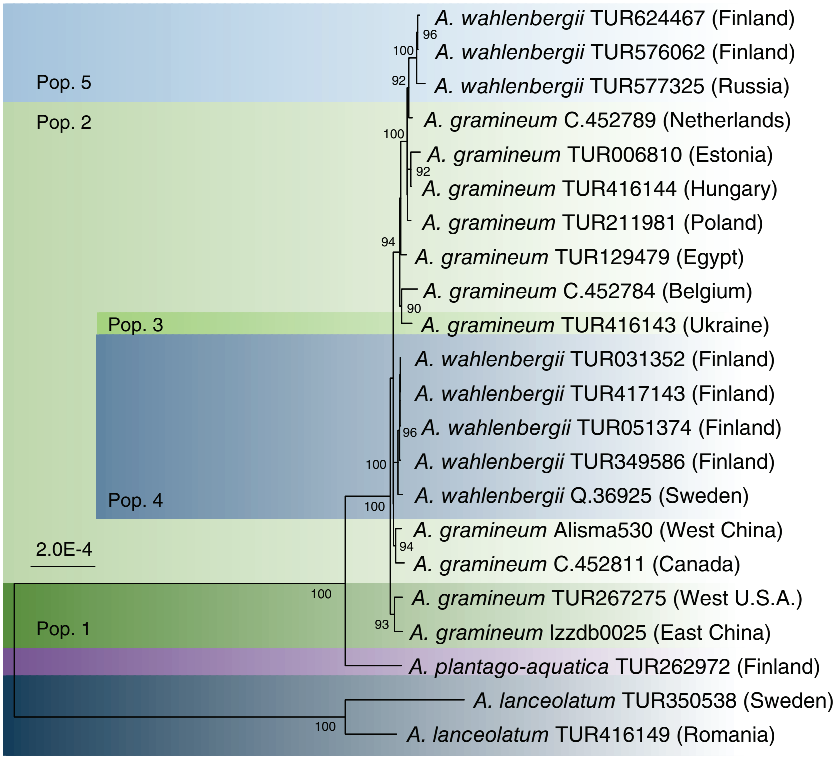

Fig. 1. IQ-TREE phylogeny of concatenated data with bootstrap support >75 indicated. Colours and population coding correspond to populations identified in the BPP analysis.

plantago-aquatica and *A. gramineum* was estimated at ca. 0.3 Ma, which was at the lower end of the IQ-TREE estimate for this node (0.22–1.47 Ma). The starBEAST analysis resolved the two *A. wahlenbergii* populations (Pop. 4 & 5) nested within *A. gramineum*, and the population formed by the samples from eastern China and the western U.S.A. (Pop. 1) was resolved as sister to the remaining populations (PP 0.89), similar to the IQ-TREE analysis (Fig. 3). The deepest split within *A. gramineum* was estimated at ca. 0.16 Ma (95% HPD: 0.08–0.24 Ma). The widespread Pop. 2 was a sister (PP 0.92) to *A. wahlenbergii* populations (with a divergence time of 0.06 Ma, 0.02–0.09 Ma), which were resolved as sisters without support (PP 0.51) and estimated divergence at 0.05 Ma (0.01–0.06 Ma). The Swedish sample of *A. wahlenbergii* was resolved in the ptDNA and mtDNA gene trees together with the plants from Bothnian Bay (PP > 0.99), consistent with the concatenated IQ-TREE analysis and the BPP population delimitation. However, 5′ETS, 3′ETS and ITS2 gene trees resolved it together with *A. wahlenbergii* from the Gulf of Finland (PP > 0.99; Fig. 4B). The remaining loci were practically invariable within the *A. gramineum*–*A. wahlenbergii* clade.

Species delimitation with Delineate. — Species delimitation with Delineate was run for 100 species trees randomly sampled from the starBEAST posterior. These analyses overwhelmingly supported the conspecificity of all studied *A. gramineum* and *A. wahlenbergii* populations, with marginal probabilities for H0 ranging from 0.77 to 0.94 (Fig. 4A). In contrast, marginal probabilities for the two *A. wahlenbergii* populations forming a species exclusively distinct from *A. gramineum* (H1) ranged from 0.00 to 0.06.

Principal component analysis of morphological data. — In the PCA of the morphological data, the first two axes explained 46.8% of the total variance (31.3% and 15.5% for axes PC1 and PC2, respectively). The first axis mainly correlated with the submerged vs. emerged habit by grouping the emerged samples of *Alisma gramineum* close to the samples of *A. lanceolatum* and *A. plantago-aquatica*, all of which were emergent individuals (Fig. 2C). This analysis did not clearly distinguish *A. wahlenbergii*, nor did it group them together.

Environmental niche models. — The Maxent model for the current climate yielded an average test AUC (area under the curve) of 0.890 with a standard deviation of 0.006. The thresholded predictions averaged over the 10 bootstrapped replicates under the present climate supported conditions suitable for *Alisma gramineum* along the coasts of the Gulf of Finland and over the range of *A. wahlenbergii* in southern Sweden. However, Bothnian Bay was not predicted as suitable area (Fig. 5). Similarly, the southernmost occurrence points in Egypt and Israel, and the populations in Central Asia were outside the predicted distribution. The disjunct area of occurrence in the state of Missouri, U.S.A., was hardly predicted to be suitable. In contrast, the northern Iberian Peninsula, Turkey, and eastern North America had high predicted suitability without actual observations, except for eastern North America, where the observed range is nevertheless far narrower than predicted.

Under the paleoclimatic models, the potential distribution showed some noteworthy patterns. During the last interglacial (ca. 130 ka), the projected range in Europe was more expansive than at present, but Asia and North America were estimated to be less suitable. During the glacial maximum (ca. 21 ka), the projected distribution withdrew south and expanded to northern Africa. Disjunct, highly suitable areas were forecasted in China and Central Asia. In Europe, the largest suitable area remained north of the Black Sea, with perhaps disjunct pockets of suitable areas around the Mediterranean. During the Heinrich Stadial (17.0–14.7 ka), the

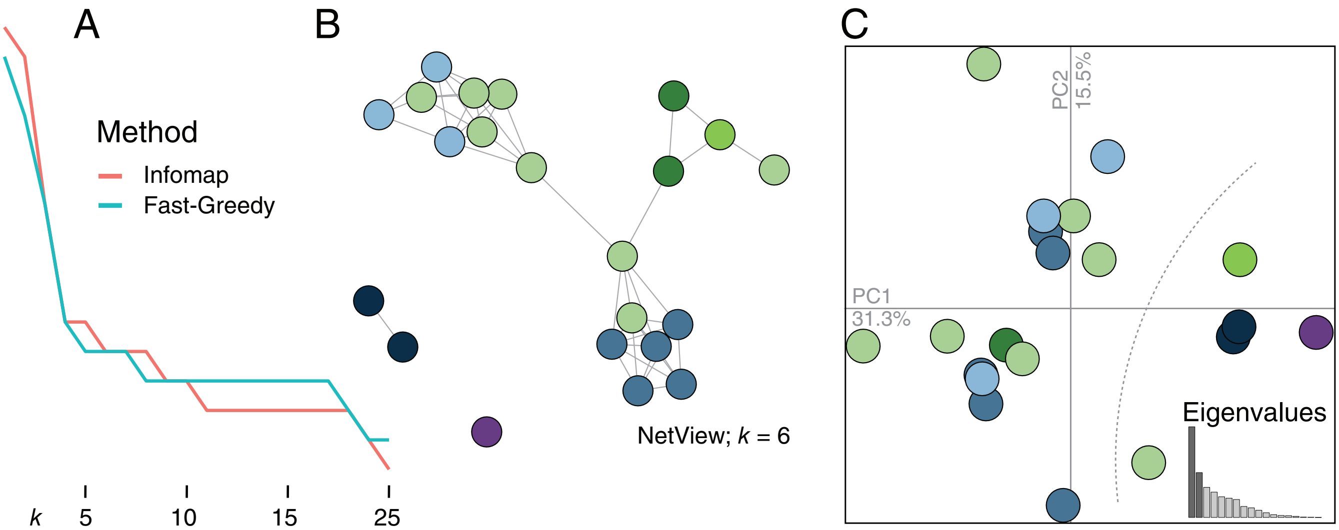

Fig. 2. **A**, *k*-selection plot; **B**, NetView network based on genetic distances at $k = 6$; **C**, PCA of the morphological data. The dotted arch splits submerged plants on the left and emerged plants on the right side. Individuals are coloured according to their population assignments by BPP using the same colour scheme as in Fig. 1.

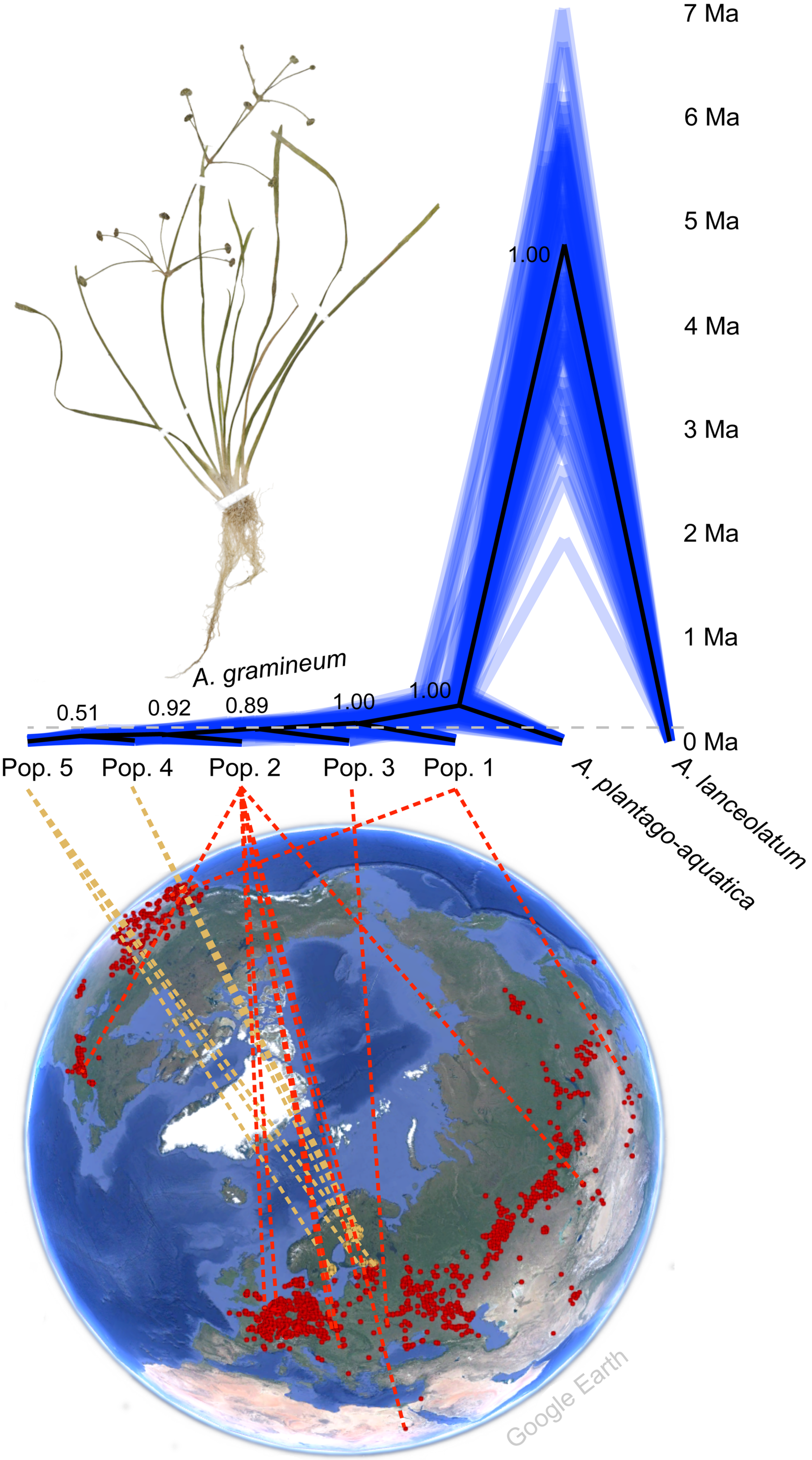

Fig. 3. DensiTree visualization of 100 randomly sampled species trees from the posterior of the starBEAST analysis. Populations delimited by BPP are numbered as shown in Fig. 1. Global distributions and sampled specimens of *Alisma wahlenbergii* and *A. gramineum* are shown. The gray dashed line marks the last interglacial 130,000 years ago. Numbers at nodes are posterior probabilities. Map: Google Earth v.7.3.6.9796.

suitable area north of the Black Sea greatly expanded, and another sizeable suitable area emerged in Beringia, but the suitable area in China nearly disappeared. The suitable area further expanded during the Bølling-Allerød Interstadial (14.7–12.9 ka), with a much wider predicted distribution in Europe and expansion into Central Asia, and with the large suitable area remaining in Beringia and new suitable areas emerging near it on the Asian side. During the Younger Dryas Stadial (12.9–11.7 ka), the suitable area in Beringia disappeared. In Fennoscandia, no suitable conditions were projected until the early Holocene (11.7–8.3 ka), when the expanding East-European area of potential occurrence reached southernmost Karelia. During the mid-Holocene (8.3–4.2 ka), the suitable area expanded around the southern Baltic Sea and through Karelia and Central Finland up to Bothnian Bay in many, but not all, of the bootstrapped replicates. During the late Holocene, the projected suitable area withdrew southwards until, according to the present-day prediction, it again expanded northwards to the Gulf of Finland.

■ DISCUSSION

Our results provide compelling evidence that *Alisma wahlenbergii* should be considered conspecific with *A. gramineum*. In the cluster analysis based on genetic distances, three clusters emerged as soon as the *k*-nearest-neighbours' graph started to plateau, representing the two outgroup species and one cluster for *A. gramineum*–*A. wahlenbergii*. The Bothnian Bay and Gulf of Finland populations were genetically quite distant within that cluster. However, raw genetic distances across the different genomes overlook much of the biologically relevant information in genetic data.

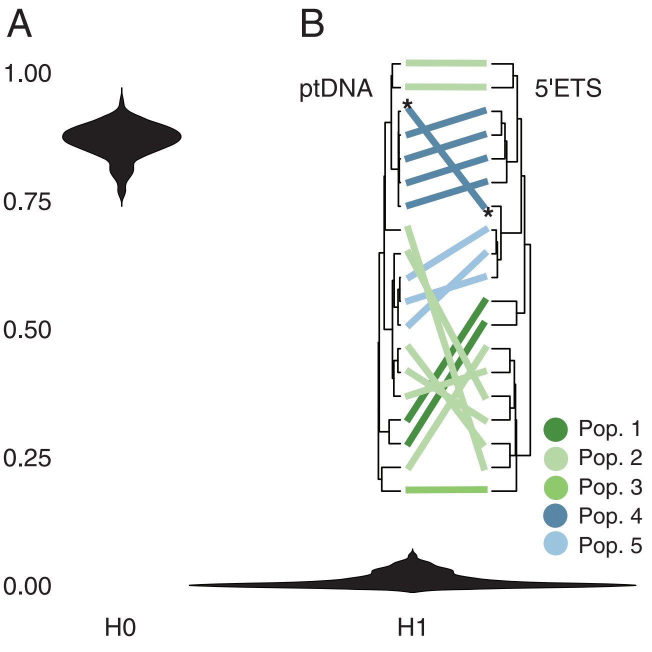

Fig. 4. **A**, Marginal probabilities of conspecificity of *Alisma wahlenbergii* and *A. gramineum* (H0), and exclusive conspecifity of the two populations of *A. wahlenbergii* (H1), computed with Delineate over 100 posterior trees from the starBEAST analysis; **B**, starBEAST gene trees for ptDNA and 5′ETS, excluding the outgroups. The Swedish sample of *A. wahlenbergii*, with a conflicting phylogenetic position, is indicated by asterisks.

For this reason, we also applied a multispecies coalescent model. Because the results of the multispecies coalescent model reflect the genetic structure, some of which is likely at population level, the model cannot be reliably used for species delimitation (Sukumaran & Knowles, 2017). Hence, we took a three-step approach, whereby data were first structured into genetic populations with BPP after which time-calibrated population phylogeny was inferred with starBEAST. We then applied Delineate to test alternative species delimitation hypotheses on this population phylogeny.

In the resulting phylogeny, samples of *Alisma wahlenbergii* were deeply nested within *A. gramineum*, and the BPP analysis suggested that plants from the Gulf of Finland should be grouped together in the same population with some samples of *A. gramineum*. Given these results, it is quite evident that such genetic isolation Björkqvist (1968) believed in when defending the species status of *A. wahlenbergii* does not exist, albeit our sampling of the nuclear genome is admittedly very poor.

Additionally, the Delineate analysis overwhelmingly supported the single-species hypothesis. This is not surprising, given that already Juzepczuk (1934), who first recognised *Alisma wahlenbergii* at species level, called for experimental studies to confirm the uncertain taxonomic status. Later, Björkqvist (1968) conducted experimental taxonomic studies in *Alisma*, but did not have *A. wahlenbergii* in cultivation. We are aware of only one unpublished experimental study on *A. wahlenbergii*. In the growing experiments, the plants from the Bothnian Bay population failed to produce aerial leaves and regularly died when exposed to emerged conditions (Virtanen, 1958). This could be taken as evidence of adaptation to obligatory submerged habit, but the Bothnian Bay population grouped together with the Swedish plants, which are able to produce aerial leaves (Björkqvist, 1968). Of course, aerial leaf production may have simply failed in the experiment, or the Swedish population may have regained this trait via hybridization, as they share similar rDNA with the plants from the Gulf of Finland, or they may represent a different stage of evolution, as Björkqvist (1968) suggested. Nevertheless, our results refute the genealogical integrity of what has been considered as *A. wahlenbergii* in the Baltic Region.

The ENM projections on past climate conditions indicated that the Bothnian Bay population may have originated via range expansion from the Ice Age Black Sea refugium towards the northwest and through Karelia up to Bothnian Bay during the mid-Holocene (8.3–4.2 ka). An unconfirmed old report from Lake Pukalus in Karelia (Krohn, 1926) is particularly interesting in this respect. Concurrent range expansion from the Black Sea refugium towards Central Asia was also projected. This scenario is fully congruent with the plastome data suggesting that the Swedish–Bothnian Bay populations

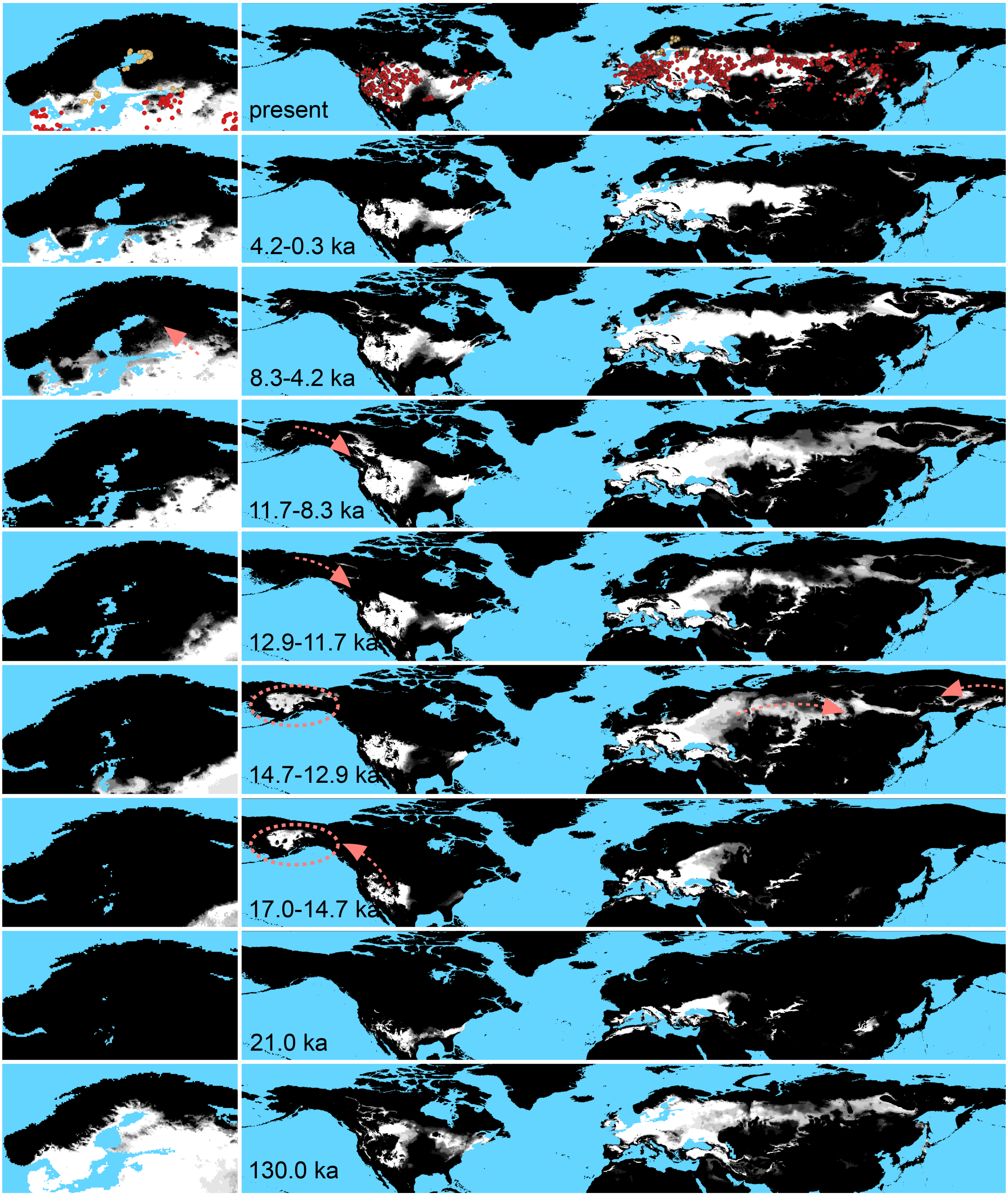

Fig. 5. Maxent model predicted under current climatic conditions and projected on Pleistocene-Holocene climate scenarios. Potential distributions averaged over 10 thresholded bootstrap replicates. Dotted red circles highlight the large suitable area in Beringia at 17.0–12.9 ka. Red arrows indicate possible dispersal routes. Dots on the present-day map indicate the occurrence points used for modelling (red, *Alisma gramineum*) and the range of *A. wahlenbergii* (orange, not used for modelling; GBIF, 2024c).

are close to the plants from western China. Unfortunately, we did not have any samples from the putative Black Sea refugium to verify this hypothesis.

In contrast, the population of the Gulf of Finland is associated with central European plants in organellar phylogenies, although rDNA suggested a closer relationship with the Bothnian Bay–western Chinese plants. Despite this, the Gulf of Finland and Bothnian Bay populations are clearly differentiated even according to rDNA and were consistently supported as distinct populations by the BPP analysis. The starBEAST analysis suggested that rDNA, mtDNA and ptDNA were segregated between these populations likely during the Ice Age, but before the Last Glacial Maximum (LGM), which could have fragmented the populations into separate refugia. Following these results, it is tempting to presume that the central European populations spread from a different Ice Age refugium, probably located somewhere around the Mediterranean, and arrived at the Baltic Sea somewhat later than the population spreading from the southeast. This would have resulted in intermixing between the populations at the contact zone, possibly somewhere around the Gulf of Finland. Our dating results are not entirely conclusive, due to their wide error margins, but does not rule out this scenario. If we assume that genetic segregation among the European populations is a result of population isolation during the LGM instead of some prior events, then we must consider the youngest age estimates allowed by our starBEAST analysis to be the most reliable. These estimates put the genetic split between the Gulf of Finland and Bothnian Bay populations in the individual gene trees around the onset of LGM, ca. 21 ka (Kucera & al., 2005). However, the minimum age estimate for the split between Swedish *Alisma wahlenbergii* from either the Gulf of Finland or Bothnian Bay populations, depending on the genetic marker, is around 10 ka. This is also the estimated minimum age for the split of the two distinct *A. wahlenbergii* populations in the starBEAST species tree, which is, given the uncertainties in dating such recent events with limited genetic data, very close to our projected mid-Holocene colonization of the Baltic Sea.

Indeed, there seems to be a gap in the current distribution of *Alisma gramineum* between Europe and western Russia, possibly reflecting expansion from different refugia. Furthermore, at least some Russian botanists consider *A. wahlenbergii* as a widespread species far beyond the Baltic Sea region in the European part of Russia (Durnikin & Zinovyeva, 2014), in line with the biogeographic hypothesis outlined here. Further sampling is needed to clarify if the western Russian population has been genetically isolated from the European population. Likewise, it would be important to sequence old samples of the now extinct *A. gramineum* populations from South Sweden to see if they represent the western lineage, in contrast to the Swedish *A. wahlenbergii*. According to our hypothesis, changing climatic conditions after the range expansion to the Baltic Sea from two different sources expelled all the northern populations around four thousand years ago, except the ones surviving buffered from the deteriorating climate by remaining submerged along the seacoast.

Under this hypothesis, the Baltic Sea populations indeed are relictual as originally suggested by Samuelsson (1922), who correlated the biogeographic history of *A. wahlenbergii* with that of *Najas tenuissima* A.Braun ex Magnus, and pointed out the occurrence of the latter one as subfossils in Karelia. Alternatively, the Baltic Sea populations may have dispersed to their current areas underwater, similarly buffered from the unsuitable climate, across the shorelines of the Baltic Sea. Indeed, in Bothnian Bay, *A. wahlenbergii* is capable of rapidly colonizing new habitats even kilometres away from the source populations (Virtanen, 1958). This hypothesis corresponds with the idea of "progressive endemism" suggested by Samuelsson (1934) after he gave up his original idea of relictual origin. We consider this "adaptive expansion" less likely than mid-Holocene range expansion as a response to suitable climate through Karelia.

Taxonomically, we consider *Alisma wahlenbergii* to be a synonym of *A. gramineum* and view the Baltic Region as a peripheral hybrid zone between different population lineages rather than an example of budding speciation. Nevertheless, even if we reject the species status of *A. wahlenbergii*, it can still be asked if these relict populations deserve taxonomic recognition at some lower rank. Indeed, since the original description of *A. gramineum* subsp. *wahlenbergii* by Holmberg (1922), the taxon has most often been treated at some infraspecific rank. The potential meaning of the infraspecific categories has been very little discussed in the context of phylogenetic systematics (but see Stuessy, 2014; Oberprieler, 2023), but genealogical, ecological, morphological, and geographical integrity have been typical ranking criteria.

Besides lacking genealogical integrity, *Alisma wahlenbergii* does not seem to be ecologically too distinct, nor coherent either. Submerged habit as such is not unusual for *A. gramineum*, and it is often reported from saline environments (Durnikin & Zinovyeva, 2013; Krasnova & al., 2013; Golub & al., 2017), so the brackish water habitats in the Baltic Sea are not unusual, and the Swedish type locality is a freshwater lake. In Bothnian Bay, the plants are only found on soft, silty bottoms, whereas the plants in Pyhtää grow on hard bottoms among rocks. On the other hand, other populations in the Gulf of Finland seem to grow on soft bottoms (Erkamo, 1942). Nevertheless, *A. wahlenbergii* does not appear to be a clearly uniform and distinct ecotype of *A. gramineum*. Furthermore, our morphometric analysis failed to recognise uniform *A. wahlenbergii* or differentiate it from the highly variable *A. gramineum*. It also remains unclear how far the name should be applied, as in Russia, it is considered widespread (Durnikin & Zinovyeva, 2014), questioning even the geographical integrity.

Besides the Baltic region other, similarly peripatric, potentially relict, or otherwise ecologically marginal populations exist. None of the projected paleodistributions suggested the presence of suitable habitats in Egypt, where the species nevertheless has been observed. This perhaps can be explained as random and short-lived populations dispersed by migrating waterfowl, or as relic populations from the Ice Ages, even if ENMs do not support this view. Similar occurrences out of the predicted range were scattered here and there in Central Asia, and they may have similar histories. Perhaps a somewhat different case is the disjunct pocket of occurrences in Missouri, U.S.A. Under the current climate, this region is predicted to have quite a low probability of occurrence, but during the Holocene, the suitability of the region has been higher at times. These populations differ ecologically from the other American populations by growing submerged in cool, fast-flowing streams, in contrast to exposed muddy shores with fluctuating water levels, and their taxonomic status has been called into question (Flora of Missouri, 2024).

Unfortunately, our sampling in North America does not shed light on this problem. We sampled one individual from the western and one from the eastern populations. The western sample was found to be related to a sample from eastern China that was not close to the sample from western China. Our ENM showed a broad area of potential occurrence in Beringia 17–12.9 thousand years ago. We suggest this region maintained the ancestral population of these samples and that eastern China was later colonized from Beringia, whereas western China was separately colonized from the west. This scenario is not contradicted by our dating results, but due to a lack of precision in age estimates cannot be considered supported either. As the split between this group and the rest of the species was estimated at the Last Interglacial (Fig. 3; 243–85 ka), it may suggest a possible vicariance between North America and Eurasia during the Ice Age and colonization of East China from North America through Beringia. Further sampling is needed to test this scenario and to verify the origins of the disjunct Missouri population. Our sample from eastern North America was related in the ptDNA tree to the presumed Black Sea refugium (i.e., *Alisma wahlenbergii* from Bothnian Bay and *A. gramineum* from western China). In the literature, the species has been described as a rapidly spreading "pesky weed" in eastern North America (Raymond & Kucyniak, 1948; Countryman, 1968). Still, the area predicted suitable for the species in eastern North America is far wider than the observed distribution. Hence, the species has an independent and more recent origin in eastern North America. It is genetically distinct enough from our Eurasian samples that anthropogenic introduction seems unlikely, but we may have simply missed more closely related Eurasian populations. Hence, further sampling from not only North America but also western Russia and Central Asia is needed to confirm the colonization history suggested here.

Luckily, our study also demonstrated that herbarium material can be successfully utilized to answer these questions. Herbariomics, the genomic studies from herbarium collections, is, therefore, a very promising avenue for botanists. Although our study provided several significant insights, it could have been even more enlightening if we had access to more comprehensive data from the nuclear genome. This is something future studies should address, in addition to more widespread sampling, especially from the regions here highlighted as potentially relevant for understanding the post-glacial dispersal history of the species.

■ AUTHOR CONTRIBUTIONS

SL conceived the original idea for the study. Z-ZL and J-MC obtained funding. SL, Z-ZL and J-MC provided field observations and specimens, SL produced and analysed the data from herbarium specimens, Z-ZL generated the DNA sequence data. SL and Z-ZL analysed and interpreted the data, and SL wrote the manuscript. All authors participated in revising the manuscript and approved the final version.

■ ACKNOWLEDGEMENTS

We thank herbaria TUR, TUR-A, H and HIB for providing material for the work, and Southeast Finland ELY Centre for the permission (KASELY/2248/2022) to collect the protected *Alisma wahlenbergii* from Pyhtää. Funding for the study was provided by the National Natural Science Foundation of China (No. 32070253). Open access publishing facilitated by Turun yliopisto, as part of the Wiley - FinELib agreement.

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Appendix 1. Voucher information and accession numbers (rDNA; ptDNA; mtDNA). All sequences generated for this study.

Alisma gramineum Lej., Poland, Wisła Wielka (1954), *Sychowa s.n.* (TUR211981), PV034693; PV366428; PV541407, PV541408, PV541409, PV541410, PV541411, PV541412, PV541413, PV541414, PV541415; Egypt, Nag Kallosa (1964), *Boulos s.n.* (TUR129479), PV034691; PV366427; PV541399, PV541400, PV541401, PV541402, PV541403, PV541404, PV541405, PV541406; U.S.A., Oregon (1979), *Wright s.n.* (TUR267275), PV034686; PV366431; PV541431, PV541432, PV541433, PV541434, PV541435, PV541436, PV541437, PV541438; Ukraine, Kyiv (1901), *Lonatschewskij s.n.* (TUR416143), PV034689; PV366434; PV541457, PV541458, PV541459, PV541460, PV541461, PV541462, PV541463, PV541464, PV541465; Estonia, Pärnumaa (1936), *Spohr s.n.* (TUR006810), PV034692; PV366424; PV541373, PV541374, PV541375, PV541376, PV541377, PV541378, PV541379, PV541380, PV541381; Hungary, Szeged (1926), *Boros s.n.* (TUR416144), PV034688; PV366435; PV541466, PV541467, PV541468, PV541469, PV541470, PV541471, PV541472, PV541473, PV541474; Canada, Québec (1987), *Blondeau 87615* (H: C.452811), PV034687; PV366419; PV541329, PV541330, PV541331, PV541332, PV541333, PV541334, PV541335, PV541336, PV541337; Belgium, Stuivekenskerke (1977), *De Raeve 77/117* (H:

Appendix 1. Continued.

C.452784), PV034694; PV366417; PV541311, PV541312, PV541313, PV541314, PV541315, PV541316, PV541317, PV541318, PV541319; Netherlands, Zandvoort (1977), *Rigelberg-Gieser s.n.* (H: C.452789), PV034690; PV366418; PV541320, PV541321, PV541322, PV541323, PV541324, PV541325, PV541326, PV541327, PV541328; China, Xinjiang, *Chen s.n. Alisma530* (HIB), PV034678; PV366416; PV541302, PV541303, PV541304, PV541305, PV541306, PV541307, PV541308, PV541309, PV541310; China, Zhangjiakou, *Li s.n. Lzzdb0025* (HIB), PV034695; PV366420; PV541338, PV541339, PV541340, PV541341, PV541342, PV541343, PV541344, PV541345. ***Alisma lanceolatum*** With., Romania, Oltenia (1970), *Cîrţu & al. s.n.* (TUR262891), PV034677; PV366429; PV541416, PV541417, PV541418, PV541419, PV541420, PV541421, PV541422, PV541423; Romania, Şelimbăr (1906), *Barth s.n.* (TUR416149), PV034676; PV366436; PV541475, PV541476, PV541477, PV541478, PV541479, PV541480, PV541481, PV541482; Sweden, Gotland (1996), *Alho & al. s.n.* (TUR350538), PV034675; PV366433; PV541448, PV541449, PV541450, PV541451, PV541452, PV541453, PV541454, PV541455, PV541456. ***Alisma plantago-aquatica*** L., Finland, Paltamo (1969), *Hyvärinen & Laine s.n.* (TUR262972), PV034696; PV366430; PV541424, PV541425, PV541426, PV541427, PV541428, PV541429, PV541430. ***Alisma wahlenbergii*** (Holmb.) Juz., Sweden, Uppland (1917), *Björkman s.n.* (H: Q.36925), PV034679; PV366421; PV541346, PV541347, PV541348, PV541349, PV541350, PV541351, PV541352, PV541353, PV541354; Finland, Pyhtää (2023), *Lehtonen & Salo 1121* (TUR624467), PV034680; PV366422; PV541355, PV541356, PV541357, PV541358, PV541359, PV541360, PV541361, PV541362, PV541363; Finland, Pyhtää (2023), *Lehtonen & Salo 1128* (TUR576062), PV034681; PV366423; PV541364, PV541365, PV541366, PV541367, PV541368, PV541369, PV541370, PV541371, PV541372; Russia, Saint Petersburg (1962), *Kozhanchikov s.n.* (TUR577325), PV034682; PV366437; PV541493, PV541494, PV541495, PV541496, PV541497, PV541498, PV541499, PV541500; Finland, Oulunsalo (1959), *Såltin s.n.* (TUR031352), PV034683; PV366425; PV541382, PV541383, PV541384, PV541385, PV541386, PV541387, PV541388, PV541389; Finland, Kalajoki (1968), *Torvi s.n.* (TUR051374), PV034685; PV366426; PV541390, PV541391, PV541392, PV541393, PV541394, PV541395, PV541396, PV541397; Finland, Simo (2004), *Juntunen & Kuoppala s.n.* (TUR417134), PV034674; PV541173; PV541483, PV541484, PV541485, PV541486, PV541487, PV541488, PV541489, PV541490, PV541491, PV541492; Finland, Raahe (1991), *Särkkä 145* (TUR349586), PV034684; PV366432; PV541439, PV541440, PV541441, PV541442, PV541443, PV541444, PV541445, PV541446, PV541447.