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RESEARCH ARTICLE

Germination and seed traits in common alder (*Alnus* spp.): the potential contribution of rear-edge populations to ecological restoration success

Inês Gomes Marques[1,2], Carla Faria[1], Sofia Isabel Rodrigues Conceição[3], Roland Jansson[4], Tamara Corcobado[5,6], Slobodan Milanović[7,8], Yann Laurent[9], Ivan Bernez[9], Simon Dufour[10], Bohumil Mandák[11,12], Hassan Ennouni[13], Abdelouahab Sahli[13], Mohammed Ater[13], Francisco Javier Dorado[14], Ana Delaunay Caperta[3], Teresa Soares David[1,15], Alejandro Solla[14], Patricia María Rodríguez-González[1]

The degradation of riparian ecosystems occurring throughout the past decades has motivated efforts aimed at the restoration of these ecosystems. The success of active revegetation approaches to restoration requires appropriate selection of reproductive material, which in turn requires knowledge of seed traits and germination. *Alnus glutinosa* (L.) Gaertn. (common alder) is a key riparian tree widely used in restoration projects, and has recently been classified as comprising three species: *A. glutinosa*; *A. lusitanica* Vít, Douda, & Mandák; and *A. rohlenae* Vít, Douda, & Mandák. To help guide restoration species selection, we assessed differences among populations of these species by (1) investigating seed weight, morphology, and germination success from a large population set and (2) modeling germination success in each species in relation to morphological traits and environmental conditions. Seeds were collected from 12 populations encompassing the latitudinal extremes of the species complex, and were then characterized and germinated. Ploidy levels and species were distinguished using cytometric analysis. Site-level climatic data and seed morphology data were used to model germination success for each species. All seed traits differed between populations and one morphological-trait (seed weight-to-area ratio) differed significantly between the three species. Germination modeling showed that the southwestern species, *A. lusitanica*, responded positively to high temperature extremes, suggesting tolerance to the climate changes projected for southern Europe. Populations of *A. lusitanica* located at the latitudinal rear edge of common alder's distribution appear to show establishment-facilitating adaptations, and therefore may contribute to ecological restoration efforts under a range of environmental conditions.

Key words: *Alnus glutinosa*, *Alnus lusitanica*, environmental cline, interspecific variation, ploidy, riparian forests

Implications for Practice

- Comparing seed traits and germination success across the three common-alder species is essential to improving seed-zone delineation and selection of plant reproductive material in ecological restoration.
- Research on within-species variation in seed germination success and related morphological traits can help improve establishment rates and contribute to preserving the adaptive potential of genetic resources.
- More knowledge on rear-edge *Alnus lusitanica* populations, which are naturally adapted to stressful climatic conditions, could help in identifying vulnerabilities and strengths in terms of projected climate changes.

Introduction

The degradation of riparian ecosystems occurring in the past decades has motivated interest in riparian restoration, and

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[1]Forest Research Centre, School of Agriculture, University of Lisbon, Tapada da Ajuda, 1349-017, Lisbon, Portugal
[2]Address correspondence to Inês Gomes Marques, email icgmarques@isa.ulisboa.pt
[3]Linking Landscape, Environment, Agriculture and Food (LEAF), School of Agriculture, University of Lisbon, Tapada da Ajuda, 1349-017, Lisbon, Portugal
[4]Department of Ecology and Environmental Science, Umeå University, Umeå, 901 87, Sweden
[5]Federal Research and Training Centre for Forests, Natural Hazards and Landscape (BFW), 1131, Vienna, Austria
[6]Phytophthora Research Centre, Mendel University, 613 00, Brno, Czech Republic
[7]Faculty of Forestry, University of Belgrade, Kneza Višeslava 1, 11 030, Belgrade, Serbia
[8]Faculty of Forestry and Wood Technology, Mendel University, Zemědělská 3, 61 300, Brno, Czech Republic
[9]Ecology and Ecosystem Health, National Institute for Agriculture, Food and Environment (INRAE), Institut Agro, Agrocampus Ouest, UMR 985 ESE, 65 rue de Saint-Brieuc, CS 84215-35042, Rennes Cedex, France
[10]Université Rennes 2, CNRS, UMR LETG, CA 24307-35043, Rennes Cedex, France
[11]Institute of Botany, Czech Academy of Sciences, Zámek 1, 252 43, Pruhonice, Czech Republic

methods to enhance the colonization and establishment of riparian plants are therefore required (González et al. 2015). In natural riparian ecosystems, seed release timing depends on a complex combination of proximity to seed source, flow regime, temperature, and ecotypic conditions (Stella et al. 2006). In degraded river systems, native riparian vegetation can be absent due to altered flow regimes and modified river margins that may no longer present suitable conditions for native seed establishment (Jansson et al. 2000; Greet et al. 2012). In addition, climate change can substantially alter local conditions and limit natural regeneration (Van Looy & Piffady 2017). To overcome these difficulties, careful choice of reproductive material in ecological restoration is of extreme importance (Mijnsbrugge et al. 2010; Erickson & Halford 2020). Colonization success, and consequently ecological restoration success, relies on good knowledge of seed germination requirements (Kildisheva et al. 2020). Restoration researchers and practitioners also increasingly consider the use of reproductive material with high genetic diversity to be important for short-term restoration success and long-term adaptive potential (Smith et al. 2007; Bischoff et al. 2010; Loss et al. 2011).

The use of reproductive material retrieved from areas geographically close to the restoration area or in previously delineated seed zones is recommended (Broadhurst & Boshier 2014). However, locally adapted populations might not offer the potential to tolerate or evolve under projected future environmental conditions (Jones 2013). One remedy for this is to use a greater number of seed sources, with a focus on including populations with evolutionary potential under projected environmental changes in order to increase restoration effectiveness (Broadhurst et al. 2008). Ongoing research on this topic focuses on preventing the loss of genetic resources and outbreeding problems (McKay et al. 2005; Brady et al. 2019). Thus, considering their potential evolutionary consequences, methodologies such as assisted migration or mixtures of seed provenances should only be considered after detailed investigation of intraspecific diversity (Gann et al. 2019).

Alnus glutinosa (L.) Gaertn. (common alder) is a major riparian tree with a wide geographical distribution (Houston Durrant et al. 2016) that plays a crucial role in supporting ecosystem health (Tarrant & Trappe 1971). Common alder has been used widely in restoration projects to increase floodplain diversity (Seer et al. 2018), to restore native woodland (Willoughby et al. 2019), to restore former mining sites (Horodecki et al. 2019), and as a pioneer species in riparian succession (Forget et al. 2012). However, germination and early survival of common alder only occurs under specific local conditions of humidity and canopy light (McVean 1953). Therefore, germination and establishment rates are frequently low, which may negatively impact restoration objectives (Löf et al. 2014; Willoughby et al. 2019).

Within the geographic range of a species, populations have been subject to different selection pressures in the past, implying that they may also play different roles in ecological restoration. For example, in species that have expanded poleward in response to postglacial warming, populations at the rear edge of the species' geographic range often have high genetic diversity and are disproportionally important in terms of the species' responses to future climate change (Hampe & Petit 2005). Recent research on the genetic conservation of Moroccan and Iberian alder populations (Lepais et al. 2013) has shown this to be true for common alder species. Common alder was previously considered to be exclusively diploid, but Lepais et al. (2013) revealed the existence of several cytotypes within the species complex. Specifically, populations in Morocco, the Iberian Peninsula (Lepais et al. 2013), and the Balkan Peninsula (Havrdová et al. 2015; Mandák et al. 2016) have been found to be tetraploid. Considering primarily the genomic differentiation among these tetraploid populations, Vít et al. (2017) described two new species closely related to *Alnus glutinosa*, namely *Alnus lusitanica* Vít, Douda, and Mandák, referring to the Iberian and Moroccan populations, and *Alnus rohlenae* Vít, Douda, and Mandák, referring to the Balkan populations. In addition to ploidy level, a recent study from Šmíd et al. (2020) found differences in seed germination rates and percentage between *A. rohlenae* and *A. glutinosa*. This recent species differentiation has so far rarely been considered by restoration practitioners and researchers (Salca 2019). However, choosing seed sources without regard to the differences between the three species could subsequently cause problems. The crossing of differing ploidies, resulting from the inadvertent collection of reproductive material from *A. lusitanica* or *A. rohlenae* and *A. glutinosa* species and subsequent seeding, could lead to outbreeding depression and fertility loss (Kramer et al. 2018). Establishment success could be particularly undermined due to the variation in triploid germination rates (Šmíd et al. 2020). Therefore, further knowledge of species-specific traits related to germination success is needed to enable the appropriate collection and use of common alder reproduction material in restoration projects.

Populations of *A. lusitanica* are subject to a wide range of environmental conditions, including subarid conditions with limited water availability, which may increase the level of threat posed to these populations (Rodríguez-González et al. 2014; Gomes Marques et al. 2018). The wide-ranging ecological preferences of *A. lusitanica* (Mandák et al. 2016) suggests populations might have been naturally selected for tolerance to challenging conditions. *Alnus lusitanica* provides a valuable opportunity to investigate the potential impacts of future climatic change throughout its natural range (De Frenne

[12]Faculty of Environmental Sciences, Czech University of Life Sciences Prague, Kamýcká 129, 165 00, Praha-Suchdol, Czech Republic
[13]Bio-Agrodiversity Team, Applied Botany Laboratory, Department of Biology, Faculty of Sciences, Abdelmalek Essaâdi University, BP 2062, 93030, Tétouan, Morocco
[14]Faculty of Forestry, Institute for Dehesa Research (Indehesa), University of Extremadura, Avenida Virgen del Puerto 2, Plasencia, Spain
[15]National Institute of Agricultural and Veterinary Research (INIAV), Av. da República, Quinta do Marquês, 2780-159, Oeiras, Portugal

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et al. 2013). Given the projected warming, it may be relevant in restoration projects to use species north of their present ranges, so that they may better tolerate the hotter and drier climate expected in the future (Gann et al. 2019).

Previous research has addressed intraspecific variation of *A. glutinosa* by studying the genetic origin and diversity of the species at local and regional scales (Mejnartowicz 2008; Beatty et al. 2015; Cubry et al. 2015; Mingeot et al. 2016) and by examining its phenology and morphological performance (Clausen 1985; DeWald & Steiner 1986; De Kort et al. 2014). Studies pertaining to populations from more limited geographic areas have mostly focused on seed biology, e.g., by describing seed traits (Aniszewska et al. 2019) and germination success (Gosling et al. 2009). Yet most of these studies have concerned Central European populations only, thus limiting the applicability of their conclusions, while other research has focused on saplings and adult trees, overlooking germination and seed traits.

This study assessed phenotypic and genotypic differences in germination success rates of *A. glutinosa*, *A. lusitanica*, and *A. rohlenae*. This can provide insight on seed-source choices in ecological restoration, thus helping ensure adaptability potential and persistence of populations under projected environmental changes. We (1) evaluated seed weight and seed morphology from a large set of common alder populations that included the three above-mentioned species and (2) modeled germination success separately for each species using morphological traits and environmental conditions. We hypothesized that seed weight, morphology, and germination would show significant variations between populations and with respect to species and environmental clines.

Methods

Population Sampling

Twelve alder populations were sampled within the geographic ranges of *Alnus glutinosa*, *A. lusitanica*, and *A. rohlenae* (Fig. 1), which extend from Northern Africa to Scandinavia (37°N to 49°N) and from the Iberian Peninsula to the Balkan Peninsula (8°W to 25°E; Table 1). *A. glutinosa* populations were sampled in Fyris, Sweden (FY); in Otava, Czech Republic (OT); in Selune, France (SE); in Riederbach, Austria (RI); in Arno, Italy (AR); and in Amitis on the Greek island of Naxos (AM). *Alnus lusitanica* populations were sampled in Torgal and Bertiandos, Portugal (TO, BE); in Furelos and Jerte, Spain (FU, JT); and in Oued Lekbir, Morocco (OU). One *A. rohlenae* population was sampled in Ibar, Serbia (IB). A minimum of 10 trees were selected per population following a longitudinal transect along each river channel, and the selected trees were georeferenced during the winter of 2017–2018. Only healthy trees were selected, and selected trees had to be separated by at least 25 m to minimize inbreeding effects (Rodríguez-González et al. 2019).

A sufficient number of mature fruiting cones were collected to ensure the sampling of at least 200 seeds from each tree. To

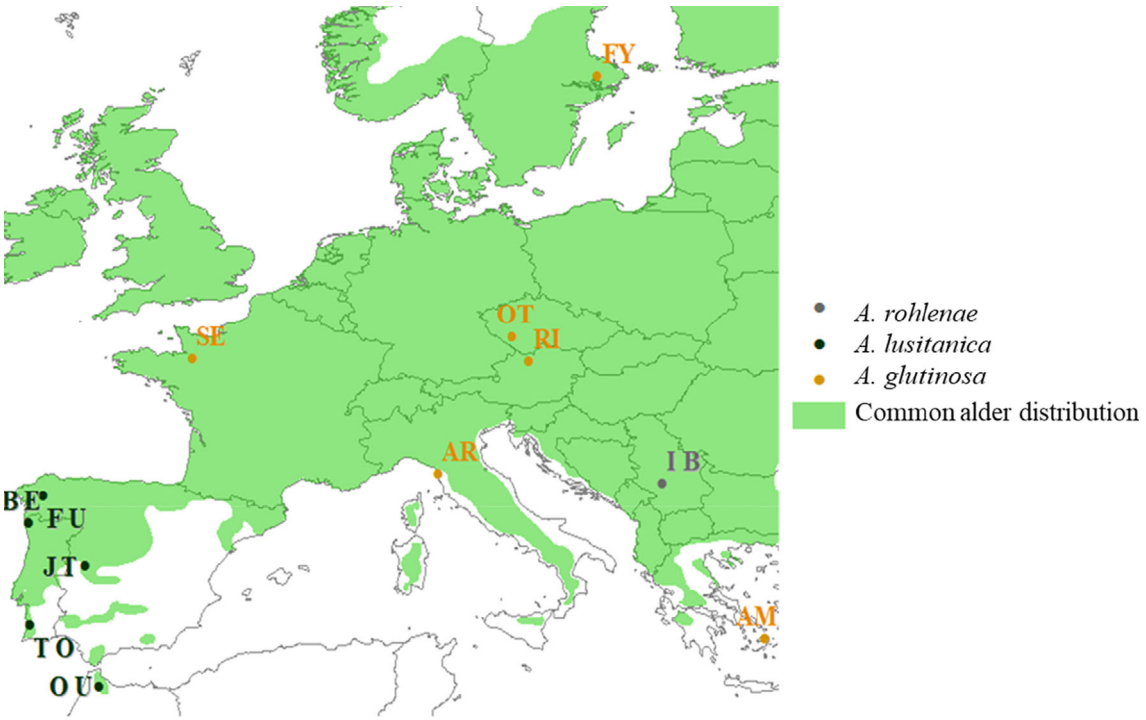


Figure 1. Locations of the 12 *Alnus* spp. (common alder) populations sampled for the study: Fyris, Sweden (FY); Otava, Czech Republic (OT); Selune, France (SE); Riederbach, Austria (RI); Arno, Italy (AR); Furelos, Spain (FU); Bertiandos, Portugal (BE); Jerte, Spain (JT); Torgal, Portugal (TO); Amitis, Naxos Island, Greece (AM); Oued Lekbir, Morocco (OU); and Ibar, Serbia (IB). The three alder species, *Alnus glutinosa*, *A. lusitanica*, and *A. rohlenae*, are represented by three different colors, while the green shading represents the natural distribution of common alder according to Caudullo et al. (2017).

Table 1. Cytotype, geographical, and environmental data of the 12 *Alnus* spp. (common alder) populations sampled in the study. Altitude values refer to the mean values for all sampled trees in each population (except for population OU, where altitude is that of the sampling transect midpoint), while latitude and longitude values refer to the coordinates of each sampling transect midpoint. Mean annual temperature and mean annual precipitation values are based on 30-year climatic data, 1986–2016.

Species	*Population*	*Cytotype*	*Country*	*Latitude*	*Longitude*	*Altitude (m)*	*Mean temperature (°C)*	*Annual precipitation (mm)*
A. glutinosa	FY (Fyris)	Diploid	Sweden	59.821640	17.668472	10	6.6	534
A. glutinosa	OT (Otava)	Diploid	Czech Republic	49.310409	13.724183	417	8.4	697
A. glutinosa	SE (Selune)	Diploid	France	48.377950	−1.077660	201	11.7	744
A. glutinosa	RI (Riederbach)	Diploid	Austria	48.290699	14.536986	314	9.7	798
A. glutinosa	AM (Amitis)	Diploid	Greece	37.118861	25.437830	18	17.5	633
A. glutinosa	AR (Arno)	Diploid	Italy	43.736550	10.310350	7	15.8	964
A. lusitanica	FU (Furelos)	Tetraploid	Spain	42.868500	−8.008200	350	11.9	1,222
A. lusitanica	BE (Bertiandos)	Tetraploid	Portugal	41.765000	−8.639510	6	14.3	1,384
A. lusitanica	JT (Jerte)	Tetraploid	Spain	40.037589	−6.068485	332	14.1	578
A. lusitanica	TO (Torgal)	Tetraploid	Portugal	37.636768	−8.620104	23	17.3	664
A. lusitanica	OU (Oued Lekbir)	Tetraploid	Morocco	35.179760	−5.374365	820	15.7	597
A. rohlenae	IB (Ibar)	Tetraploid	Serbia	43.402014	20.678141	411	9.6	864

facilitate seed extraction, the collected cones were oven dried at 35°C for a minimum of 24 hours until completely open. After extraction, seed lots (for each tree) were stored for at least 2 weeks in sealed containers at 4–5°C with 30–40% air humidity. Each seed lot was then divided into two subsamples. The first subsample was germinated, and the leaves of the 1-year old seedlings were used for cytogenetic analysis. The second subsample was stored for later morphological analysis of the seeds.

Cytogenetic Analysis for Ploidy Assessment

Flow cytometry was used to assess the ploidy levels of populations and facilitate species identification, by comparing genome size of the common alder samples with a reference standard. Common alder leaves were collected from a total of 111 saplings originating from at least three mother trees per population. Nuclei were isolated following the procedure of Galbraith et al. (1983): 0.5 cm^2 of fresh leaf tissue from each alder sapling and from the internal reference standard (*Bellis perennis* L., 2C nuclear DNA content set to 4.20 pg) was chopped with a razor blade and placed inside a Petri dish containing 1 mL of woody plant buffer (Loureiro et al. 2007). The suspension was filtered through a 50 µm nylon mesh, and propidium iodide (50 µg/mL) was added to stain the DNA. To avoid staining of double-stranded RNA, 50 µg/mL of RNAse (Fluka, Buchs, Switzerland) was also added to the suspension. After a 5-minute incubation period, the samples were analyzed in a Partec CyFlow Space flow cytometer (Sysmex Partec, Görlitz, Germany) equipped with a green solid-state laser (Cobolt Samba 532 nm, operating at 30 MW; Cobolt, Stockholm, Sweden) to measure the relative fluorescence of stained nuclei. Results were obtained using Partec FloMax software (v.2.9; Sysmex Partec, Görlitz, Germany). About 5,000–5,500 nuclei were examined in each sample. The DNA-ploidy level was inferred as the position of the sample G1 peak relative to that of the internal reference standard. The genome size of each individual (in picograms; sensu Greilhuber et al. 2005) was obtained using the following equation:

$$\begin{aligned}\text{2C nuclear DNA content} = {} & (\text{Individual G1 peak mean} / \\ & \text{Reference-standard G1 peak mean}) \\ & \times \text{Reference-standard genome size}\end{aligned}$$

Seed Traits and Germination Assessment

Subsamples of 100 fresh seeds from each tree of all sampled populations were used to analyze seed weight and morphological traits. First, each 100-seed subsample was weighed (Weight). Then, the projected area (Area), curved length (Clength), and length-to-width ratio (Length/Width) of individual seeds were obtained using WinSEEDLE 2019 software. The relationship between seed weight and seed size was expressed as the seed weight-to-area ratio (Weight/Area), calculated by dividing mean seed weight by mean seed area. Morphological seed traits were analyzed for all populations except the Morocco population, which had an insufficient total number of seeds for subsampling.

A 31-day germination trial was conducted in April 2018 in a greenhouse with an air-cooling system to prevent air temperatures exceeding 26°C and a periodic fog watering system to prevent seed desiccation. Seeds from all species were sown in tree-nursery growing trays with cells of 300 cm^3, using a germination medium of sand and peat in a 1:2 mixture. To avoid environmental heterogeneity effects in the sowing area, subsamples of 60 seeds for each tree were divided into six replicates of 10 seeds each, which were distributed randomly in three different homogeneous areas (blocks), so that each block included two replicates for each tree lot. Each replicate was established in groups of 10 growing-tray cells in sequence. The germination process was assessed three times per week for 31 days (ISTA 2003) and germination was recorded when root emergence was observed. Seeds that had not germinated by the last day of the germination trial were recorded as "censored" (i.e., considered as missing germination events due to the experiment's time limit).

Climatic Variables

Climatic data series from the 30 years previous to seed collection (1986–2016) were obtained for each of the 12 populations from the closest 0.5° × 0.5° grid point of the Climate Research Unit's global gridded database (CRU TS4.03; Harris et al. 2014). Monthly rainfall and maximum and minimum temperature data were used to calculate various climatic variables (Table S1), including annual variables and variables for the spring months of March, April, and May (MAM) when germination of our target species would most likely occur across their natural geographic ranges (McVean 1955*a*). The altitude of each sampled tree was obtained from the European Digital Elevation Model (v 1.1; https://land.copernicus.eu/imagery-in-situ/eu-dem/eu-dem-v1.1, last access: 29 April 2020), and the mean altitude of each population (except the Moroccan population, for which only the altitude of the middle point of the transect was available) was calculated. Climatic variables and altitude were used in principal component analyses (PCAs) and Pearson's correlation analyses to detect similarity patterns and in regression models to characterize germination environmental conditions.

Statistical Analysis

Statistical analysis based on species and on populations within species was performed to assess inter- and intraspecific variation in the three alder species. Patterns in morphological and environmental characteristics of *A. glutinosa* and *A. lusitanica* were analyzed by means of Pearson's correlations and PCA.

Linear mixed models were used to assess variations in weight and morphological traits of seeds. "Weight", "Area", "Weight/Area" ratio, and "Length/Width" ratio were dependent variables, "species" was a fixed factor, and "population" was a random factor nested within "species." Normality and homoscedasticity of the dependent variables were confirmed, and "Length/Width" was log-transformed prior to analysis. Tukey's test with a confidence interval of 95% was used to test for differences in mean values of morphological traits between species. Linear mixed models were implemented using the *nlme* package (Pinheiro et al. 2016). All statistical analyses were performed in R (v. 3.5.1; R Core Team 2018).

Seed germination rates were calculated using the Kaplan–Meier estimate, and differences in germination success between species and between populations were examined using the log-rank test in the *survival* package (Thearneau & Grambsch 2000; Therneau 2015). Pairwise differences were determined with the *survminer* package (Kassambara et al. 2021). The germination success followed a beta distribution with continuous values on the interval [0, 1], which was confirmed by visual comparison between data and test distribution quantiles using the *qqtest* package (Oldford 2016). Thus, beta regression was used to model germination success of *A. glutinosa* and *A. lusitanica* using the morphological and environmental characteristics of each population as explanatory variables (Ferrari & Cribari-Neto 2004). Explanatory variables for germination modeling were selected through exclusion of strongly correlated variables ($p < 0.001$) from Pearson's correlation analysis employing all populations. Regressions were fitted using a forward stepwise procedure: first, the single explanatory variable that presented the lowest Akaike information criterion (AIC) value was fitted, and second, the remaining variables were added until there was no decrease in AIC value, thereby reaching the maximum optimized model (Bandara et al. 2019). Model performances were evaluated using AIC and pseudo-r^2 values. Beta regression lines were drawn for *A. glutinosa* and *A. lusitanica*, relating germination success and the PCA loadings of the first axis for morphological ($PC1_m$) and environmental ($PC1_e$) variables. Pseudo-r^2 values were calculated for each regression line. Beta regression was performed using the *betareg* package (Cribari-Neto & Zeileis 2010).

Results

Estimation of DNA Ploidy in Seedlings

The genome size analysis revealed two cytotypes (Table S2), consistent with the revised species taxonomy of Vít et al. (2017). The Iberian, Balkan, and Moroccan populations were tetraploid ($2n = 4x = 56$ chromosomes), and the remaining populations were diploid ($2n = 2x = 28$ chromosomes). DNA amounts for tetraploids and diploids (mean 2C-values ± SD) were 2.603 ± 0.104 and 1.247 ± 0.085, respectively (Table S2). Based on these estimations, six

Table 2. Mean values (± SE) of morphological variables of the three *Alnus* (common alder) species. Means followed by different superscript letters (a, b, and c) are significantly different ($p < 0.05$; Tukey test).

Seed trait	A. glutinosa	A. lusitanica	A. rohlenae
Weight (mg)	1.13 ± 0.04 [a]	1.06 ± 0.04 [a]	0.91 ± 0.05 [a]
Area (mm^2)	4.47 ± 0.11 [b]	3.47 ± 0.08 [a]	4.84 ± 0.20 [b]
Weight/Area (mg/mm^2)	0.25 ± 0.01 [b]	0.29 ± 0.01 [a]	0.19 ± 0.01 [c]
Length/Width (mm/mm)	1.31 ± 0.01 [a]	1.27 ± 0.02 [a]	1.35 ± 0.04 [a]

populations were defined as *Alnus glutinosa* (diploid), five populations as *A. lusitanica* (tetraploid), and one population as *A. rohlenae* (tetraploid; Table 1).

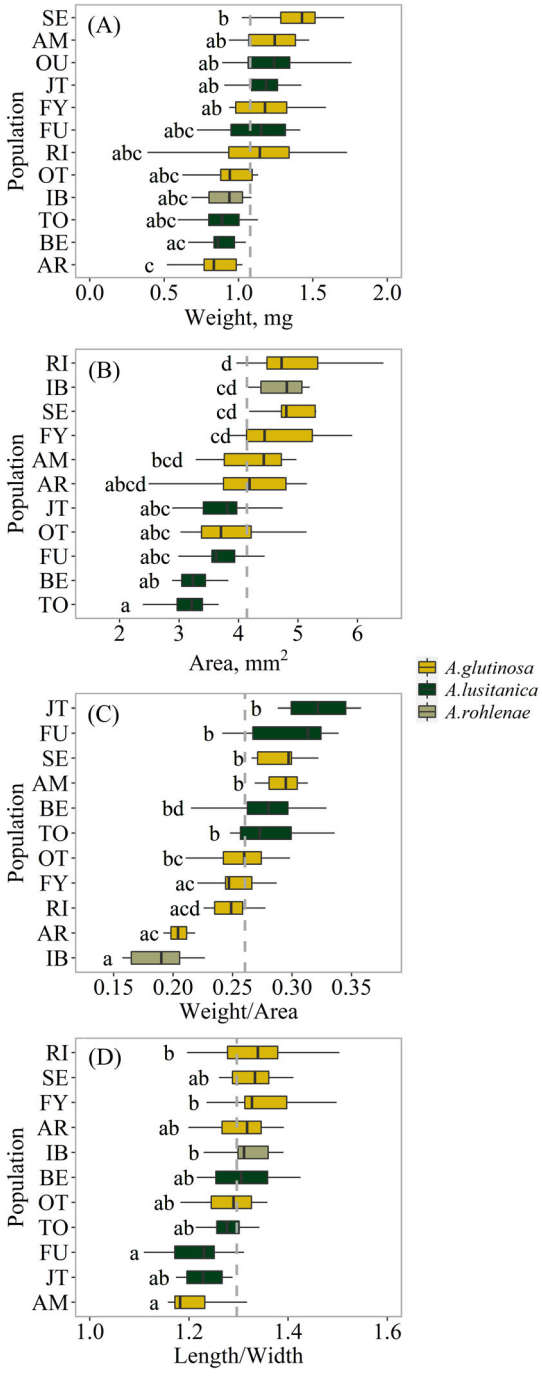


Figure 2. Boxplots of seed weights (A), seed areas (B), seed weight-to-area ratios (C), and seed length-to-width ratios (D) of the 12 sampled *Alnus* spp. (common alder) populations. The three alder species, *Alnus glutinosa*, *A. lusitanica*, and *A. rohlenae*, are represented by three different colors. Populations with distinct combination of lowercase letters (a–d) are significantly different (95% confidence interval). Vertical dashed lines indicate the mean value of each trait across all 12 populations.

Seed Weight, Morphology, and Germination

Species and populations within species were distinguishable by Area and Weight/Area seed variables ($p \leq 0.05$; Table S3). Weight/Area mean values were significantly different between *A. glutinosa*, *A. lusitanica*, and *A. rohlenae* ($p < 0.05$; Table 2). Significant differences were found between *A. glutinosa* and *A. lusitanica* populations in terms of the median values of several morphological traits ($p < 0.05$; Fig. 2A–D). Similarities in morphological-trait median-values between populations from similar latitudinal positions were also observed. For example, the high-latitude *A. glutinosa* populations FY and RI exhibited similar median Weight, Weight/Area, and Length/Width values ($p < 0.05$; Fig. 2A, 2C, & 2D), the Iberian Atlantic *A. lusitanica* populations TO and BE consistently showed similar morphological-trait median values (Fig. 2A–D), and the *A. glutinosa* population AR and the *A. rohlenae* population IB, both mid-latitude populations, had the two lowest median Weight/Area values (Fig. 2C). The *A. glutinosa* population AR had the lowest median Weight value (Fig. 2A).

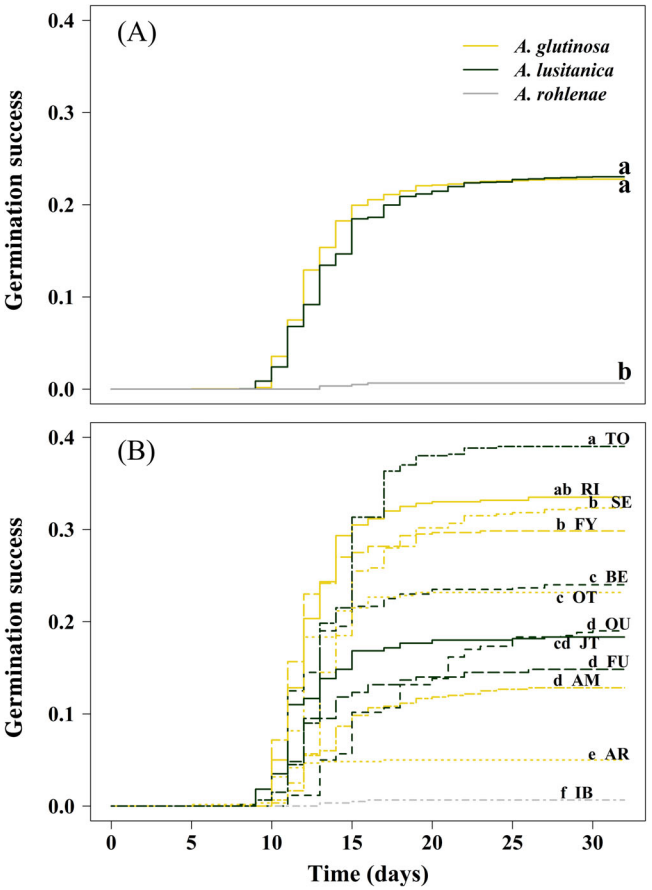


Figure 3. Seed germination success rates of the three *Alnus* (common alder) species, *Alnus glutinosa*, *A. lusitanica*, and *A. rohlenae* (A) and of the 12 sampled alder populations (B). The three alder species are represented by three different colors. Uppercase letters refer to the population locations listed in Table 1. Populations with distinct combination of lowercase letters (a–d) are significantly different between germination curves ($p < 0.05$).

Germination success of *A. glutinosa*, *A. lusitanica*, and *A. rohlenae* were 0.228, 0.230, and 0.070, respectively (Fig. 3A), with significant differences between *A. rohlenae* and the other two species ($p < 0.05$). Germination success ranged from 0.01 (*A. rohlenae* population IB) to 0.39 (*A. lusitanica* population TO; Fig. 3B). Overall, the most northerly *A. glutinosa* populations (RI, SE, and FY) had significantly higher germination success (0.33, 0.32, and 0.30, respectively) than the other *A. glutinosa* populations ($p < 0.01$; Fig. 3B). The lowest germination success values were observed in the *A. glutinosa* population AR and the *A. rohlenae* population IB, although germination success values were significantly different between these two populations ($p < 0.01$). Two of the higher latitude *A. glutinosa* populations (OT and FY) and the two populations with lowest germination success (*A. rohlenae* population IB and *A. glutinosa* population AR) germinated earlier than all the other populations (maximum germination success reached at 16, 17, 19, and 23 days for populations OT, FY, IB, and AR, respectively).

Variation in Seed Morphology and Germination along Environmental Clines

According to the 30-year climatic data obtained for the locations of the 12 sampled populations, most of the *A. lusitanica* locations (TO, JT, and OU) and two of the Mediterranean *A. glutinosa* locations (AM and AR) showed a combination of higher mean temperatures with lower annual precipitation (Tables 1 & S4). The northernmost *A. glutinosa* population (FY) had the lowest temperatures and annual precipitation. The northwestern Iberian *A. lusitanica* populations (BE and FU) had the highest values for annual precipitation while the more central Iberian *A. lusitanica* population (JT) had the lowest precipitation values. The southwestern Iberian *A. lusitanica* population (TO) had the highest mean annual temperature.

Both *A. glutinosa* and *A. lusitanica* populations showed significant correlations between temperature-based variables and the morphological trait Length/Width, while precipitation was significantly correlated with the Weight and Weight/Area traits (Fig. 4). In terms of the Pearson's correlations between morphological traits and environmental conditions, the largest differences between *A. glutinosa* and *A. lusitanica* were observed for the precipitation and temperature variables. The Weight and Weight/Area traits were more negatively correlated with precipitation for *A. glutinosa* than for *A. lusitanica*. Similarly, the Length/Width traits was more negatively correlated with monthly and annual temperatures for *A. glutinosa* than for *A. lusitanica*.

The variances explained by the first principal components of the morphological PCA ($PC1_m$) and the environmental PCA ($PC1_e$) were 54.2% and 42.6%, respectively (Fig. 5). The variables with higher loadings were Weight/Area for $PC1_m$, and Min Cold Month and Max Warm Month for $PC1_e$ (Table S5).

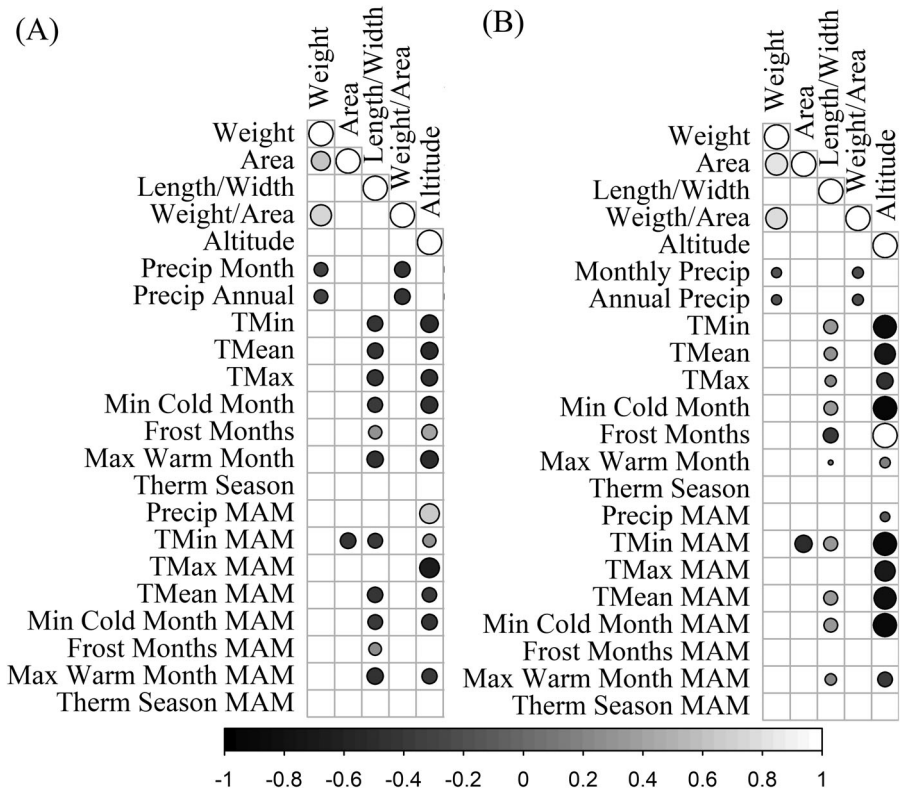


Figure 4. Pearson's correlation between morphological and environmental variables for *Alnus glutinosa* (A) and *A. lusitanica* (B). Circle shading represents Pearson's correlation values (black = −1; white = 1), while circle size is proportional to correlation absolute values. Only significant correlations ($p < 0.05$) are shown. Abbreviations of climatic variables are defined in Table S1.

The northernmost *A. glutinosa* populations (FY, SE, and RI) showed a negative relationship with the Weight/Area variable (Fig. 5A). The *A. glutinosa* population OT was in a central position in the $PC1_m$ plot, closer to the *A. lusitanica* populations BE and TO. The *A. glutinosa* population AM and the *A. lusitanica* populations FU and JT were clustered together in the $PC1_m$ plot, positively related to Weight/Area values. The *A. glutinosa* and *A. lusitanica* populations from locations with higher mean temperatures (TO, AR, AM, JT, and OU) were clustered together in the $PC1_e$ plot (Fig. 5B), while the remaining *A. glutinosa* and *A. rohlenae* populations (FY, OT, RI, IB, and SE) formed a separate cluster, related to higher altitude values. The Iberian *A. lusitanica* populations formed two distinct clusters, with the high-latitude FU and BE populations associated with high precipitation values, and the low-latitude TO and JT populations associated with high temperature values.

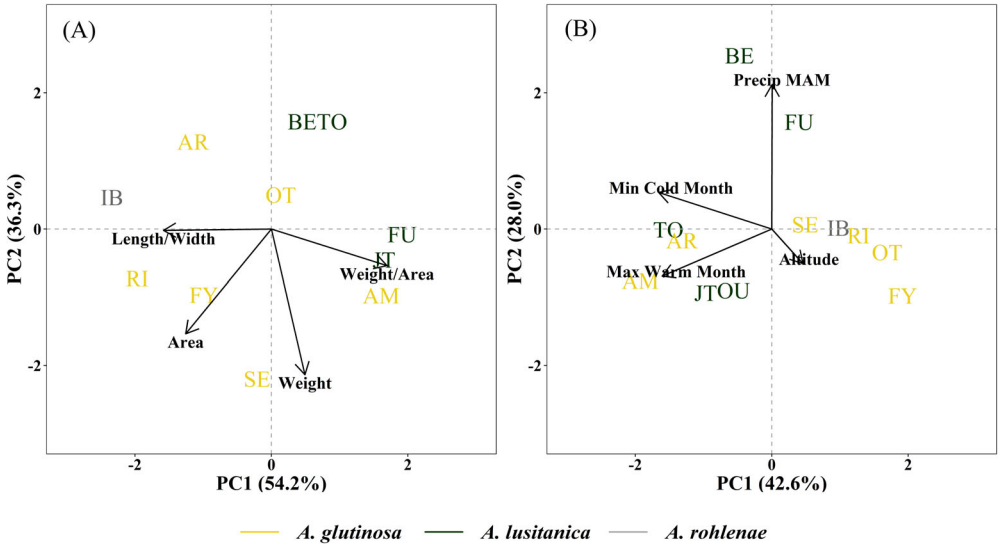


Figure 5. Principal component analysis applied to seed morphology variables (A) and environmental variables (B). "Precip MAM" is mean monthly precipitation between March and May, "Min Cold Month" is the minimum temperature of the coldest month in the year, and "Max Warm Month" is the maximum temperature of the warmest month in the year. Uppercase letters refer to the 12 population locations listed in Table 1, while the yellow, green, and gray text of the location codes represents *Alnus glutinosa*, *A. lusitanica*, and *A. rohlenae* populations, respectively.

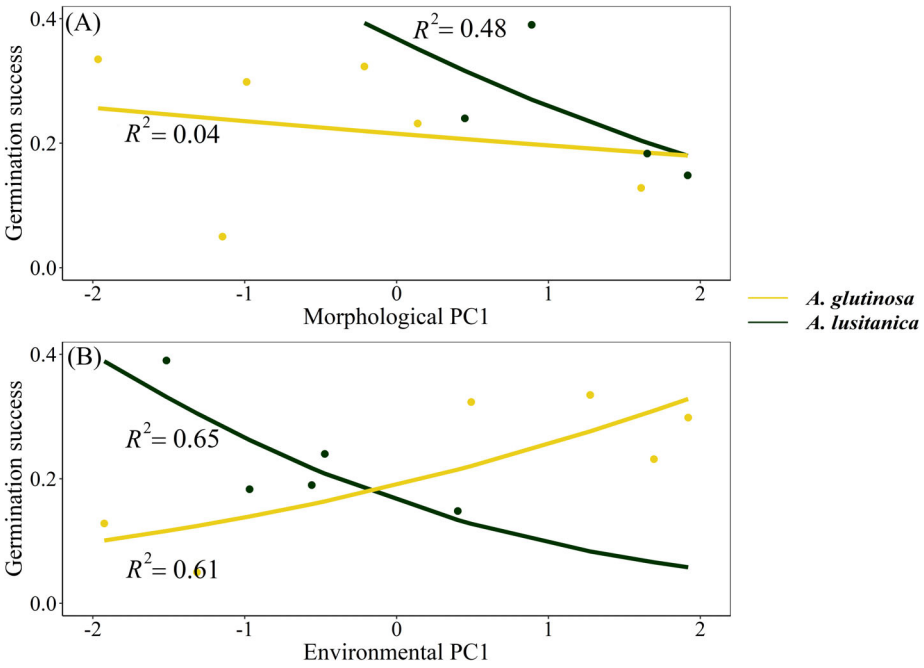


Figure 6. Beta regression curves between germination success and the first principal component axis (PC1) of the principal component analyses applied to seed morphology variables (A) and environmental variables (B) in *Alnus glutinosa* and *A. lusitanica* populations.

Germination Modeling

As several variables were strongly correlated with one another, the number of explanatory variables used for modeling germination success was reduced to eight, of which four were environmental (Min Cold Month, Max Warm Month, Precip MAM, and Altitude; Table S1) and four were morphological (Weight, Area, Weight/Area, and Length/Width). In terms of germination success, the three species presented different responses to environmental variables both in the beta regressions and beta lines.

The best-fitting beta regression (i.e., that with the highest pseudo-r^2 value) explained 38% of the germination success of *A. lusitanica* and 32% of that of *A. glutinosa* (Table S6). The germination success of *A. lusitanica* was explained by the positive effect of Altitude and Min Cold Month (Table S6). Only *A. glutinosa* showed a positive effect of a morphological trait (Weight) on germination success, combined with the negative effect of Max Warm Month (Table S6). The beta regression lines between the principal component with the greatest amount of variation explained (PC1) of the PCA plots and germination success showed stronger relationships in *A. lusitanica* (pseudo-r^2 values of 0.48 and 0.65 for the $PC1_m$ and $PC1_e$ regressions, respectively) than in *A. glutinosa* (pseudo-r^2 values of 0.04 and 0.61 for the $PC1_m$ and $PC1_e$ regressions, respectively; Fig. 6A & 6B). A changing in the $PC1_e$ loadings showed opposite effects on germination success for *A. lusitanica* and *A. glutinosa* (Fig. 6), with the Min Cold Month and Max Warm Month variables positively affecting germination success for *A. lusitanica* and negatively affecting germination success for *A. glutinosa* (Fig. 6) due to the $PC1_e$ loadings of these two temperature variables (Table S5). A decrease in the $PC1_m$ loadings had a clear negative effect on germination success in *A. lusitanica* (Fig. 6). This decrease in the $PC1_m$ loadings can be explained by the negative effect of the Weight/Area variable, due to the higher $PC1_m$ variable loading (Table S5).

Discussion

This study addressed seed morphological traits and germination success of three alder species (*Alnus glutinosa*, *A. lusitanica*, and *A. rohlenae*), and the results revealed differences between the three species in terms of these seed-related variables. The species were sampled from a wide range of climatic conditions across Europe and northern Morocco, with variations in these conditions occurring both between species and between populations within species. The observed variations in seed morphological traits and germination success were associated with environmental clines. This provides key information regarding the use of these species in ecological restoration and under projected climatic changes, and the study's results are therefore relevant to decision-making in ecological restoration projects.

Interspecific Patterns of Seed Germination and Seed Traits

Interspecific variation in seed morphological traits (e.g., Area and Weight/Area) differed among the alder species, allowing modeling of the relationship between germination success and seed morphological traits. For instance, the variables most strongly related to germination success were Weight in *A. glutinosa* and Weight/Area in *A. lusitanica*. This indicates a positive relationship between seed mass and/or size and plant establishment. Greater seed mass and larger seed size may positively influence germination and plant establishment due to the heavier and/or larger seeds containing a greater amount of reserves (Leishman et al. 2009). The populations showing the lowest Weight/Area values (the *A. rohlenae* population IB and the *A. glutinosa* population AR) showed the lowest germination success. These larger but lighter seeds may have smaller amounts of resources for germination, which could negatively affect the colonization capacity of these populations in restoration sites.

Establishment success in ecological restoration projects requires an adequate choice of plant material, ideally based on species-specific knowledge (Erickson & Halford 2020). Normally, local seed-sources are recommended for planting or seeding in restoration, and local sources should be available within a reasonable distance if natural regeneration is required (Broadhurst & Boshier 2014). This ensures that propagules are adequately adapted to the local environmental conditions of restoration sites. The recent division of the common alder species complex into three distinct species (*A. glutinosa*, *A. lusitanica*, and *A. rohlenae*; Vít et al. 2017) raises specific concerns in choosing alder seed sources. First, in areas where different cytotypes occur in close geographical proximity, hybrids are likely to occur. Examples include the triploid populations reported in mainland Greece and Bosnia and Herzegovina (Mandák et al. 2016) and locations in the Dinaric Alps where *A. glutinosa* and *A. rohlenae* co-occur (Mandák et al. 2016; Vít et al. 2017; Šmíd et al. 2020). The existence of such cytotype contact-zones may have important implications for seed collection and for the subsequent use of differing genetic materials with variable germination success rates. Recent research on triploid hybrid sterility in the Dinaric Alps has pointed out high germination variability (Šmíd et al. 2020), raising concerns about the colonization capability of future alder populations and restoration plant-material sources. Furthermore, under projected climate changes, the geographic ranges of all three species are likely to shift. This calls for further study to determine which species and populations should be used to ensure that longer-term restoration objectives are not compromised by novel climatic conditions and avoid species hybridization and introgression problems.

Seed Germination Success in Relation to Environmental Conditions

According to the beta regressions performed, extreme high and low monthly temperatures showed contrasting relationships to germination success across the different species. Relationships between germination success and annual minimum and maximum temperatures were positive for the *A. lusitanica* populations and negative for the *A. glutinosa* populations. This could be explained by the higher mean annual temperatures typical to the locations where the southernmost populations of

A. lusitanica were sampled (OU, TO, JT, and BE). These regionally specific environmental determinants of germination success suggest local adaptation, providing further evidence in support of the use of local seed sources in riparian restoration projects. The positive germination response of *A. lusitanica* to higher temperature extremes could also indicate selection pressures favoring tolerance of a wider range of environmental conditions, most likely resulting from the geographical and genetic isolation of *A. lusitanica* tetraploid populations.

In the future, higher maximum and minimum temperature extremes are projected for Northern, Southern, and Eastern Europe, with a higher prevalence of maximum extremes in Southern Europe and decline of cold extremes in Northern Europe (Nikulin et al. 2011). Four of the *A. glutinosa* populations (FY, OT, RI, and SE) may therefore face plant-establishment constraints in the future. Low temperatures break seed dormancy, an important mechanism in avoiding unfavorable seed-germination conditions (Finch-Savage & Leubner-Metzger 2006). The importance of low temperature extremes on seed germination and dormancy in *A. glutinosa* has been reported in terms of the positive effect of autumn low temperatures on germination in British alder populations (Gosling et al. 2009), while another British study on direct seeding of alder reported no effect of dormancy on germination (Willoughby et al. 2019). In our study, germination was within the range of previously reported values for Spanish, Scandinavian, and British alder populations (McVean 1955*b*; Schalin 1967; Gosling et al. 2009; Elices et al. 2013), suggesting variation in dormancy was not a significant factor in germination success. Overall, the extent of intra- and interspecific variation of this seed trait is still unclear, presenting opportunities for further research.

The projected climatic changes in northern *A. glutinosa* sites suggest that natural establishment success may be at risk due to high temperature or drought events. This risk should be considered in the ecological restoration of river margins, as it may imply that longer germination and establishment periods will be required for natural regeneration, or that repeated seeding or planting will be necessary. Studies on natural colonization and specific measures aimed at genetic resource protection should therefore be prioritized. However, the positive relationship between the germination success of *A. lusitanica* populations and the temperature extremes observed in this study also suggests that this species exhibits a large tolerance to the environmental conditions expected in the future. Therefore, the conservation of the rear-edge *A. lusitanica* populations analyzed in this study should be prioritized, as these populations may serve as genetic resources for ecological restoration projects designed with projected climate changes in mind.

Knowledge about variation between and within species is essential to seed-zone delineation and to conserving and promoting genetic diversity in populations (De Kort et al. 2014, 2016). This is particularly important as genetically impoverished populations, which are already less resilient to environmental change, may suffer from maladaptation and outbreeding depression if new genotypes are introduced (Brady et al. 2019). The *Alnus* genus comprises trees of key importance in riparian ecosystems over a wide geographical area. In-depth studies of variation within and between *Alnus* species is essential both ecologically and economically to ensure the availability of appropriate plant material in riparian ecosystem restoration. Here, we demonstrate the importance of considering genomic, morphological, and environmental information in the correct differentiation between *A. glutinosa* and *A. lusitanica*. Integrating this information into management decisions will help reduce the risk of ecological restoration failure by ensuring that the use of undesirable seed sources is avoided. In addition, better knowledge of the tolerance of seeds and seedlings to environmental conditions different from those in the locations from where they are sourced is needed to better understand the advantages, limitations, and consequences of restoration approaches such as assisted migration (Bucharová et al. 2019). Further research on intraspecific variation is required to ensure the use of populations resilient to biotic threats and to anthropogenically induced hydrological and morphological changes in rivers and wetlands (Solla et al. 2010; Belletti et al. 2020).

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Supporting Information

The following information may be found in the online version of this article:

Table S1. Climatic and local variables considered for each of the 12 *Alnus* spp. (common alder) populations sampled in the study.

Table S2. Descriptive analysis of nuclear DNA (2C value) content estimates for each of the 12 *Alnus* spp. (common alder) populations sampled in the study.

Table S3. Linear mixed model results for morphological seed traits of the six *Alnus glutinosa* and five *A. lusitanica* populations.

Table S4. Descriptive analysis of climatic variables for each of the 12 *Alnus* spp. (common alder) populations sampled in the study.

Table S5. Principal component analysis axes loadings applied to seed morphological and environmental variables.

Table S6. Results of beta regressions used to model germination success in the six *Alnus glutinosa* populations (FY, OT, SE, RI, AR, AM) and the five *A. lusitanica* populations (FU, BE, JT, TO, OU).

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