Drivers of ecological niche breadth in partially mycoheterotrophic orchids and implications for future distributions

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Alexandra Evans

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Summary

Defining the spatial distribution of species is a fundamental topic in ecology and an important part of understanding both the history and future of biodiversity. The ecological niche, or set of environmental conditions that supports the presence of a species, has great potential to aid in estimating the occurrence of species in a landscape. The breadth of the ecological niche represents the range of habitats tolerated by a species, with habitat generalists having a broad niche breadth and able to occur in a greater variety of conditions than habitat specialists restricted by narrow niche breadth. However, the relationship between species occurrence and niche breadth is complicated by species traits such as dispersal mode and interaction with other organisms, as well as stochastic effects involving environmental changes over time, which in turn can dictate the evolutionary divergence of taxa. This is of particular interest for the Orchidaceae, as one of the most species-rich and widespread angiosperm families but with species that are spatially restricted by species interactions and habitat adaptations. Orchids have the potential to colonise a huge area with their highly dispersible seeds, which is at odds with the small ranges and populations recorded for most orchid species, and the high potential for gene flow offered by these seeds is expected to counteract the strong adaptive traits and taxonomic divergence characteristic of the family.

This thesis focused on the taxonomically complex genus of terrestrial orchids *Epipactis* to investigate the role that niche breadth plays in shaping distributions of species subject to conflicting evolutionary pressures and spatial patterns. The reliance of orchid seeds on mycorrhizal fungi to germinate, iterative switching of mating system during the evolutionary history of the genus, and periods of reproductive isolation have likely contributed to the spatial distribution and divergence of the *Epipactis*. However, the extreme variety of range size and habitat among closely related *Epipactis* species, as well as in other orchid genera, is not well understood. I used Maxent ecological niche modelling to quantify and visualise the spatial distribution of suitable habitat in Europe of fourteen

Epipactis species based on species occurrence points and satellite imagery of climate, bedrock, soil, elevation and land-use variables. Levins' B metric of ecological niche breadth and Schoener's D metric of niche overlap were calculated in geographic and environmental space from the ecological niche models, and species range size was defined using alpha-hull range definition. Niche breadth and range size varied considerably between *Epipactis* species, with habitat generalists such as *E. helleborine* being widespread throughout many habitats in Europe and specialists like *E. dunensis* restricted to coastal dunes. Habitat associations also varied considerably, but in general *Epipactis* species occurrence was linked to forest cover and calcareous bedrock. Selfing species were hypothesised to have larger ranges and broader niche breadth than outcrossing species, not being limited by partner availability, but mating system did not have a significant effect on the current differences in distribution between *Epipactis* species.

Mycorrhizal fungus presence dictates the locations that orchids can colonise and contribute to the adult fitness of mycoheterotrophic orchids, which led to the investigation of the relationship between environmental niche breadth and fungal communities in *Epipactis* species. The genetic material of mycorrhizal fungal taxa present in the roots of nine *Epipactis* species was sampled and identified using genetic sequencing and metabarcoding, and fungal diversity was summarised with taxon counts and multivariate analyses. Species that associated with more fungal taxa had wider niche breadths, while a smaller number of fungal associates likely limited the distribution of habitat specialists. Although all *Epipactis* species associated with a wide variety of fungal taxa, some species had distinct communities and those with overlapping ecological niches had more similar fungal communities, indicating that the type of fungal taxa associating with an orchid may contribute to environmental niche divergence.

To investigate how niche breadth influences *Epipactis* distributions with climate change, I projected the ecological niche models onto climate scenarios estimated for 2061-2080. Increased temperature in southern Europe caused all species to

suffer a decrease in habitat at the southern edge of their ranges, but eight of the fourteen species increased their ranges by expanding their leading edge northwards. Niche breadth did not predict a change in range size, as availability of certain land-use types such as forest can limit both habitat generalists and specialists. Finally, to gain insight into the current distribution and evolutionary divergence of *Epipactis*, I investigated how historical climatic changes influenced habitat availability and gene flow of *E. helleborine*. An ecological niche model was projected onto climate data from the Last Glacial Maximum (LGM) to identify glacial refugia, and a single-nucleotide polymorphism (SNP) dataset was used to define and compare the genetic structure of *E. helleborine* populations distributed through Europe. Populations likely survived glaciation in refugia around the Mediterranean, south-western Europe and Carpathians, after which they followed the expansion of forests northwards, with high genetic admixture between populations aided by highly dispersible seeds. Populations in Greece and Italy diverged to some extent in response to geographical isolation and adaptation to local elevation and temperature, indicating a high adaptive potential of the species.

These results demonstrate how niche breadth may have evolved in tandem with mycorrhizal associations in *Epipactis* despite high gene flow, and contributed to distribution differences between species. *Epipactis* can track a changing climate if the distribution of suitable habitats coincides with its future climatic envelope, which may increase genetic isolation of populations. *E. helleborine* has the potential to adapt to increasing temperatures, although it is uncertain if this will be relevant in light of the rapid rate of contemporary climate change. Future research into the spatial distribution and functional roles of fungal taxa, as well as ecological niche definition at higher resolutions, may yield more insight into the drivers of range size, habitat associations, mutualisms and divergence in *Epipactis* and other orchids.

Samenvatting

Het definiëren van de ruimtelijke verspreiding van een soort is een fundamenteel onderwerp in de ecologie en een belangrijk onderdeel voor het begrijpen van zowel de geschiedenis en de toekomst van de biodiversiteit. De ecologische niche, een reeks omgevingscondities die de aanwezigheid van een soort ondersteunen, heeft een groot potentieel om te helpen bij het schatten van het voorkomen van soorten in een landschap. De breedte van de ecologische niche vertegenwoordigt het scala aan habitats die door een soort worden getolereerd, waarbij habitat- generalisten een bredere niche breedte vertonen en in een grotere verscheidenheid aan omstandigheden kunnen voorkomen dan habitatspecialisten, die beperkt zijn door een nauwe niche-breedte. De relatie tussen het voorkomen van soorten en de nichebreedte wordt echter bemoeilijkt door soortkenmerken zoals verspreidingswijze en interactie met andere organismen, evenals stochastische effecten met veranderingen in het milieu in de loop van de tijd, die op hun beurt de evolutionaire divergentie van taxa kunnen bepalen. Dit is van bijzonder belang voor de Orchidaceae, als een van de meest soortenrijke en wijdverspreide angiospermfamilies, maar met soorten die ruimtelijk beperkt zijn door soortinteracties en habitataanpassingen. Orchideeën hebben het potentieel om een enorm gebied te koloniseren met hun sterk verspreidbare zaden, wat in strijd is met de kleine verspreidingsgebieden en populaties die voor de meeste orchideeënsoorten zijn waargenomen, en het hoge potentieel voor genenoverdracht dat door deze zaden wordt geboden, zal naar verwachting de sterke adaptieve eigenschappen en taxonomische divergentie kenmerkend voor de familie tegengaan.

Deze thesis richtte zich op het taxonomisch complexe geslacht van terrestrische orchideeën *Epipactis* om de rol te onderzoeken die nichebreedte speelt bij het vormgeven van verspreidingen van soorten die onderhevig zijn aan tegenstrijdige evolutionaire druk en ruimtelijke patronen. De afhankelijkheid van orchideeënzaden van mycorrhiza-schimmels om te ontkiemen, het iteratief wisselen van paringssysteem tijdens de evolutionaire geschiedenis van het

geslacht en perioden van reproductieve isolatie hebben waarschijnlijk bijgedragen aan de ruimtelijke verspreiding en divergentie van de *Epipactis*. De extreme variatie in verspreidingsgebied en leefgebied tussen nauw verwante *Epipactis*-soorten, evenals in andere orchideeëngeslachten, wordt echter niet goed begrepen. Ik heb ecologische nichemodellering van Maxent gebruikt om de ruimtelijke verdeling van geschikte habitats in Europa van veertien *Epipactis*-soorten te kwantificeren en te visualiseren op basis van de verschijningspunten van soorten en satellietbeelden van klimaat-, gesteente-, bodem-, hoogte- en landgebruikvariabelen. Levins' B-metriek van ecologische nichebreedte en Schoener's D-metriek van niche-overlap werden berekend in geografische en ecologische ruimte vanuit de ecologische nichemodellen, en de grootte van het soortbereik werd gedefinieerd met behulp van de alfa-hull-bereikdefinitie. Nichebreedte en verspreidingsgrootte varieerden aanzienlijk tussen *Epipactis*-soorten, waarbij habitat-generalisten zoals *E. helleborine* wijdverspreid waren in veel habitats in Europa en specialisten zoals *E. dunensis* beperkt tot kustduinen. Habitatassociaties varieerden ook aanzienlijk, maar over het algemeen was het voorkomen van *Epipactis*-soorten gekoppeld aan bosareaal en kalkhoudend gesteente. Er werd verondersteld dat zelfbestuivende soorten een groter verspreidingsgebied en een bredere nichebreedte hebben dan uitkruisende soorten, niet beperkt door de beschikbaarheid van partners, maar het paarsysteem had geen significant effect op de huidige verschillen in verspreiding tussen *Epipactis*-soorten.

De aanwezigheid van mycorrhiza-schimmels leidt de locaties waar orchideeën kunnen koloniseren en draagt bij aan de fitness van mycoheterotrofe orchideeën, wat leidde tot het onderzoek naar de relatie tussen nichebreedte in het milieu en schimmelgemeenschappen bij *Epipactis*-soorten. Het genetische materiaal van mycorrhiza-schimmeltaxa aanwezig in de wortels van negen *Epipactis*-soorten werd bemonsterd en geïdentificeerd met behulp van genetische sequentiebepaling en metabarcodering, en de schimmeldiversiteit werd samengevat met taxontellingen en multivariate analyses. Soorten die geassocieerd waren met meer schimmeltaxa hadden bredere nichebreedten, terwijl een kleiner aantal

schimmelpartners waarschijnlijk de verspreiding van habitatspecialisten beperkte. Hoewel alle *Epipactis*-soorten geassocieerd zijn met een grote verscheidenheid aan schimmeltaxa, hadden sommige soorten verschillende gemeenschappen en hadden soorten met overlappende ecologische niches meer vergelijkbare schimmelgemeenschappen, wat aangeeft dat het type schimmeltaxa dat geassocieerd wordt met een orchidee kan bijdragen aan divergentie van milieu-niche.

Om te onderzoeken hoe nichebreedte de verspreiding van *Epipactis* beïnvloedt bij klimaatverandering, heb ik de ecologische nichemodellen geprojecteerd op klimaatscenario's geschat voor 2061-2080. Door de hogere temperatuur in Zuid-Europa leden alle soorten aan een afname van hun leefgebied aan de zuidelijke rand van hun verspreidingsgebied, maar acht van de veertien soorten vergrootten hun verspreidingsgebied door hun voorrand naar het noorden uit te breiden. Niche-breedte voorspelde geen verandering in de omvang van het verspreidingsgebied, aangezien de beschikbaarheid van bepaalde typen landgebruik, zoals bos, zowel habitat-generalisten als specialisten kan beperken. Ten slotte heb ik, om inzicht te krijgen in de huidige verspreiding en evolutionaire divergentie van *Epipactis*, onderzocht hoe historische klimaatveranderingen de beschikbaarheid van habitats en de genenstroom van E. helleborine beïnvloedden. Een ecologisch nichemodel werd geprojecteerd op klimaatgegevens van het Last Glacial Maximum (LGM) om glaciale refugia te identificeren, en een single-nucleotide polymorphism (SNP) dataset werd gebruikt om de genetische structuur van *E. helleborine*-populaties verspreid over Europa te definiëren en te vergelijken. Populaties overleefden waarschijnlijk de ijstijd in refugia rond de Middellandse Zee, Zuidwest-Europa en de Karpaten, waarna ze de uitbreiding van bossen naar het noorden volgden, met een hoge genetische vermenging tussen populaties geholpen door zeer verspreidbare zaden. Populaties in Griekenland en Italië liepen tot op zekere hoogte uiteen als reactie op geografische isolatie en aanpassing aan lokale hoogte en temperatuur, wat wijst op een hoog aanpassingsvermogen van de soort.

Deze resultaten laten zien hoe de breedte van niches mogelijk is geëvolueerd samen met mycorrhiza-associaties in *Epipactis* ondanks een hoge genenstroom, en heeft bijgedragen aan verspreidingsverschillen tussen soorten. *Epipactis* kan een veranderend klimaat volgen als de verspreiding van geschikte habitats samenvalt met zijn toekomstige klimatologische omhulling, wat de genetische isolatie van populaties kan vergroten. *E. helleborine* heeft het potentieel om zich aan te passen aan stijgende temperaturen, hoewel het onzeker is of dit relevant zal zijn in het licht van de hoge snelheid van de hedendaagse klimaatverandering. Toekomstig onderzoek naar de ruimtelijke verspreiding en functionele rollen van schimmeltaxa, evenals ecologische nichedefinitie bij hogere resoluties, kan meer inzicht opleveren in de drijvende krachten achter de grootte van het verspreidingsgebied, habitatassociaties, mutualismen en divergentie in *Epipactis* en andere orchideeën.

List of abbreviations

AMOVA – Analysis of Molecular Variance
ANOVA – Analysis of Variance
AR – allelic richness
AUC – area under the receiver-operator curve
$B2_{env}$ – Levins' B2 metric of niche breadth projected in environmental space
$B2_{geo}$ – Levins' B2 metric of niche breadth projected in geographic space
BAM (diagram) – biotic, abiotic, movement
bp – base pairs
CCSM – Community Climate System Model
CLC – Corine Land Cover
DNA – deoxyribonucleic acid
dNTP – deoxynucleoside triphosphate
ENM – ecological niche model
F_{st} – fixation index
GIS – Global Information System
GT – gigatonnes
GUI – graphical user interface
H_e – expected heterozygosity
H_o – observed heterozygosity
km – kilometer
LGM – Last Glacial Maximum
LI – Last Interglacial
m – meter
MCMC – Markov Chain Monte Carlo
mg – milligram
MH – Mid-Holocene
MIROC-ESM – Model for Interdisciplinary Research on Climate - Earth System Model
mm – millimetre
MTSS – Maximum Test Sensitivity plus Specificity
NMDS – nonmetric multi-dimensional scaling
OMF – orchid mycorrhizal fungi
OTU – operational taxonomic unit
PCA – principal component analysis
PCR – polymerase chain reaction
PERMANOVA – permutation multivariate analysis of variance
RDA – redundancy analysis
SBPC – soil biochemical principal component
SNP – single-nucleotide polymorphism
SPPC – soil physical principal component
SSP – Shared Socio-economic Pathway
TSS – test skill statistic
µl – microliter
µM – micromolar
°C – degrees Celsius

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Chapter 1

General introduction

Spatial distributions and the ecological niche

The spatial distribution of organisms, and the drivers, mechanisms and implications associated with its definition, is a fundamental topic in biology (Guisan and Thuiller 2005). By defining the ecological processes that cause individuals in a species to occur in one environment but not another, we can predict where species occur through time and over a landscape and through this can gain an understanding of dispersal and adaptation (Sheth *et al.* 2020). The establishment of a population in an area occurs through colonization, survival into adulthood and ability to reproduce. Global patterns of plant distribution are limited through these processes by certain ecological characteristics such as photosynthesis, nutrient acquisition and reproduction opportunities. Because of this, spatial distribution is firstly determined by dispersal and then, for the dispersed individuals to successfully establish in an area, the structure of the environment and interactions with other organisms must be able to support it (Gaston 2003; Soberón 2007; Boulangeat *et al.* 2012). These abiotic and biotic components of a species' habitat form its ecological niche, the quantification of which is critical in estimating the distributions of species (Brown *et al.* 1996).

The ecological niche is a concept that has received much attention and has been assigned many definitions over the last century; in the broadest sense it is the set of environmental characteristics that make up the habitat of a species (Soberón and Nakamura 2009). To take it a step further, the breadth of this niche describes the range of conditions that supports the presence of the species in its habitat and therefore acts as a quantification of a species' mean habitat requirements (Levins 1968; Dolédec *et al.* 2000; Gaston 2003). A habitat generalist species has a broad niche breadth and can tolerate a wide range of conditions and therefore inhabit a wide range of environment types. A habitat specialist, able to tolerate less variability in environmental conditions, will only be found in certain habitat types with specific and more strongly-defined limitations. For this reason, habitat generalists tend to have larger spatial ranges than specialists due to the greater number of environments that can be inhabited (Brown *et al.* 1996; Slatyer *et al.* 2013). However, this pattern holds only when niche breadth is calculated as tolerance of a set of environmental factors (Slatyer *et al.* 2013), and differs at global and regional scales (**Fig. 1**; Kambach *et al.* 2019). Additionally, traditional metrics of niche breadth are limited by spatial autocorrelation and the availability of a habitat type in a landscape, which has resulted in inconsistent results in

the literature (Slatyer *et al.* 2013; Sheth *et al.* 2020).

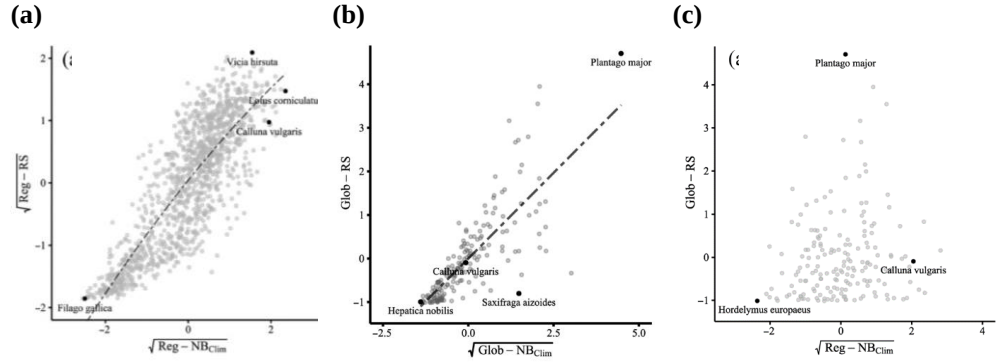


Figure 1. A strong relationship between niche breadth (x) and range size (y) is demonstrated by alpine plant species when investigated at regional **(a)** and global **(b)** scales, but global range size is not estimated by regional niche breadth (c) (Kambach *et al.* 2019).

Even when suitable habitats are available in a landscape, the realised distribution of a species often does not exactly match the distribution of its fundamental niche, either because it has not dispersed into it or, in some cases, it extends past it (Townsend Peterson 2011; Saupe *et al.* 2012; **Fig. 2**). This, and the fact that closely related species can differ substantially in niche breadth and range size (Brown *et al.* 1996; Anacker and Strauss 2014) indicate that species-specific traits interact with niche effects in shaping species distributions.

There is mounting evidence to suggest that the traditional definition of abiotic niche is insufficient to predict species distributions realistically, which has resulted in a shift to include context of species interactions and life history traits (Bruno *et al.* 2003; Alexander *et al.* 2022). In plants, dispersal mode is critical for both range limitation and expansion because seed dispersal is the primary mechanism by which the distributions of sessile plant populations increase (Nathan *et al.* 2008). Small, highly dispersible seeds may allow greater access to a variety of habitats, but smaller seeds also have lower recruitment success (Ehrlén and Eriksson 2000). Once a seed disperses, establishment is dependent on the ecological niche breadth of the species and extrinsic factors can cause range contraction and reproductive isolation even in highly dispersible plant species (Sheth *et al.* 2020). The trend of plant species with narrow niche breadth being more sensitive to habitat changes is consistent even when differences in dispersal mechanism are taken into account

(Broennimann *et al.* 2006). Selfing species are less limited by the availability of mates in an area, and this reduced limitation on range size allows a wider niche breadth to develop (Baker 1955; Randle *et al.* 2009; Grant and Kalisz 2019). However, the lower genetic variation of selfing species reduces their ability to adapt to a large range of habitats (Holt and Gaines 1992; Honnay and Jacquemyn 2007) and Park *et al.* (2018) concluded that mating system is not a strong predictor of plant niche breadth in >400 species.

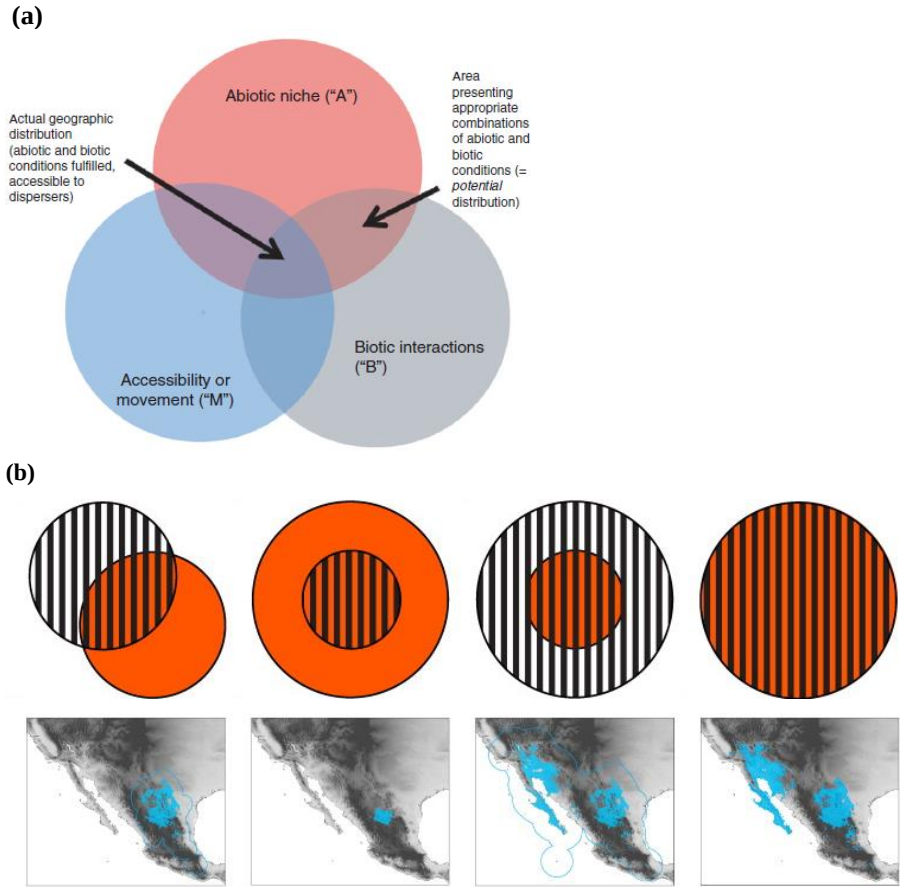


Figure 2. A BAM (biotic, abiotic, movement) diagram (a) representing the potential spatial range of a hypothetical species in a region that has been accessible to the species "M", which is constrained by the distribution of the abiotic niche "A" and biotic interactions "B". Taken from Townsend Peterson (2011). Different BAM scenarios and corresponding spatial ranges for a hypothetical species (b) where abiotic niche distribution "A" is represented by the orange circle and accessible space "M" is represented by the hatched circle. The blue area on the maps represents the area inhabited and the blue outline represents the accessible space "M". From left to right, scenarios include a partial overlap between niche and accessible space, accessible space limitation, abiotic niche limitation, and full overlap between niche and space. Taken from Saupe et al. (2012).

The relationship between niche breadth and range size is relevant when predicting how species respond to environmental change (Thuiller *et al.* 2005, 2008). This is an important topic in light of current global change, considering predicted changes to size and location of the ranges of many plant species (Broennimann *et al.* 2006; Kelly and Goulden 2008; Franklin *et al.* 2016). However, predicting the effects of climate change on plant distributions depends on the species and landscape, and can result in both range expansions and range contractions, invasions and extinctions, as well as latitudinal and elevation shifts in distributions (Bradley *et al.* 2010; Franklin *et al.* 2016; Alarcón and Cavieres 2018). Understanding and characterising the niche breadth of a species has the potential to aid in predicting its response to climate change. Species adapted to a narrow range of environmental conditions are less likely to establish in new areas with different conditions if their current habitats become unsuitable, while generalists are better equipped to shift or expand their ranges when conditions change (Thuiller *et al.* 2005; Slatyer *et al.* 2013). Habitat specialists are at greater risk of extinction than generalists due to a lower tolerance to environmental change and a higher chance of the entire area of a small habitat being affected by an event that might only affect part of a generalist's range (Thuiller *et al.* 2005).

The niche breadth and range size of a species can also reflect historical climate change, which can shape population dynamics, distribution and genetic structure (Comes and Kadereit 1998). For example, the significant climatic changes associated with glaciation caused range contractions, population isolation and local adaptation in many species (Svenning *et al.* 2008) and resulted in the current distribution of habitat specialists in glacial refugia (Tribsch 2004; Keppel *et al.* 2012; Reisch and Rosbakh 2021). Populations of species occupying a larger area were more likely to have access to refugia and, if they retained their wide niche breadth, were able to expand into new environments during the postglacial climate amelioration (Saupe *et al.* 2015; Qiao *et al.* 2016). To some extent, understanding how species were able to survive historic climate change can be used to estimate responses to future climate change, as species that successfully tracked the climate during this period may be able to do it again if they still retain a wide niche breadth (Wiens and Graham 2005; Hampe *et al.* 2013; Bai *et al.* 2018).

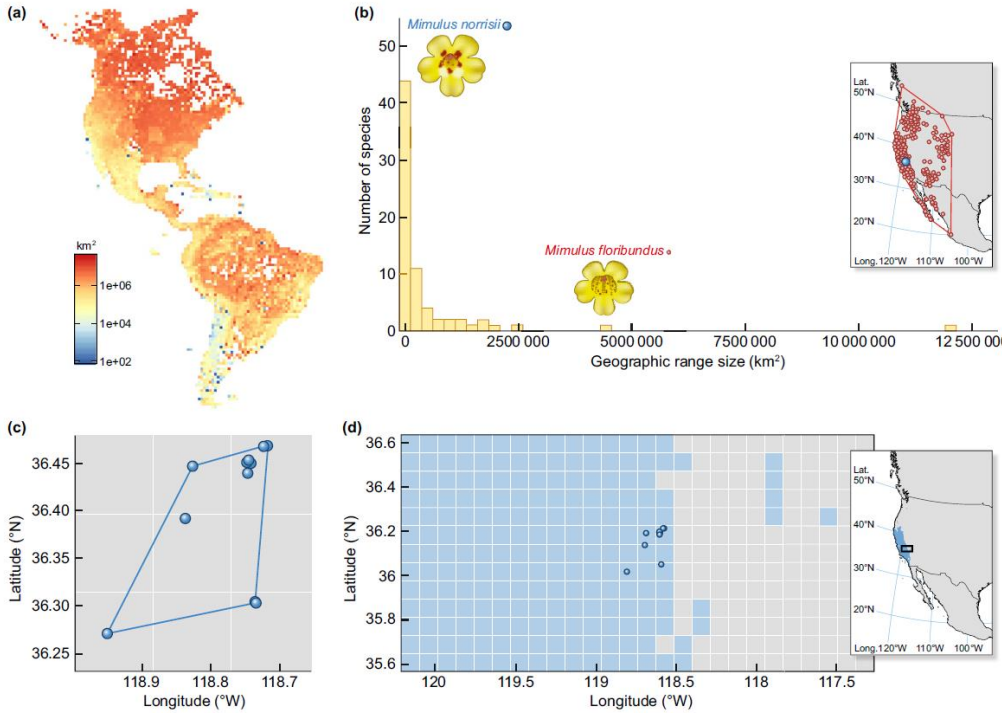


Figure 3. Species occurrence in space can be measured in different ways, taken from Sheth *et al.* 2020: **(a)** Variation in mean range size of New World plants (redrawn from Morueta-Holme et al., 2013) where red grid cells represent a large mean range size of species in the cell and blue represents a small mean range size. **(b)** Frequency distribution of range size for two closely related *Mimulus* species with contrasting range sizes (redrawn from Sheth et al., 2014). **(c)** Extent of occurrence (area of a polygon drawn around the outermost areas where a species occurs) and area of occupancy (number of 30-second grid cells), based on locality data derived from herbarium specimens (blue dots) of *Mimulus norrisii*. **(d)** Climatically suitable habitat (blue shading) estimated by a niche model for *M. norrisii*, with recorded occurrence (blue dots).

The abiotic niche of temperate orchids – effects on distribution and divergence

The Orchidaceae is one of the most diverse families of flowering plants with 707 genera and occurs on all continents except Antarctica (Givnish *et al.* 2015; POWO 2023). Considering the extremely broad distribution of the Orchidaceae, it is likely that the high species diversity is at least partly a result of the diversity of environments it occupies. Despite the broad spatial distribution at the family level, only 2.6% of orchid species occur in more than one broad geographical area (Givnish *et al.* 2016) and populations tend to be patchily distributed (Otero and Flanagan 2006). While the Orchidaceae is a model plant family of diversity and pushing ecological limits (Bronstein *et al.* 2014), some morphological and ecological characteristics that are shared by all orchids limit their distributions to some extent. They are herbaceous and either epiphytic (specifically in the tropics) or terrestrial. Orchid species can be autogamous (self-pollinating) or rely on pollinators for allogamous fertilisation, and can also reproduce vegetatively through rhizomes to produce clones. Many orchid species have coevolved with pollinators to produce a variety of floral morphologies and pollinator reward systems, which is considered to be one of the mechanisms driving the diversification of the family (Givnish *et al.* 2015). If fertilization is successful, seed pods containing millions of tiny seeds are produced and the seeds can be dispersed great distances by the wind, although a large proportion land near the mother plant (Arditti and Ghani 2000; Jersáková and Malinová 2007). These seeds lack an endocarp and require association with mycorrhizal fungi to germinate in a substrate (Rasmussen 2008). The ecological niche of an orchid is a product of the limitations that these life history traits place on the environments they can inhabit, but simultaneously it is these ecological constraints that have resulted in their extraordinary adaptations and diversity, which make orchids ideal organisms to study the relationship between ecological niche, spatial distribution and evolution.

Climate

As with other plants, an important component of an orchid's ecological niche is climate and the presence of terrestrial orchids in a landscape, in terms of distribution, species richness and abundance, is strongly affected by climatic gradients (Blinova 2008; Zhang *et al.* 2015a; van der Meer *et al.* 2016; Gaskett and Gallagher 2018; Tsiftsis *et al.* 2019). Orchid

success and occurrence is linked to fairly mild, stable temperatures and is negatively associated with areas that experience temperature extremes, both during the growing season and dormant period (Blinova 2008; Zhang *et al.* 2015b). This is likely a contributing factor to the lower occurrence of orchids in exceptionally cold and dry areas in the north of Europe (Tsiftsis *et al.* 2019). However, many European orchid species have successfully colonised colder environments (Delforge 2006; Acharya *et al.* 2011; Djordjević and Tsiftsis 2022). Extreme temperatures have a negative effect on germination, growth and flowering time (Light and MacConaill 1998; Rasmussen *et al.* 2015) and therefore it is understandable why areas that are more likely to experience large temperature fluctuations are less likely to successfully support orchid colonisation and persistence. Precipitation has a similar effect to temperature on orchid occurrence as, like temperature, precipitation affects growth and flowering time of orchids and therefore may dictate their presence in an area depending on local precipitation (McCormick *et al.* 2009; Acharya *et al.* 2011; Djordjević *et al.* 2016b). This has resulted in species richness being tied to higher precipitation, for example in Greece (Tsiftsis *et al.* 2019). Hot weather in the growing season can reduce water availability, reducing reproductive success in orchids such as *Himantoglossum* and *Ophrys* and therefore drought has a limiting effect on orchid occurrence (Wells 1991; Pfeifer *et al.* 2006). Additionally, the climate of an area can influence the presence of organisms that associate with orchids, for example pollinating insects (Zhang *et al.* 2015b; Štípková *et al.* 2020) and presence of cold-adapted forest trees (Djordjević *et al.* 2020).

As well as climatic gradients over space, climatic changes over time can be reflected in the distribution and population genetics of plant species. Past glaciation caused distributions of European plants to contract and become disjunct, with populations separated by uninhabitable land becoming reproductively isolated (Comes and Kadereit 1998; Lafontaine *et al.* 2018). Many populations survived glacial periods in refugia distributed around the Mediterranean which were relatively buffered from glacial temperatures and, being reproductively isolated, diverged genetically (Bhagwat and Willis 2008; Keppel *et al.* 2012; Reisch and Rosbakh 2021). With postglacial warming temperatures, distributions expanded northwards into newly suitable habitats, a trend that is continuing in the current warming climate (Bhagwat and Willis 2008; Normand *et al.* 2011; Schwartz 2012). For example, ongoing increases in temperature in northern latitudes has allowed the orchid

Himantoglossum hircinum to expand its range at its leading edge, while increasing drought in the south is predicted to negatively affect the persistence of populations at the rear edge of the species' range (Pfeifer *et al.* 2010). This trend is also relevant in terms of niche breadth, as orchid species with specialist habitat requirements and smaller ranges being more negatively affected (Vogt-Schilb *et al.* 2015; Geppert *et al.* 2020). However, studies quantifying niche breadth and its effects on orchid response to climate change are scarce.

Elevation

An important component of an orchid's abiotic niche is elevation (Djordjević and Tsiftsis 2022). This is often correlated with climate and as such, plant distributions may shift in elevation in response to climate changes (Telwala *et al.* 2013). This is particularly noticeable in mountainous areas, where the effects of gradients and contrasts in temperature and precipitation on the distribution can be seen over a much shorter space. The effect of this high heterogeneity on distributions is demonstrated by differences in range size and climatic niche between alpine and subalpine plants (Yu *et al.* 2017). This is also evident in climatic changes over time, as has been seen in recent range shifts to higher elevations of cold-adapted forest trees (Jump *et al.* 2009; Telwala *et al.* 2013) and European orchid species (Steinbauer *et al.* 2018). This 'climate tracking' towards higher elevations can result in smaller, isolated ranges due to the reduced amount of habitat available towards the tops of mountains (Jump *et al.* 2009) which can result in reproductive isolation, local adaptation and divergence (Reisch and Rosbakh 2021). Such periods of isolation have resulted in a high diversity of orchid species in mountainous areas, with areas such as the Andes and Mediterranean basin being central to orchid diversification (Peakall 2007; Médail and Diadema 2009; Givnish *et al.* 2016). Glacial refugia in Europe are often identified in mountainous areas around the Mediterranean, due to the buffering they offered from glaciation, but these areas are also expected to serve as refugia for plant diversity from currently warming temperatures (Bhagwat and Willis 2008; Keppel *et al.* 2012; Hampe *et al.* 2013).

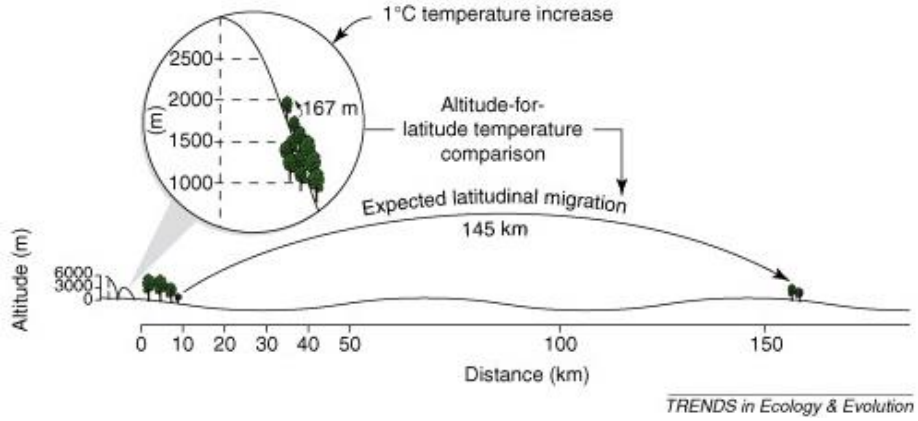


Figure 3. The relationship between climate and elevation on distribution changes over a landscape, demonstrated by predicted shifts of trees in response to climate change (Jump *et al.* 2009)

Substrate

The physical and chemical properties of a substrate significantly affect the presence of orchids in an area (Djordjević *et al.* 2020; Djordjević and Tsiftsis 2022). In Europe, terrestrial orchids occur mainly on calcareous bedrock and orchids that occur here tend to demonstrate lower physiological stress induced by metal presence than those on ultramafic soils, indicated by lower levels of proline (indicating drought stress), phenolic compounds, malondialdehyde and antioxidant activity, as well as higher concentrations of chlorophyll and carotenoids (Jakovljević *et al.* 2023). However this pattern is less pronounced towards the east where many orchids occur on other types of substrates that are available there such as metamorphic and ophiolitic rock, and the diversity of geological substrates is considered to be one of the drivers of the exceptional plant diversity in the Balkans (Stevanović and Stevanović 1995; Tsiftsis *et al.* 2008, 2019). A notable association, and another example of orchids occurring in extreme habitats, is the occurrence of orchids on serpentine rock, which is considered an unusual environment for plants due to high levels of heavy metals and low nutrients, and low water retention (Djordjević *et al.* 2016a). Soil structure was a strong predictor of orchid species richness in central Europe (Hrivnák *et al.* 2020), likely because the physical composition of soil dictates soil moisture which is a component of the orchid ecological niche and has been shown to affect the distribution of species (Bowles *et al.* 2005; McCormick and Jacquemyn 2014; Djordjević *et al.* 2016b). As with climate, most

orchid species are supported by moderate soil moisture conditions, but species such as the coastal dune orchid *Epipactis dunensis* and many Mediterranean species have successfully adapted to dry soils while others occur in wet soils such as the fen orchid *Liparis loeselii* (Delforge 2006; Djordjević *et al.* 2016b). Soil nutrient levels affect plant growth and, particularly for orchids, can affect germination success and therefore limit spatial distribution. In this aspect, orchids are less likely to occur in areas of high nitrogen and phosphate which can decrease germination through mycorrhizal fungus disruption (Bunch *et al.* 2013; Li, Yang, *et al.* 2021). Furthermore, substrates with lower nitrogen concentrations or high metal concentrations offer orchids an environment with reduced competition with many other plant species that proliferate in nitrogen-rich soils (Rasmussen 1995; Djordjević *et al.* 2016a). Within the limits of nutrient poor soils, different proportions of nitrogen, phosphate, carbon and magnesium that exist between habitats are considered to have some effect on differences in orchid communities due to different degrees to which species can tolerate these nutrients (Djordjević and Tsiftsis 2022). Similarly, most orchids occur in a fairly neutral soil pH which promotes mineral uptake, while gradients in soil acidity have been linked to differences in orchid assemblages between habitats (Landi *et al.* 2009; Djordjević *et al.* 2016b). However, a number of orchid species including habitat specialists have been found in Estonian mine tailing hills usually associated with high levels of sulphur and toxic metals (Shefferson *et al.* 2008), once again demonstrating the occurrence of orchid in unexpected habitat conditions.

Land use

The structure of a landscape in terms of land-use or land cover is also a relevant predictor of orchid presence. As this is linked to climate, substrate, and elevation, orchids that are generalists in those niche components will, by definition, be able to inhabit multiple land cover types. However, many terrestrial orchid species in Europe only occur in areas with certain vegetation types (**Fig. 4**), particularly grassland and forest, and orchid communities can differ between different grasslands and forests (Hagsater *et al.* 1996; Delforge 2006). The presence of forests is also dependent on climate and therefore is linked to orchid presence in a similar way; for example, coniferous forests tend to have lower orchid species richness because these forests occur in colder areas, but a higher proportion of cold-adapted orchids (Jacquemyn, Waud, *et al.* 2017; Hemrová *et al.* 2019; Djordjević and Tsiftsis 2022). Although orchid species presence in Europe is greatest in deciduous forests

(Djordjević and Tsiftsis 2022), both specialists and generalists can be found in environments such as coastal dunes, marshes and meadows (Delforge 2006). Some orchid species also occur in areas of anthropogenic land-use such as agricultural land or gardens, either if conditions are similar to those in their natural habitats or because of a wide niche breadth can tolerate these environments. For example, orchid taxa of *Himantoglossum* are often found near roads, presumably because of a similarity in vegetation type to grasslands, as well as water runoff, reduced competition and seed dispersion opportunities that roads offer (Delforge 2006; Fekete *et al.* 2017, 2019). Land-use change through increased agriculture and urbanisation tends to have a negative effect on European orchid range size and survival (Vogt-Schilb *et al.* 2015; Kull *et al.* 2016; Štípková and Kindlmann 2021), while the persistence of grassland species depends on activities such as mowing which increases light availability and reduces competition (Janečková *et al.* 2006).

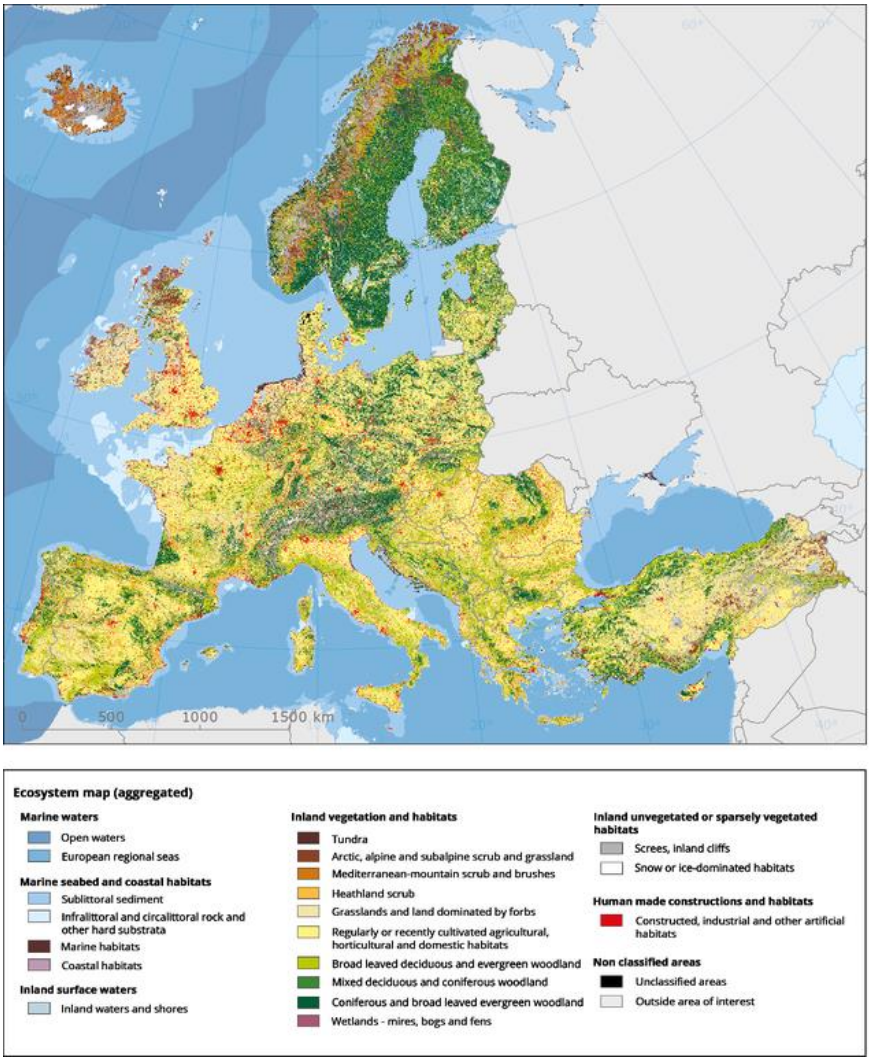


Figure 4. Map of European land-cover derived from Corine Land Cover (European Environment Agency 2017).

Distribution and ecological niche of orchids in the context of life history traits

The patterns of orchid occurrence in relation to the abiotic niche indicate that moderate environmental conditions support the greatest number and abundance of orchid species in Europe but that habitat specialists can occur at the limits of the abiotic niche (Djordjević *et al.* 2020). The general distribution of orchids is limited by fairly specific conditions, while on the other hand, orchids show an exceptional ability to adapt to environments that extend past these limits. This is because the distribution of species is too complex to be explained purely by the distribution of abiotic environmental variables (Bruno *et al.* 2003; Sheth *et al.* 2020). Association with other organisms can extend the limits of the geographic range beyond what is predicted by the abiotic niche; for example, mutualisms allowed grasses to colonise habitats that would usually be too dry to support the species (Afkhami *et al.* 2014). However, obligate mutualisms can also limit the range of species (Townsend Peterson 2011). Therefore, to understand and realistically predict orchid distributions, the abiotic niche needs to be studied alongside the biotic aspects of its life history (Flores-Tolentino *et al.* 2020).

Mating system

The ability to self-fertilise has evolved many times in plants because it offers plants the clear benefit of being able to reproduce without the limitation of mate availability (Baker 1955). Autogamous plant species can reproduce and establish populations in new areas with fewer founding individuals, and can maintain population growth more easily than outcrossers (Baker 1955; Goodwillie *et al.* 2005; Randle *et al.* 2009). This has resulted in the general pattern among plants of autogamous species having larger ranges and wider niche breadths (Grossenbacher *et al.* 2015; Grant and Kalisz 2019). However, populations of allogamous species may have a greater potential to adapt to more habitats from the introduction of new genetic material (Holt and Gaines 1992; Hamrick and Godt 1996; Honnay and Jacquemyn 2007) and therefore increase the range and niche breadth of the species. Approximately 31% of orchid species are autogamous (self-pollinating) while the rest are allogamous and rely on the availability of mates for outcrossing (Peter 2009). These two mating systems have evolved iteratively throughout orchid evolutionary history and are considered to be part of a spectrum rather than two distinct states, with many orchids able

to self-pollinate facultatively when frequency of outcrossing is low (Catling 1990; Sramkó *et al.* 2019). It is plausible that autogamous and facultative autogamous species would have larger distributions and wider niche breadths than species constrained by the availability of mates, but evidence for this in orchids is scarce. Autogamy has been shown to be linked with reproductive isolation in European orchids (Brys *et al.* 2014; Brys and Jacquemyn 2016) but it is uncertain how this affects the niche breadth relative to adaptive potential of these orchids. Considering the effects of plant mating system on genetic diversity and the potential effects on range size, this is a significant research gap in orchid ecology.

Seed dispersal and mycorrhizal interactions

One of the most characteristic aspects of orchid ecology is the production of small seeds that rely on mycorrhizal fungi interactions to germinate (**Fig. 5**; Taylor *et al.* 2003; Rasmussen 2008; Smith and Read 2010). Orchid dust seeds are tiny, numerous and dispersed by wind which gives them a high potential to colonise many areas and travel far distances (Arditti and Ghani 2000). Most orchid seeds land near the mother plant, but small proportion that disperse further are likely numerous enough to contribute to range expansion (Jersáková and Malinová 2007). Other plant species that have small light seeds tend to have larger spatial distributions due to the lower physical restraints imposed on the movement of these seeds compared to heavier seeds. Additionally, wind dispersion eliminates the reliance on other organisms to disperse the seeds, and therefore their distributions are not limited to the distributions and habits of hosts. This raises the paradox of orchid dispersal (Givnish *et al.* 2016) – if orchid seeds are able to disperse distantly and in large numbers, why do orchid species tend to have small distributions (Otero and Flanagan 2006)? Limitations in the abiotic niche contribute to range limitation to a degree, but it does not fully account for how some orchid species occur in environments that are outside of the limits of this niche. Furthermore, in addition to contributing to a large distribution, the high dispersal potential of orchid seeds means that genetic material can easily be transferred between populations (Phillips *et al.* 2012). However, this tends to work against adaptive characters becoming fixed in a population and the consequent speciation (Levin 2010; Paul *et al.* 2011), which does not account for the high species diversity and adaptive traits of orchids (Tremblay *et al.* 2005; Givnish *et al.* 2016).

Although the dispersion potential of dust seeds is high, their lack of an endocarp means that they rely on mycorrhizal fungi to germinate, and therefore they are limited to areas where these fungi are present (Smith and Read 2010; McCormick and Jacquemyn 2014). All orchids are obligately mycoheterotrophic in the germination phase, when soil fungi transfer carbon and nutrients to the orchid (Dearnaley *et al.* 2012; Jacquemyn and Merckx 2019). The distribution of orchids at this phase of the life cycle is therefore tied to the distribution of fungi with which they can form mycorrhizal associations (Hollick *et al.* 2007; McCormick *et al.* 2018; Tedersoo *et al.* 2020). The distribution of these fungi appears to be fairly widespread and therefore the distribution of fungal taxa is not expected to limit orchid distribution on a global scale (McCormick and Jacquemyn 2014) but at the local scale, the identity of fungal taxa may differ between habitats with distinct soil properties and tree presence and influence the occurrence of orchid species (McCormick *et al.* 2012; Bunch *et al.* 2013).

With the development of leaves, many orchids switch to autotrophy but some continue to utilise mycorrhizal associations into adulthood (Wang *et al.* 2022). Most of these species use both photosynthesis and mycorrhizae (mixotrophy or partial mycoheterotrophy) to gain carbon and nutrients (Dearnaley *et al.* 2012; Schiebold *et al.* 2018) but some orchids such as *Cephelanthera subaphylla* and the albino variant of *Epipactis helleborine* remain fully mycoheterotrophic (Sakamoto *et al.* 2016; Suetsugu *et al.* 2017). The association of these orchids with ectomycorrhizae which also associate with trees can allow for a fungus-mediated transfer of carbon from the photosynthesizing tree to the orchid. Even orchids which are considered fully autotrophic have been found to associate with mycorrhizal fungi, presumably because of the nutrient benefits this provides in addition to carbon (Gebauer and Meyer 2003; Wang *et al.* 2022). Partial mycoheterotrophy represents a continuum between the extremes of full autotrophy and full mycoheterotrophy and ongoing research indicates that is more prevalent in orchids (Schiebold *et al.* 2018; Jacquemyn and Merckx 2019) and other plant (Giesemann *et al.* 2020, 2021) than was previously thought. Partial mycoheterotrophy offers photosynthesizing orchids a competitive advantage in areas with other autotrophic plants and during dormancy periods (Jacquemyn and Merckx 2019; Shefferson, 2009). While the use of fungi for germination is well understood, the effects of fungal community structure on adult orchids is less well defined than at the germination phase (Dearnaley *et al.* 2012; Selosse and Martos 2014; Jacquemyn, Waud, *et al.* 2017).

Fungal communities differ between seedling and adult (Bidartondo and Read 2008; Waud *et al.* 2017; Li *et al.* 2022) and temporal turnover of fungal communities is common in orchids (Ventre Lespiaucq *et al.* 2021) and therefore it is plausible that fungal community structure may have different effects on orchid distribution at difference phases of the life cycle.

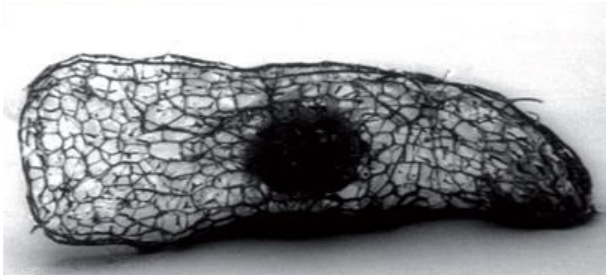
Orchids associate with a diversity of fungal mutualists, including those that form root pelotons (**Fig. 5(c)**, so-called orchid mycorrhizal fungi) from the Basidiomycota, as well as ectomycorrhizae and saprotrophic fungi from both the Basiodiomycota and Ascomycota (Dearnaley *et al.* 2012; Wang *et al.* 2022). Considering that fungal taxa have diverse ecologies, this creates the potential for a large range of ecological services that orchids can utilise (Dearnaley *et al.* 2012). For example, some taxa reduce the inhibitory effect of nitrates on orchid seed germination (Figura *et al.* 2021), which may be particularly useful in nitrate-rich soils and therefore orchids able to associate with these fungi would be more common in such an environment. Furthermore, the number of mycorrhizal fungus taxa an adult orchid associates with has the potential to contribute to orchid habitat distributions through improved population persistence, as mycorrhizal interactions can offer fitness benefits to adult orchids such as increased below-ground survival and seedling growth (Gonneau *et al.* 2014). Such mycorrhizal interactions therefore have the potential to increase an orchid's chances of survival in a habitat, while the ability to utilise a variety of such fungi would presumably allow fungal generalist orchids to persist in more habitats that contain these fungi than fungal specialists, resulting in a larger area occupied (Swarts *et al.* 2010; Downing *et al.* 2020).

However, specialising in fungal interactions may potentially have a similar effect on distribution as specialised habitat associations. Specialist fungal interactions have been shown to result in higher carbon and nutrient transfer (Bonnardeaux *et al.* 2007; van der Heijden *et al.* 2015) and the related fitness benefit of this adaptation on establishment and persistence in a habitat could hypothetically result in expansion or at least greater stability in range size (Sheth *et al.* 2020). However, some large range species such as *Cypripedium calceolus* have a narrow fungal range (Shefferson *et al.* 2007), indicating the effect of fungal specificity on orchid distributions is not always clear (McCormick *et al.* 2012, 2018).

(a)



(b)



(c)

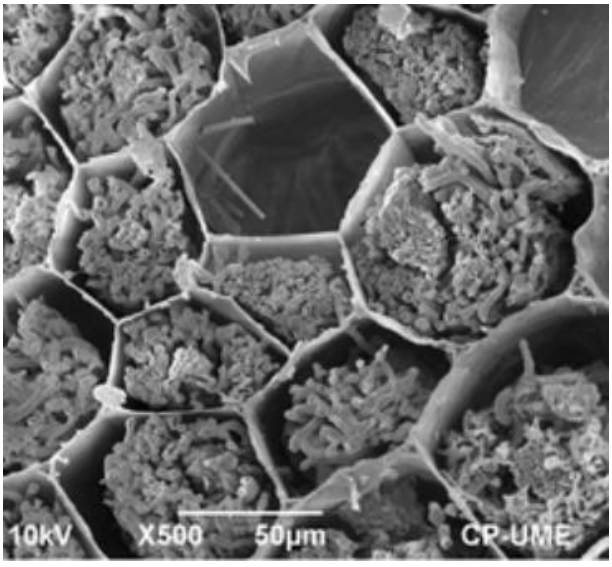


Figure 5. The tiny, numerous seeds of an orchid are wind dispersed (Photograph: Ruben Sauleda) **(a)**.

A scanning electron micrograph of a seed of the orchid *Limodorum abortivum* shows a net-like seed coat encapsulating the embryo **(b).** Taken from Barthlott *et al.* (2014).

The absence of an endosperm necessitates association with mycorrhizal fungi in orchid seed germination, and many adult orchids continue to be mycoheterotrophic through fungal colonisation of roots, seen here as fungal pelotons in *Vanilla planifolia* root cells **(c)**. Taken from González-Chávez *et al.* (2018).

Research question and study species

Despite the importance of the ecological niche for predicting species distribution responses to environmental change, studies on the relationship between niche, distribution and divergence are complicated by inconsistent definition and quantification of these concepts in the literature. Furthermore, the quantification of ecological niche breadth and its effect on distribution and divergence of terrestrial temperate orchids are rare in the literature. It is clear that to gain a realistic picture of orchid distribution and its relationship with divergence, one has to look at the interaction between the abiotic niche and life history and how this both limits and promotes dispersal and establishment of populations. This project aims to shed light on the ecology of temperate orchids in this regard, using multiple lines of evidence and methods to investigate the ecological niche and distribution of the genus *Epipactis* as a study system. *Epipactis* species are distributed across Europe, Asia and north Africa and have a variety of ecologies, habitats, range sizes and morphologies to match (Delforge 2006), making it an ideal genus to compare the effects of environmental gradients on distribution and adaptation. This variation has resulted in many species being described (Delforge 2006). However, recent taxonomic revision has led to many species being recategorised as subspecies and ecotypes (Sramkó *et al.* 2019; Bateman 2020). These phylogenetic analyses resulted in ten legitimate species (**Fig. 6**). I include four additional *Epipactis* taxa that were not investigated in these studies but which have support for species classification according to other studies: *Epipactis microphylla* (Hollingsworth *et al.* 2006; Zhou and Jin 2018), *E. tremolsii* (Bernardos *et al.* 2004), *E. kleinii* (Crespo *et al.* 2001) and *E. fageticola* (Gévaudan *et al.* 2001). The morphometric and phylogenetic evidence of these studies points to these taxa being ecologically distinct enough to be on the level of species, and therefore it is practical to include them here for the purposes of ecological comparisons. Bateman (2020) similarly noted that it is ecologically relevant and practical to consider *Epipactis helleborine* a species even though it is non-monophyletic when studied in the context of the genus, particularly because it is considered the ancestral species of the genus.

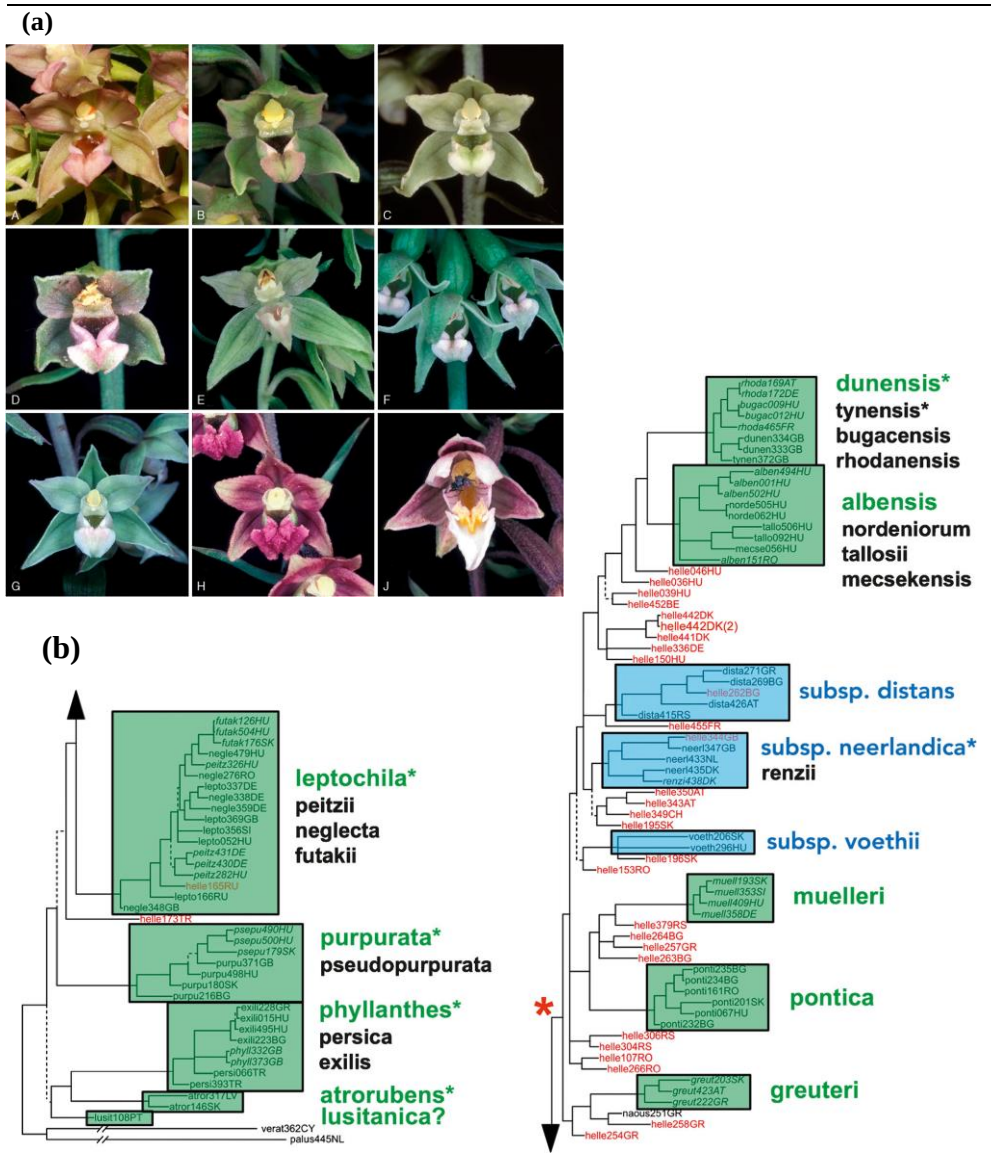


Figure 6. flower morphology **(a)** and phylogeny **(b)** of *Epipactis* species confirmed by the phylogenetic results of Bateman (2020), with *E. helleborine* samples shown in red and *E. palustris* as an outgroup. A-C: *E. helleborine helleborine*, D: *E. dunensis*, E: *E. leptochila*, F: *E. phyllanthes*, G: *E. purpurata*, H: *E. atrorubens*, J: *E. palustris*. Taken from Bateman (2020).

Of these fourteen species, *E. albensis*, *E. atrorubens*, *E. fageticola*, *E. kleinii*, *E. leptochila*, *E. muelleri*, *E. microphylla*, *E. phyllanthes* and *E. purpurata* tend to occur in broad-leafed forests and mixed broad-leafed/deciduous forests, while *E. lusitanica* and *E. tremolsii* are more often found in xeric shrubland (Delforge 2006). *Epipactis palustris* tends to occur in wetter areas such as marshes and coastal floodplains but can also be found in forests, while *E. dunensis* occurs exclusively in coastal dunes (Delforge 2006). *E. helleborine* has been recorded in all of these habitats, as well as anthropogenic environments such as gardens and roadsides. Despite the variation in habitat type, *Epipactis* species tend to occur in varying proximity to trees, presumably which act as ectomycorrhizal hosts (Bidartondo *et al.* 2004). Research has been done on the mycorrhizal communities (Ogura-Tsujita and Yukawa 2008; Jacquemyn *et al.* 2016), reproduction (Brys and Jacquemyn 2016; Rewicz *et al.* 2017) and population genetics (Ehlers and Pedersen 2000; Jacquemyn *et al.* 2020) of some *Epipactis* species, the details of which are discussed in the context of the relevant chapters to follow, while quantitative studies on spatial distribution and ecological niche remain scarce.

The major objective of the study is to characterise the ecological niche of *Epipactis* species in Europe and assess how the interaction of abiotic environment, mating system and mycorrhizal fungus communities contribute to distribution differences between species, as well as predicted historical and future distribution changes (**Fig. 7**).

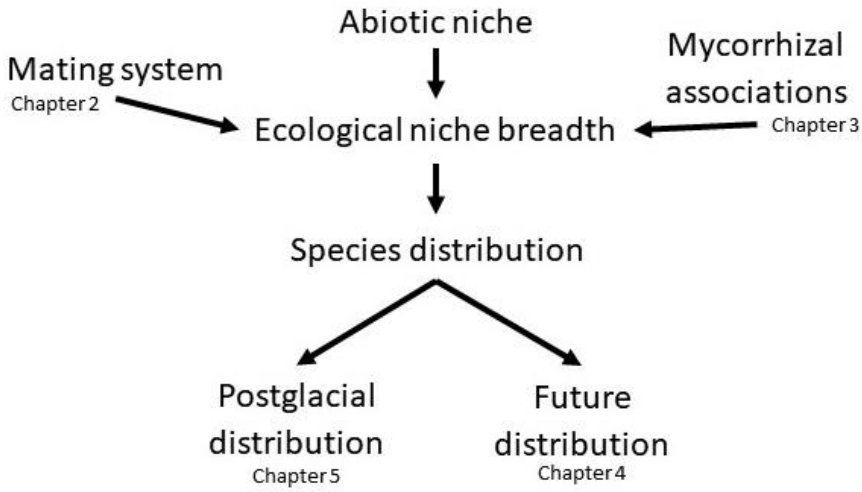


Figure 7. Schematic of the main topics covered in the dissertation. The abiotic niche of *Epipactis* is quantified in terms of niche breadth and applied throughout the thesis. The influences of mating system and mycorrhizal associations on niche breadth are assessed and used to explore spatial distribution differences between species. Climate models are used to investigate historical distribution patterns of *Epipactis helleborine* and predict future habitat distributions.

Chapter 2 uses ecological niche modelling to quantify the niche breadth and habitat distribution of *Epipactis* species. Species occurrence data and environmental data are used to create niche models with Maxent to estimate the distribution of suitable habitat in Europe and assess how environmental gradients contribute to different species distributions. Niche breadth is defined for each species based on the niche models to represent the relative habitat specialization of the species. I test the hypothesis that species able to self-pollinate have wider niche breadths and larger distributions by comparing niche breadth and area of habitat between autogamous, allogamous, and facultative auto- and allogamous species. The results are used to explore the contribution of species traits to spatial distribution differences between species, and the model created provides context and a basis of understanding of habitat-use patterns in *Epipactis* species for subsequent chapters.

Chapter 3 identifies the mycorrhizal communities associating with adult *Epipactis* species using genetic sequencing and metabarcoding. I explore the relationship between fungal community structure and orchid niche breadth calculated in Chapter 2 using multivariate analyses, and quantify the diversity of fungal taxa that with which each orchid species

associates. I investigate whether habitat generalist and specialist orchids differ in number and type of fungal associates. These results are used to assess whether differences in mutualistic associations contribute to distribution differences between closely related temperate orchid species.

Chapter 4 investigates how the habitat distribution of *Epipactis* species will change in response to predicted future climate change using ecological niche modelling and climate models. I test the hypothesis that habitat specialists differ from habitat generalists in the size and location of habitat area predicted for 2061-2080. I explore the implications of using niche breadth as a predictor for climate tracking in orchids and identify the risks and opportunities available to temperate orchid species in light of global change.

Chapter 5 focusses on *Epipactis helleborine* and investigates how historical distribution changes are reflected in current population genetic structure. Ecological niche modelling is used to identify glacial refugia and possible opportunities for postglacial recolonization. A Single Nucleotide Polymorphism (SNP) dataset is used to identify genetic clusters and quantify genetic variation of *E. helleborine* populations across Europe to characterise gene flow and isolation. Outlier analysis and redundancy explore adaptation of populations to local environments. The results are used to create a picture of the historical spatial distribution and evolutionary trajectory of *E. helleborine* populations in Europe, to provide context for the differences observed in current populations and to identify the potential for future climate tracking and adaptation.

Chapter 6 summarises and integrates the main results of the data chapters and assesses how they contribute to the story of *Epipactis* ecology, and identifies which questions remain for future research. I summarise the general habitat-use patterns of *Epipactis* and discuss notable differences between species. I critically evaluate the concepts of specialism and generalism and demonstrate how using different niche definitions and spatial resolutions can affect the interpretation of results, in this thesis specifically and landscape ecology in general. I assess the contributions of mating system and mycorrhizal associations to the spatial distribution of *Epipactis* species as well as the implications of past and future climatic changes, and identify key environmental drivers of range change and niche breadth evolution in temperate orchids.

Chapter 2

Impact of mating system on range size and niche breadth in *Epipactis* (Orchidaceae)

Adapted from *Evans, A. and Jacquemyn, H., 2020. Impact of mating system on range size and niche breadth in Epipactis (Orchidaceae). Annals of Botany, 126(7), pp.1203-1214.*

Abstract

Background and Aims The geographical distribution of plant species is not only fundamentally linked to environmental variables, but also to key traits that affect the dispersal, establishment and evolutionary potential of a species. One of the key plant traits that can be expected to affect standing genetic variation, speed of adaptation and the capacity to colonize and establish in new habitats, and therefore niche breadth and range size, is the plant mating system. However, the precise role of mating system in shaping range size and niche breadth of plant species remains unclear and different studies have provided contrasting results. In this paper, we tested the hypothesis that range size and niche breadth differed with mating system in the orchid genus *Epipactis*.

Methods We modelled the ecological niches of 14 *Epipactis* species in Europe using occurrence records and environmental satellite data in Maxent. Niche breadth and niche overlap in both geographic and environmental space were calculated from the resulting habitat suitability maps using ENMTools and geographic range was estimated using α-hull range definition. Habitat suitability, environmental variable contributions and niche metrics were compared among species with different mating systems.

Key Results and Conclusions We did not detect significant differences in niche breadth, occurrence probability or geographical range between autogamous and allogamous *Epipactis* species, although autogamous species demonstrated notably low variation in niche parameters. We also found no significant differences in niche overlap between species with the same mating system or different mating systems. For all *Epipactis* species, occurrence was strongly associated with land-cover, particularly broad-leafed and coniferous forests, and with limestone bedrock. These results suggest that the mating system does not necessarily contribute to niche breadth and differentiation, and that other factors (e.g. mycorrhizal specificity) may be more important drivers of range size and niche breadth in *Epipactis* and orchids in general.

Key words: distribution range, ecological niche modelling, *Epipactis,* mating system, Maxent, niche divergence, orchids.

Introduction

Understanding the various factors that determine the geographic distribution and niche breadth of plant and animal species is one of the key topics in ecology and evolutionary biology (Brown *et al.* 1996; Sexton *et al.* 2017). Niche breadth, or the range of habitat conditions encompassed by a species' ecological niche, is defined as the distance between the mean habitat conditions of a species and the mean habitat conditions across the study area (Dolédec *et al.* 2000). Habitat generalists tolerate a wide range of environmental conditions, or have broad niches, in comparison with habitat specialists, which have narrow niches and inhabit a smaller array of environments. In general, the range size and niche breadth of a species are limited by traits that affect its dispersal, population dynamics, and evolutionary potential (Gaston 2003). Species that possess traits that facilitate dispersal and colonization of new habitats or that have high genetic variation, and hence high evolutionary potential, can be expected to have larger distribution ranges and wider niche breadths than species that lack these traits. Because species within a single genus may strongly differ in key plant traits, the range sizes and niche breadths of even closely-related species can differ substantially (Brown *et al.* 1996; Grossenbacher and Whittall 2011; Anacker and Strauss 2014).

One of the traits that can be expected to have a large impact on range size and niche breadth is plant mating system (Geber 2008; Randle *et al.* 2009; Petanidou *et al.* 2012; Grossenbacher *et al.* 2015; Grant and Kalisz 2019). Because selfing species tend to have lower standing genetic variation than outcrossing species (Hamrick and Godt 1996; Honnay and Jacquemyn 2007), this may restrict their ability to adapt to changing environmental conditions (Fisher 1958; Holt and Gaines 1992) and therefore decrease their distribution ranges and niche breadths. The transition from allogamy to autogamy has been considered to be an evolutionary dead-end because of selfers' inability to adapt to new environments as a result of inbreeding depression (Stebbins 1957; Takebayashi and Morrell 2001; Gamisch *et al.* 2015). On the other hand, unlike outcrossing species, selfing species do not rely on the availability of pollinators and compatible mates to reproduce in a new environment and may therefore be able to expand their ranges more easily (Baker 1955). Additionally, the gene flow caused by outcrossing can limit the concentration of certain alleles that lead to the evolution of adaptive traits (Levin 2010;

Paul *et al.* 2011), resulting in outcrossing species being less equipped to adapt to new environments and consequently having narrower niche breadths and smaller ranges than autogamous species (Grossenbacher *et al.* 2015). Because selfing acts as a reproductive isolation barrier (Brys *et al.* 2014, 2016), it will limit the influx of maladaptive genes and may increase the rate of adaptive evolution (Levin 2010) and thus increase niche expansion.

At present, few studies have investigated the relationship between plant mating system and niche breadth and these studies have revealed contrasting results. For example, Grant and Kalisz (2019) recently showed that selfing species had significantly wider niche breadths than outcrossing species. Similarly, Randle *et al.* (2009) and Grossenbacher *et al.* (2015) showed that selfing species had larger distribution ranges than outcrossing species. In contrast, a study conducted by Park *et al.* (2018) of more than 400 species indicated that mating system was not a strong predictor of niche breadth for plant species. Similarly, Johnson *et al.* (2014) showed that although the geographic ranges of selfing *Fragaria* species were larger than those of outcrossing species, there was no effect of mating system on ecological niche breadth. Given these contrasting predictions, it is not entirely clear how mating system affects the distribution and niche breadth of plant species and is likely variable among taxa.

In the Orchidaceae, selfing is relatively common compared to other plant families, with 31% of species able to self-pollinate (Peter 2009), but its influence on distribution and niche breadth remains unclear. Despite the large number of seeds produced by orchids and their high dispersal potential (Arditti and Ghani 2000), most orchid populations tend to be small and isolated (Otero and Flanagan 2006), suggesting that their distributions must be limited by factors other than dispersal ability (McCormick and Jacquemyn 2014). The distribution of orchids, as with other plants, is influenced by a combination of biotic and abiotic factors that determine successful fertilization and seed production, seed dispersal and germination, seedling establishment, and survival to adulthood. Fruit set in orchids is generally low (Tremblay *et al.* 2005), and the evolution to selfing clearly provides reproductive assurance and allows orchids to reproduce in habitats where pollinators are scant or lacking (Brys and Jacquemyn 2016) and therefore may allow rapid

colonization of novel habitats and increased niche breadth. Evolution to selfing also represents an important barrier to gene flow and may facilitate genetic and morphological divergence between taxa, ultimately leading to speciation. However, the extent to which variation in mating system affects the distribution range and niche breadth of orchids remains unknown.

In this study, we used ecological niche modelling to investigate the distributions of *Epipactis* species in Europe and to compare the components that define their ecological niches. The genus *Epipactis* contains a complex of allogamous and autogamous species, among which species limits are not always easy to define (Hollingsworth *et al.* 2006; Sramkó *et al.* 2019). Moreover, *Epipactis* species display wide variation in geographic distribution (Ehlers and Pedersen 2000; Efimov 2008) and therefore represent an ideal study system in which to study the effects of mating system variation on distribution range and niche breadth. Mating system transitions and changes in pollination strategy that allow establishment and survival of newly colonized habitats has been invoked to explain the diversification of the genus (Hollingsworth *et al.* 2006; Tranchida-Lombardo *et al.* 2011). However, little is known about how mating system affects range size and niche breadth. Specifically, we tested the hypothesis that autogamous species have broader niches than allogamous species by comparing niche breadth and range size between species with different mating systems. The results of these investigations may shed light on the contribution of mating system to the divergence of a taxonomically-complex genus through differential adaptation to abiotic environmental components.

Materials and methods

Study species

Fourteen *Epipactis* (Orchidaceae) species were selected for analyses based on having at least 30 occurrence records available. Due to the taxonomically complex nature of the genus *Epipactis* and the difficulties that arise as a result of the existence of morphologically distinct ecotypes, multiple names likely exist for the same species; indeed, Bateman (2020) considers there to be no legitimate *Epipactis* species at the local level. To reduce the likelihood of analysing a single species under two names, we used the recent phylogeny of the genus created by Sramkó *et al.* (2019) to choose ten species that are distinct enough from one another to be considered species with sufficient certainty: *E. albensis, E. atrorubens, E. dunensis, E. helleborine, E. leptochila, E. lusitanica, E. muelleri, E. palustris, E. phyllanthes* and *E. purpurata*. Additionally, Sramkó *et al.* (2019) indicated that *E. rhodanensis* and *E. bugacensis* fall under *E. dunensis* and so we analysed the location records of *E. rhodanensis* and *E. bugacensis* as part of *E. dunensis*. *Epipactis microphylla* and *E. tremolsii* were also included because there is strong support for their being monophyletic species in phylogenetic and morphometric research (Bernardos *et al.* 2004; Hollingsworth *et al.* 2006; Zhou and Jin 2018). The species status of *E. kleinii* and *E. fageticola* has yet to be tested with genomic analyses; however we include these here as *E. kleinii*, a new name for *E. parviflora*, is considered to be distinct by Crespo *et al.* (2001) and morphological and ecological analyses suggests that *E. fageticola* is likely also a species (Gévaudan *et al.* 2001). Included are species that are very localised, such as *E. tremolsii* which has been recorded only in the western Mediterranean area (Bernardos *et al.* 2004), as well as widespread species, such as *E. helleborine* which can be found from western Europe to China (Xing *et al.* 2020).

Occurrence data

Records of each species' occurrence from 2000 to 2019 for the continent of Europe were obtained from the online database GBIF (www.GBIF.org; **Supplementary material S1**). We discarded records with missing GIS coordinates, ambiguous species identification or

with coordinates with a spatial resolution lower than ~ 100 m (3 decimal places), as well as those outside of the extent of the ecogeographic variables chosen (i.e., the terrestrial European Union, see below). The number of resulting species records varied between 31 (*E. lusitanica*) and 45354 (*E. helleborine*), with the majority of species having over 100 records. Records for each species were spatially filtered by aggregating records in a 10 km^2 grid cells and extracting the centre coordinates of each grid cell in which the species was recorded, to reduce the effects of spatial clustering resulting from sampling bias (Boria *et al.* 2014). Processing of occurrence data was performed in QGIS v3.4.9 (QGIS Development Team 2019).

Ecogeographic variables

The ecological niche of orchids has been shown to be a function of precipitation and temperature, altitude, soil composition, bedrock, and vegetation type. We therefore used a total of nine raster-format predictor variables with < 0.5 correlation with one other. Two of the 19 bioclimatic variables available from the WorldClim online database (RJ Hijmans *et al.* 2005) (http://worldclim.org/version1) were chosen for use in our model, mean annual temperature and annual precipitation. Two topsoil datasets were acquired through the European Soil Data Centre (ESDAC), one consisting of six physical measurements of soil texture and water availability (Hiederer, 2013; https://esdac.jrc.ec.europa.eu/content/european-soil-database-derived-data) and one consisting of six biochemical levels and pH (Ballabio et al., 2019; https://ec.europa.eu/eurostat/web/lucas/data/primary-data/2018). A PCA was performed on both sets of variables, and the rasters of the first two axes were used as predictors (**Supplementary material S2**). Two categorical variables were acquired: dominant bedrock from the ESDAC database (Van Liedekerke *et al.* 2006); https://esdac.jrc.ec.europa.eu/resource-type/european-soil-database-maps) and Corine Land Cover (CLC) from the Copernicus program of the European Environmental Program (Heymann, 1994; https://land.copernicus.eu/pan-european/corine-land-cover/clc2018). An elevation raster was also included as a predictor variable (Amatulli et al., 2018; https://www.earthenv.org/topography). All rasters had a spatial resolution of 1 km except for land cover, which had a resolution of 100 m and was resampled to match the resolution of the other rasters. Raster processing was performed in RStudio v3.6.2 (R

Core Team 2021) using the 'raster' (RJ Hijmans *et al.* 2005) and 'RStoolbox' (Leutner *et al.* 2017) packages.

Ecological niche modelling

We used the program Maxent v3.4.1 (Phillips *et al.* 2017) to create ecological niche models for the fifteen *Epipactis* species. Maxent uses species occurrence data and environmental rasters to calculate a Gibb's *p* value for each pixel of the study area, representing the probability that the pixel has suitable conditions for the species (Phillips 2005). Maxent then creates habitat suitability maps by projecting the ecological niche model onto the geographic space of the study area. In addition to these maps, Maxent produces a table of the percentage contribution of each predictor variable to the distribution of suitable habitat of each species. The choice of Maxent settings was informed by Barbet-Massin *et al.* (2012) and Merow *et al.* (2013). Each model was run using a random seed and 100 bootstrap replicates with 75% of the data used to train the model and 25% to test it. The variables 'land-use' and 'bedrock' were specified as categorical variables through the Maxent GUI (see Merow *et al.* (2013) for more details on how Maxent handles categorical variables). The rest of the settings were left as the default (convergence threshold of 0.00001, regularisation threshold of 1 and a maximum of 10000 background points) and allowed for linear, quadratic, product and hinge features to be chosen automatically, producing a cloglog output. Model performance was assessed using AUC (area under the receiver-operator curve), with values closer to 1 indicating good fit.

A cluster analysis was performed on the Maxent output table of variable contributions to investigate whether species could be grouped based on similarity in habitat preference. Five agglomerative clustering methods were performed on a Euclidean distance matrix of the data, and the best method was chosen based on having the smallest cophenetic correlation value. The number of clusters to use was assessed using the silhouette width and Mantel approaches. The contribution of each variable to the species' estimated distribution maps was then plotted as a heat map. These analyses were performed in RStudio v3.6.2 (R Core Team 2021) using the 'cluster' (Maechler *et al.* 2012), 'gclus' (Hurley and Hurley 2019) and 'pheatmap' (Kolde and Kolde 2015) packages.

Geographical range

The area of each species' current spatial range in Europe (unlike the projected distribution based on environmental variables described above) was determined by creating an α-hull polygon around each species' occurrence points (Edelsbrunner *et al.* 1983; Burgman and Fox 2003). This was done by performing Delaunay triangulation on a species' locality points, calculating the mean line length and multiplying it by an α value of 5, and removing any lines longer than this number. This was performed using the 'alphahull' package (Rodríguez Casal and Pateiro López 2010) in RStudio v3.6.2 (R Core Team 2021). The resulting polygons were then exported to QGIS v3.4.9 (QGIS Development Team 2019), clipped to the boundary of Europe to exclude oceans and seas from the distributions, and the areas extracted for each species. GBIF data mainly consist of records resulting from random field sampling and sampling restricted to certain areas, and so are unlikely to encompass the entire geographic ranges of species. For example, *E. albensis* is only evident in France in our data but has been identified in Romania by Molnár and Sramkó (2012). Therefore we stress that these alpha-hull areas are conservative, and are used here purely as European range estimates and for use in comparisons among species.

Niche breadth and niche overlap

The niche breadth in geographical space was calculated for each species as the mean Levins' *B* value (Levins 1968) from 30 replicate habitat suitability maps of each species with the software ENMTools v1.3 (Warren *et al.* 2010). The ENMTools package was further used to calculate Schoener's *D* value in environmental space, representing niche overlap, or similarity in habitat requirements (Schoener 1968; Warren *et al.* 2008), for each species pair. We also calculated niche breadth (*B2* value) and overlap in environmental space for each species using the ENMTools R package (v 0.2) (Warren *et al.* 2017). *B2* represents the specificity in environmental conditions of a species' habitat, in comparison to the specificity of the geographic distribution of the potential niche resulting from these ecological associations represented by Levins' *B* values (see Peterson et al., 2011 for detail on environmental and geographic space). Levins' *B* and Schoener's *D* values range from 0 to 1, with values closer to 0 representing narrow niche breadth and

a small degree of niche overlap, and values closer to 1 representing wide niche breadth and a high degree of niche overlap, respectively. Niche overlap among species was visualised with a correspondence analysis plot using the 'ca' package (Nenadic and Greenacre 2007) in RStudio v3.6.2 (R Core Team 2021).

Statistical analysis

Mean distribution area, Gibb's *p* value (habitat suitability), variable contributions, Levins' *B* value (niche breadth in geographic space), *B2* value (niche breadth in environmental space) and Schoener's *D* value (niche overlap) were compared among species with different mating systems and among species clusters using Kruskal-Wallis and Dunn tests with a Holm correction, or ANOVA and Tukey tests if the data were normally-distributed. We further tested the relationships between mean niche breadth, habitat suitability and geographical range using linear regression performed on log-transformed data. All statistical analyses were performed in RStudio v3.6.2 (R Core Team 2021).

Results

The AUC values of the Maxent models were above 0.8 for all species except *E. helleborine*, which had an AUC of 0.74. The mean habitat suitability (Gibb's *p* value) predicted by the Maxent model ranged from 0.002 ± 0.007 (standard deviation) for *E. lusitanica* to 0.34 ± 0.253 for *E. helleborine* (**Table 1**; **Fig. 1**), with an average of 0.072 ± 0.028 (standard error). The areas of the α-hull distributions varied greatly between species, with values between 0.54 (*E. albensis*) and 938.877 (*E. helleborine*) decimal degrees (dd) (**Table 1**; **Fig. 1**) and a mean area of 223.347 ± 84.557 dd. Levins' *B* values (niche breadth) ranged from 0.047 ± 0.004 (*E. lusitanica*) to 0.642 ± 0.001 (*E. helleborine*) (**Table 1**), with a mean of 0.240 ± 0.045 for all the species. Mean Gibb's *p* values (habitat suitability) and Levins' *B* values were moderately correlated ($R^2 = 0.66$, $p < 0.001$). Mean geographical range increased with Levins' *B* ($R^2 = 0.63$, $p < 0.001$) and Gibb's p values ($R^2 = 0.721$, $p < 0.001$). Mean Gibb's *p* values and Levins' *B* were not correlated with *B2* (niche breadth in environmental space) ($R^2 = -0.055$, $p = 0.584$ and $R^2 = -0.055$, $p = 0.113$ respectively) and mean geographical range had a weak positive association with *B2* ($R^2 = 0.178$, $p = 0.003$).

Environmental variables contributed to mean *Epipactis* habitat suitability to different degrees ($\chi^2 = 54.31$, d.f. = 8, $p < 0.001$). The mean contributions of bedrock (26.78 ± 1.41) and land-cover (32.07 ± 5.39) were both higher than those of elevation (3.53 ± 1.27), precipitation (6.95 ± 1.81), soil biochemical components (4.00 ± 1.04 and 5.02 ± 2.25) and the second soil physical component (1.72 ± 0.58) and bedrock was also higher than temperature (11.33 ± 3.37) and the first soil physical component (8.61 ± 2.01). Broad-leafed forest, coniferous forest and non-irrigated arable land were dominant land types associated with species occurrence. Of the bedrock types, limestone was associated with most species and boulder clay was also commonly utilised.

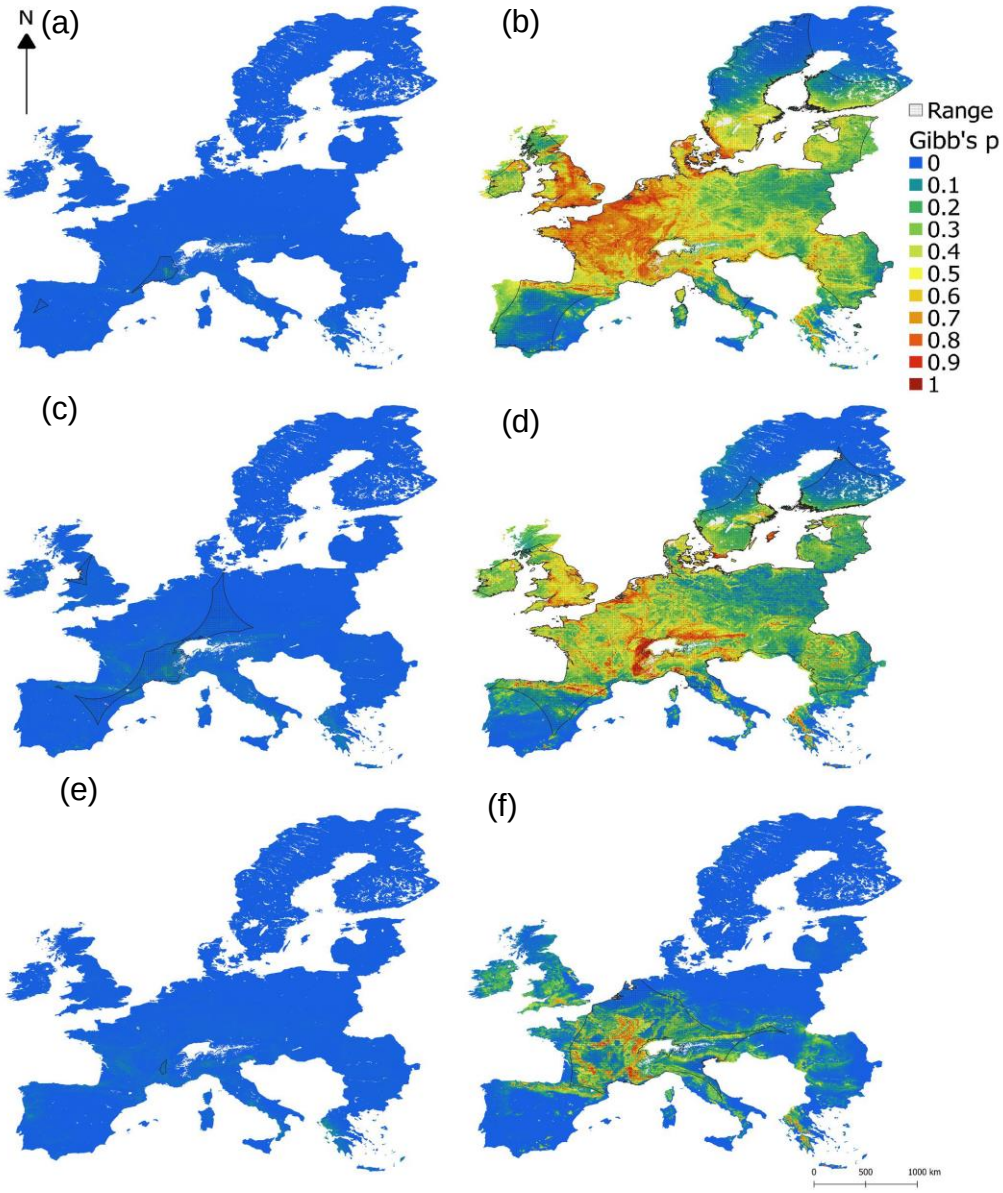


Figure 1. Habitat suitability maps of two allogamous *Epipactis* species, (a) *E. fageticola* and (b) *E. helleborine*, two facultative autogamous species, (c) *E. dunensis* and (d) *E. palustris*, and two autogamous species, (e) *E. albensis* and (f) *E. muelleri* in Europe, predicted by Maxent using occurrence and environmental data, overlain with α-hulls demonstrating the species' current realised occurrence. High Gibb's *p* values (in red and orange) represent more suitable habitat conditions where the probability of species presence is higher, while low values (in blue and green) represent lower habitat suitability and occurrence probability.

Table 1. Distribution and niche metrics for each *Epipactis* species. Mean Gibb's *p* values represent the average occurrence probability or habitat suitability of a species over the study site. Levins' *B* values closer to 1 indicate wide ecological niche breadth (habitat generalist species) and values closer to 0 indicate narrow niche breadth (habitat specialist species). Mating systems include autogamy, facultative autogamy (f. autogamy) and allogamy.

Species	Range size (decimal degrees)	Mean Gibb's *p* value (standard deviation)		Levins' B (standard error)		*B2*	Mating system
E. albensis	0.544	0.006	(0.0099)	0.219	(0.0592)	0.957	Autogamy
E. atrorubens	707.340	0.207	(0.2199)	0.467	(0.0022)	0.662	Allogamy
E. dunensis	37.768	0.007	(0.0125)	0.223	(0.0080)	0.710	F. autogamy
E. fageticola	5.692	0.003	(0.0060)	0.186	(0.0115)	0.660	Allogamy
E. helleborine	938.877	0.340	(0.2534)	0.642	(0.0010)	0.643	Allogamy
E. kleinii	23.300	0.003	(0.0069)	0.138	(0.0077)	0.532	Allogamy
E. leptochila	115.518	0.019	(0.0616)	0.093	(0.0015)	0.526	F. autogamy
E. lusitanica	6.118	0.002	(0.0067)	0.047	(0.0041)	0.989	Allogamy
E. microphylla	165.713	0.052	(0.1116)	0.183	(0.0015)	0.789	F. autogamy
E. muelleri	113.653	0.075	(0.1450)	0.214	(0.0018)	0.835	Autogamy
E. palustris	802.640	0.244	(0.2291)	0.523	(0.0021)	0.615	F. autogamy
E. phyllanthes	29.914	0.004	(0.0086)	0.159	(0.0141)	0.863	Autogamy
E. purpurata	110.733	0.046	(0.0924)	0.191	(0.0017)	0.459	Allogamy
E. tremolsii	69.051	0.006	(0.0099)	0.078	(0.0019)	0.448	Allogamy

The unweighted average linkage agglomerative clustering method was used to group species based on habitat similarity. Three species clusters were created based on similarities in habitat variables (**Fig. 2**). Cluster 1 was most distinguishable in terms of variable contributions, having a significantly stronger association with land cover and a weaker association with temperature than the other two groups (**Table 2**). Cluster 2 had a stronger association with the second biochemical soil component (pH and potassium concentration) than cluster 1, although this difference was of only marginal significance. The mean range size of species in cluster 1 (19.44 ± 6.35 dd) was significantly smaller than that of cluster 2 ($p = 0.011$; 870.76 ± 48.17 dd). Cluster 1 had a mean habitat suitability probability (0.004 ± 0.001) that was lower than cluster 2 ($p < 0.001$; 0.292 ± 0.03) and cluster 3 ($p < 0.001$; 0.058 ± 0.025). Cluster 2 had a higher Levins' B value (niche breadth) (0.58 ± 0.042) than cluster 1 ($p = 0.016$; 0.185 ± 0.015) and cluster 3 ($p = 0.008$; 0.182 ± 0.049). There were no differences in mean *B2* niche breadth among clusters ($p = 0.705$).

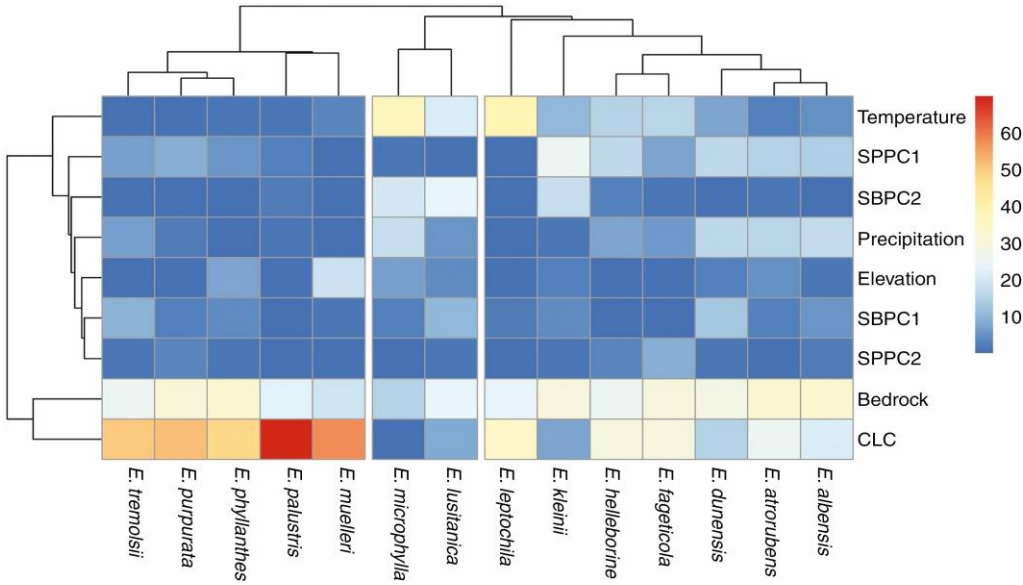


Figure 2. *Epipactis* species clustered based on habitat similarity. Red and orange cells represent strong associations between species occurrence and the corresponding environmental variable, while blue represent weak associations. Variables tested were mean annual temperature, two axes of a PCA of soil biochemical variables (SBPC1 & 2), two axes of a PCA of physical soil characteristics (SPPC1 & 2), elevation, annual precipitation, dominant bedrock material and land-cover (CLC).

Table 2. Differences in mean environmental variable contribution among three species clusters (C1, C2, C3). Mean contribution values in bold are significantly higher than those in italics of the same environmental variable.

Environmental variable	p-value	F statistic	Post-hoc pairwise tests p-values			Mean % contribution (se)		
			C1–C2	C1–C3	C2–C3	C1	C2	C3
Bedrock	0.067	3.483	0.225	0.553	0.057	26.308 (2.283)	19.404 (3.22)	29.223 (1.177)
Land cover	0.002	11.12	0.002	0.317	0.008	**55.439 (3.546)**	*4.186 (2.903)*	**23.345 (3.307)**
Elevation	0.348	1.163				5.474 (3.147)	5.338 (0.913)	1.619 (0.581)
Precipitation	0.165	2.134				1.958 (1.102)	11.547 (4.216)	9.209 (2.617)

Among mating systems, there was no significant difference in mean geographical range (F = 1.34, d. f. = 2, p = 0.30), habitat suitability (F = 0.35, d. f. = 2, p = 0.71), Levins' *B* niche breadth (F = 0.04, d. f. = 2, p = 0.96) or *B2* niche breadth (F = 3.19, d. f. = 2, p = 0.081), but autogamous species demonstrated notably low variation in these measures compared to the other mating systems (**Fig. 3**).

The degree of niche overlap (Schoener's *D*) varied between 0.348 (*E. purpurata* - *E. phyllanthes*) and 0.817 (*E. helleborine* – *E. palustris*) (mean *D* = 0.550 ± 0.0946) (**Table 3**; **Fig. 4**). No difference in mean niche overlap value was detected when comparing among species pairs (i.e. allogamous - allogamous overlap, allogamous - autogamous overlap, etc.; F =0.53, d. f. = 5, p = 0.75). *Epipactis helleborine*, *palustris*, *muelleri*, *dunensis*, *leptochila*, *fageticola* and *lusitanica* had niches that were closer to the mean ecological niche, while *E. microphylla*, *purpurata*, *kleinii*, *tremolsii*, *albensis* and *phyllanthes* had more distinctive niches (**Fig. 4**).

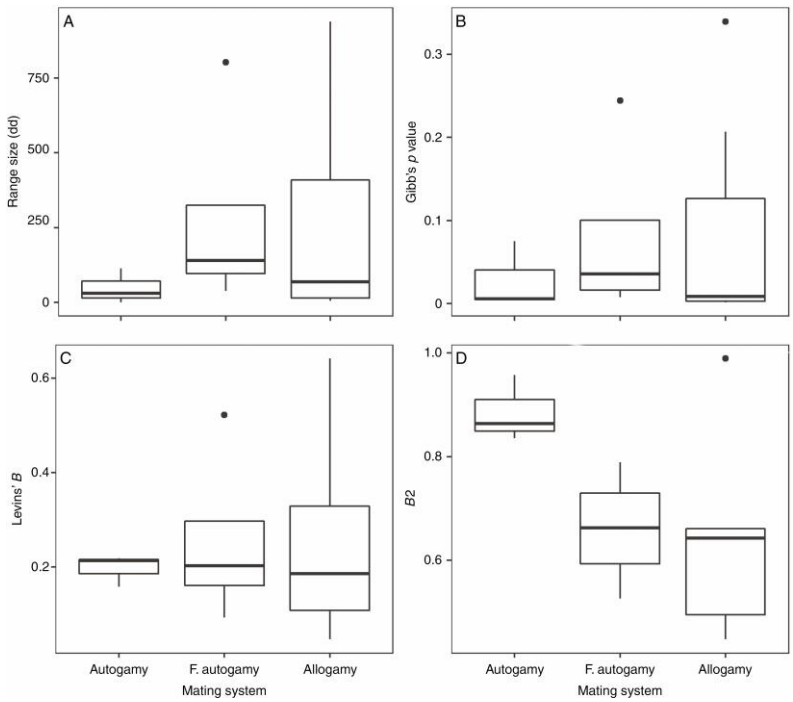


Figure 3. Differences in probability of occurrence, distribution range, and niche breadth among autogamous, facultative autogamous and allogamous *Epipactis* species.

Table 3. Schoener's *D* values representing niche overlap between *Epipactis* species for all species pairs. Values closer to 1 indicate a high level of overlap, while low values indicate a low level of overlap.

Species	*E. purpura*	*E. phyllant*	*E. palustri*	*E. muelleri*	*E. microph*	*E. lusitanic*	*E. leptochi*	*E. kleinii*	*E. hellebor*	*E. fageticо*	*E. dunensi*	*E. atrorub*	*E. albensis*
E. atrorubens	x	x	x	x	x	x	x	x	x	x	x	1.000	0.586
E. dunensis	x	x	x	x	x	x	x	x	x	x	1.000	0.589	0.443
E. fageticola	x	x	x	x	x	x	x	x	x	1.000	0.708	0.621	0.505
E. helleborine	x	x	x	x	x	x	x	x	1.000	0.683	0.711	0.639	0.495
E. kleinii	x	x	x	x	x	x	x	1.000	0.513	0.611	0.526	0.772	0.446
E. leptochila	x	x	x	x	x	x	1.000	0.424	0.593	0.532	0.588	0.538	0.517
E. lusitanica	x	x	x	x	x	1.000	0.525	0.519	0.704	0.665	0.607	0.621	0.527
E. microphylla	x	x	x	x	1.000	0.482	0.486	0.450	0.468	0.476	0.457	0.706	0.380
E. muelleri	x	x	x	1.000	0.515	0.627	0.646	0.476	0.694	0.639	0.663	0.505	0.489
E. palustris	x	x	1.000	0.723	0.470	0.695	0.598	0.494	0.817	0.663	0.654	0.691	0.480
E. phyllanthes	x	1.000	0.517	0.565	0.382	0.580	0.520	0.437	0.521	0.500	0.537	0.777	0.529
E. purpurata	1.000	0.348	0.506	0.538	0.447	0.523	0.463	0.449	0.539	0.464	0.465	0.539	0.460
E. tremolsii	0.485	0.578	0.467	0.518	0.481	0.548	0.509	0.580	0.476	0.556	0.491	0.558	0.591

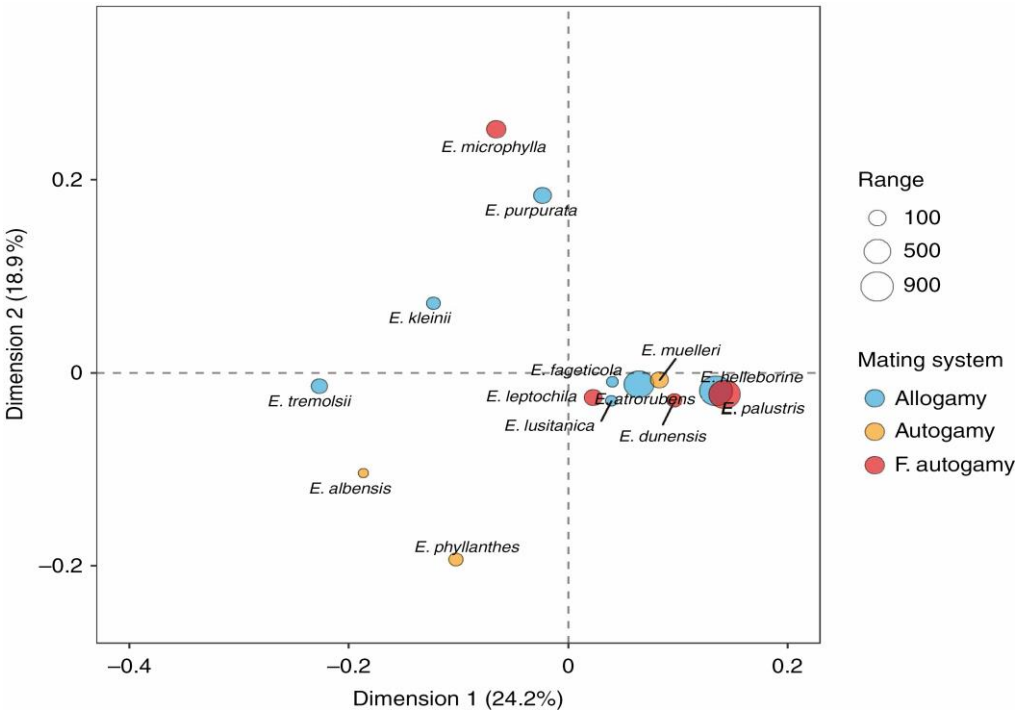


Figure 4. Correspondence plot of *Epipactis* species niche overlap showing niche similarity among mating systems and in relation to geographic range size. Species with points closer together have higher niche overlap.

Discussion

With the use of ecological niche models (ENMs) and α-hull range definition, we characterised the ecological niches of 14 *Epipactis* species to test the hypothesis that range size and niche breadth were related to mating system. *Epipactis* is a terrestrial orchid genus that contains a wide range of allogamous, facultative autogamous and full autogamous species (Claessens and Kleynen 2011). *Epipactis* species occupy a wide variety of soils and habitat types and differ substantially in distribution area in Europe, with some species having very broad ranges (e.g. *Epipactis helleborine*, *E. atrorubens*, *E. palustris*) and others having very narrow distribution ranges (e.g. *E. albensis*, *E. lusitanica*), making it a perfect genus to test the impact of mating system on distribution range and niche breadth. Our results showed that mating system did not affect range size, habitat suitability or niche breadth of *Epipactis* species, supporting earlier findings that mating system not always predicts niche breadth (Johnson *et al.* 2014; Park *et al.* 2018, but see Randle *et al.* (2009) and Grant and Kalisz (2019)). However, variation in niche parameters was much wider in facultative autogamous and allogamous species than in autogamous, which showed very little variation.

The effect of mating system on niche parameters

Selfing species generally have a lower genetic diversity than outcrossing species (Hamrick and Godt 1996; Honnay and Jacquemyn 2007) which, in turn, may restrict the ability of selfing species to adapt to local environmental conditions and therefore lead to small ranges and narrow niche breadths. *Epipactis* is not different in this respect. Population genetic studies have shown that autogamous species tend to have low levels of population genetic diversity (Ehlers and Pedersen 2000; Jane Squirrell *et al.* 2002; Hollingsworth *et al.* 2006; Sramkó *et al.* 2019). For example, populations of *E. dunensis*, *E. leptochila* and *E. muelleri* have been shown to be completely homozygous and uniform for all loci studied (Squirrell *et al.* 2002a). Similarly, Ehlers and Pedersen (2000) showed that the obligate autogamous *E. phyllanthes* was completely monomorphic at all loci examined, while the allogamous and widespread *E. helleborine* showed high levels of polymorphism. However, despite these large differences in genetic diversity between autogamous and allogamous *Epipactis* species, our results did not show significant

differences in niche breadth between mating systems. Nonetheless, huge variation in distribution range and niche breadth was observed among facultative autogamous and allogomous species, while this was less the case for autogamous species. Considering that *Epipactis* autogams transition from allogams (Brys and Jacquemyn 2016; Sramkó *et al.* 2019), it is possible that the larger ranges that are sometimes associated with facultative autogamous species reflect a temporary stage in the transition from outcrossing to selfing and that selfing species will ultimately have more restricted niches than allogamous species (Park *et al.* 2018) but this would require further investigation to ascertain.

Selfing can also act as a reproductive barrier (Brys *et al.* 2014, 2016), and therefore reduce the influx of maladaptive genes and increase the rate of adaptive evolution (Levin 2010). If orchid seeds arrive in a novel habitat and manage to establish, selfing can be expected to maintain these pre-adapted genotypes and consequently conserve a wider niche breadth of the species. However, this assumes that seeds from a single species are able to colonize multiple habitats. This is not necessarily the case, because seed germination in orchids strongly depends on mycorrhizal fungi and these fungi may represent an important bottleneck in the colonization process (Bidartondo and Read 2008; Swarts *et al.* 2010; Dearnaley *et al.* 2012, but see Těšitelová *et al.* 2012). On the other hand, local adaptation can be expedited by low or moderate levels of gene flow by increasing additive genetic variance and allowing post-colonization local adaptation (Peterson and Kay 2015). Facultative allogamous species may therefore be more likely to experience niche shifts than fully autogamous species. Our results are somewhat in line with this hypothesis, as facultative autogamous species displayed larger variation in range size and niche breadth than autogamous species.

Environmental variables affecting the distributions of Epipactis

To model the niche of each orchid species, we included a wide range of different variables that previously have been shown to influence the distribution of orchids (Bowles *et al.* 2005; Tsiftsis *et al.* 2008; McCormick *et al.* 2009; Acharya *et al.* 2011; Djordjević *et al.* 2016a; Štípková *et al.* 2017). These variables related to temperature and precipitation, soil, land cover type, bedrock and elevation. Land cover and bedrock appeared to be the most important variables for predicting mean species habitat

suitability. These results are largely in line with previous studies that have modelled the distribution of *Epipactis* species. For example, Tsiftsis *et al.* (2008) concluded that in Macedonia, the most important environmental variables linked to orchid occurrence were habitat types. Similarly, Djordjević *et al.* (2016) showed that temperature and bedrock were important predictors of mean *Epipactis* occurrence, and certain species such as *E. helleborine* and *E. lusitanica* demonstrated strong associations with mean annual temperature. However, the contributions of these variables to differences in habitat suitability and niche breadth were not related to mating system. These results are in contrast with findings of Grant and Kalisz (2019), who showed that the environmental variables that contributed most to differences in niche breadth between selfing and outcrossing species were precipitation, seasonality, and elevation. Their results showed that average annual precipitation was higher in outcrossing species, suggesting that selfing species are generally more tolerable to dry or water-limited environments, while selfing species occurred at higher elevations than outcrossing species. None of these variables, however, appeared to have a large influence on the distribution of *Epipactis* species.

The *Epipactis* species with wider niche breadths in geographic space tended to have larger geographic ranges than those with narrow niche breadths. This pattern was clear even with though data limitations resulted in measures of geographic range being conservative, especially for the species with large ranges. The greater number of habitats that generalists are able to use allows them to disperse farther than species specialised to inhabit a small number of environments, a pattern that can be seen in the distributions of many taxa (Thompson *et al.* 1999; Slatyer *et al.* 2013; Yu *et al.* 2017). However, in terms of environmental space, the *Epipactis* species with wide niches did not necessarily have large ranges. Species with large ranges can have narrow niches if they are adapted to habitats that happen to be common in the study area, and species with small ranges can have narrow niches if they are limited by factors other than those included in their niche definition (e.g. geographical barriers) (Peterson *et al.* 2011; Saupe *et al.* 2015). Most of the *Epipactis* species with small ranges demonstrated distinctly strong associations with forests, and the dependence on forests that they developed in their evolutionary histories may have made them less able to utilise other environments, limiting their ranges. The

existence of both forest-dependent and generalist *Epipactis* species indicates that there may be some species traits not investigated in this study which affects their distributions.

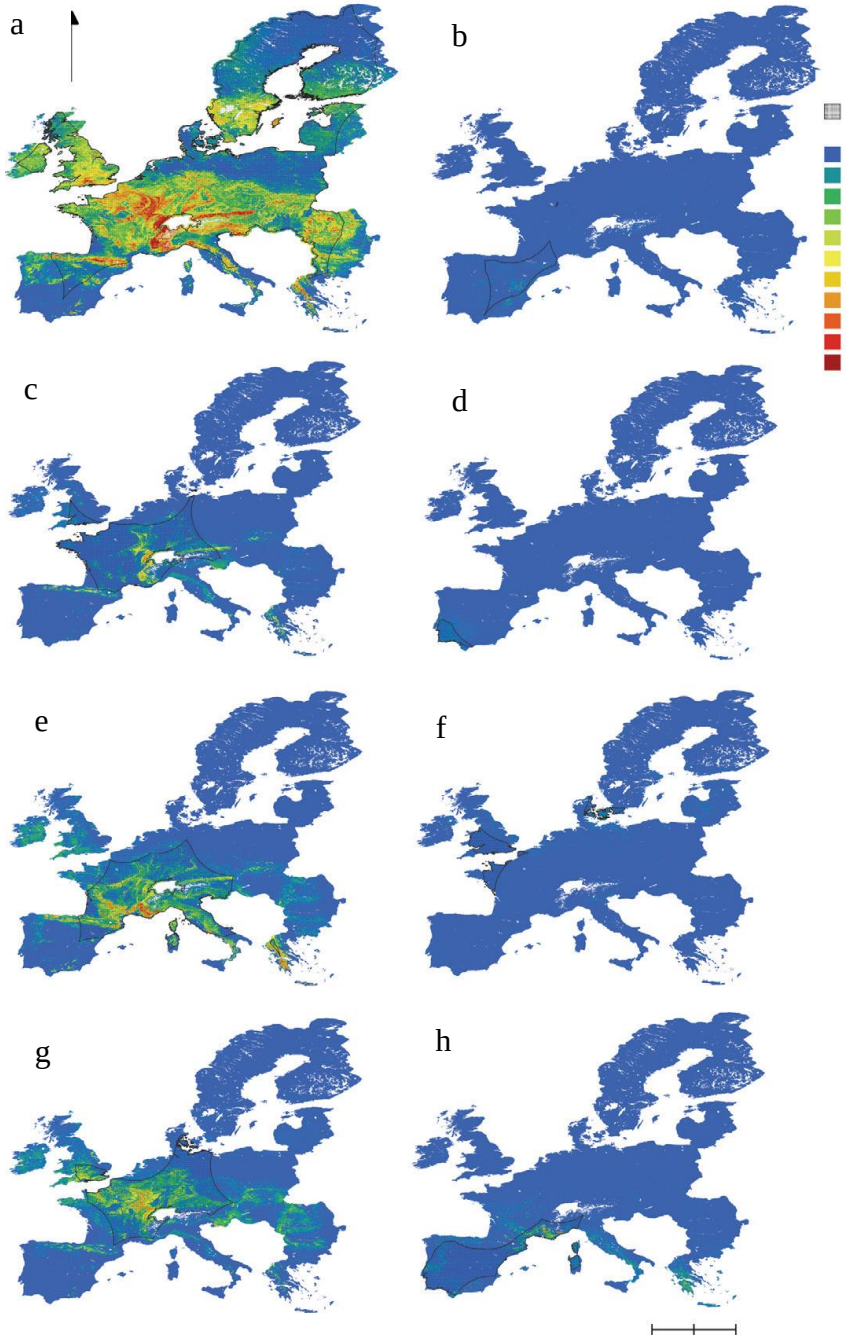
Other factors contributing to Epipactis distributions

An aspect of *Epipactis* niches that should be investigated to create a clearer picture of the drivers of their distributions is the influence of biotic interactions, in particular, mycorrhizal fungi. The lack of an endosperm in orchid seeds means that terrestrial orchids need fungi in the soil to germinate and establish (Smith and Read 2010) and therefore the presence of fungal species that provide nutrients for developing seeds likely restricts *Epipactis* distributions (Ogura-Tsujita and Yukawa 2008; McCormick and Jacquemyn 2014). Species that are capable of associating with a wide variety of mycorrhizal fungi can be expected to have a wider distribution range and niche breadth than species that associate with a small number of fungi. For example, the widespread *E. helleborine* and *Gymnadenia conopsea* have recently been shown to associate with a wide range of OMF across their entire distributions and their mycorrhizal partners differed substantially between geographic regions (Xing *et al.* 2020). Associating with different sets of mycorrhizal fungi can also induce niche differentiation (Jacquemyn, Brys, Merckx, *et al.* 2014; Jacquemyn *et al.* 2015) and act as a reproductive barrier in a similar way to selfing. For example, Jacquemyn *et al.* (2016) showed that *Epipactis* species of dune and forest habitats associated with distinct mycorrhizal communities and that these differences in mycorrhizal communities severely limited gene flow between the two habitats. Immigrant seeds had a significantly lower probability of protocorm formation than native seeds (Jacquemyn *et al.* 2018). Hybrid seeds originating from crosses between the two ecotypes also showed significantly lower protocorm formation than pure seeds, further contributing to reproductive isolation. These results suggest that differences in niche breadth may also be caused by differences in mycorrhizal associations. Furthermore, the cluster of species characterised by having small ranges in our results had strong associations with forest land cover, and Bidartondo and Read (2008) concluded that mycorrhizal fungi that are restricted to forests limit the dispersal of their orchid symbionts. However, Těšitelová *et al.* (2012) found that neither the availability of mycorrhizal fungi nor abiotic habitat conditions limited germination in *Epipactis*, even in species that demonstrate habitat specializations in adulthood. Further research, therefore,

is needed regarding both seedlings and adult plants to see whether species with narrow niche breadths in mycorrhizal associations have significantly smaller distribution ranges than species with broad niche breadth in mycorrhizal partners.

Conclusion

To conclude, our results do not support the hypothesis that autogamous species have larger ranges and wider ecological niches than facultative autogamous or allogamous species. For *Epipactis* species in Europe, bedrock and land cover type appeared to be more important environmental variables than climatic conditions in predicting distributions. This suggests that plant traits other than mating system are more important in defining the ecological niche of *Epipactis* species. Because germination and seedling establishment are critical stages in the life cycle of orchids, differences in interaction with mycorrhizal fungi may be as, or more, important than mating system in defining the distribution range and niche breadth of orchids. Future research should investigate in more detail that, in addition to abiotic environmental structure, biotic interactions such as those with mycorrhizal fungi are important drivers of *Epipactis* distributions.



Appendix 1. Habitat suitability maps overlain with α-hull range estimations of *Epipactis* species not illustrated in Fig. 1: *E. atrorubens* (a), *E. phyllanthes* (b), *E. leptochila* (c), *E. lusitanica* (d), *E. microphylla* (e), *E. kleinii* (f), *E. purpurata* (g), *E. tremolsii* (h).

Chapter 3

Ecological niche breadth and overlap are related to variation in mycorrhizal communities in *Epipactis*

Abstract

Background and aims While it is generally accepted that physiological tolerances determine where organisms can potentially occur, interactions with other organisms, specifically mutualists, may be equally important in determining plant population dynamics and range limits. This is particularly applicable to orchids, which depend on interactions with mycorrhizal fungi for successful germination and subsequent establishment of seedlings. At present, we do not know how and to what extent range size and niche breadth of orchids are related to variation in mycorrhizal communities associating with orchids. In this study, we tested the hypothesis that ecological niche breadth and overlap are related to the diversity and composition of orchid mycorrhizal fungal communities in the orchid genus *Epipactis*.

Methods We used ecological niche modelling to quantify the ecological niche breadth and niche overlap of nine *Epipactis* species in Europe. High-throughput sequencing was used to identify orchid mycorrhizal fungus (OMF) taxa in *Epipactis* root samples from 43 populations in France and Spain and species diversity and community structure were investigated with multivariate analyses.

Key Results and Conclusions Niche breadth varied considerably between species (0.28 for *E. kleinii* to 0.77 for *E. helleborine*) and most species showed moderately low niche overlap (mean overlap: 0.33 ± 0.20). All studied *Epipactis* species associated with a wide variety of mycorrhizal fungi (mean number of fungal taxa per species: 65 ± 17). *Epipactis* species associated with a higher fungal diversity occupied a large range of habitat conditions while *Epipactis* species with more specialised fungal associations were also more specialised in their habitat requirements. In addition, fungal community structure was significantly more similar in *Epipactis* species with similar ecological niches. Overall, our results demonstrate that the environmental niche in *Epipactis* is related to fungal diversity and community composition and more generally that biotic interactions may contribute to shaping patterns of range size among orchids.
Keywords: Niche breadth, orchid mycorrhizal fungus, mutualisms, ecological niche model, high-throughput sequencing

Introduction

Investigating the mechanisms that shape species distributions is central to understanding why species are found where they are and how their distribution will change over time. Within an order, family, or even genus, range size and niche breadth can vary by several orders (Brown *et al.* 1996; Gaston 2003), but what drives this variation is not always clear. Seed dispersal is a primary determinant of plant occurrence, followed by a complex combination of environmental factors such as climate and substrate which allow seeds to germinate, adults to grow and reproduce, and populations to establish (Sheth *et al.* 2020). The ecological niche breadth, or range of environmental characteristics which support occurrence, can differ between species and can be reflected in differences in range size between species (Slatyer *et al.* 2013; Sexton *et al.* 2017). Habitat specialists are species with a narrow niche breadth that tolerate less variation in their habitat range compared to habitat generalists, which occur in a wider range of habitats (Thuiller *et al.* 2005; Sheth *et al.* 2020).

In addition to the abiotic components of a species' habitat, there is mounting evidence that biotic interactions play an important role in shaping distribution patterns (Bruno *et al.* 2003; Van der Putten *et al.* 2010; Pigot and Tobias 2013; Alexander *et al.* 2022). The geographic range of a plant species can be extended beyond the usual limits imposed by the abiotic niche by mutualist relationships, but can also be restricted by the absence of mutualists (Fowler *et al.* 2023). For example, plant species that are able to self-pollinate tend to have larger ranges than outcrossing species, because the occurrence of the former is less limited by the availability of mates in a habitat (Baker 1955; Grossenbacher *et al.* 2015). Similarly, biotic interactions may enhance or impede a species' tolerance to abiotic stress and hence influence its abiotic niche breadth and range size (Sheth *et al.* 2020). For example, the range of a plant species can extend beyond the limits imposed by the abiotic niche with the help of fungal associations, such as the range expansion of grasses with fungal endophytes into environments that would otherwise be too dry to inhabit (Afkhami *et al.* 2014). Considering that the majority of plant species associates with mycorrhizal fungi and that fungal associations can differ in type between plant species and habitats (Read 1991; van der Heijden *et al.* 2015; Jacquemyn and Merckx 2019), it is likely that

differences in fungal communities in combination with ecological niche breadth contribute to the variation of geographical range among plant species.

With >27.000 species, the Orchidaceae is one the largest angiosperm families (Givnish *et al.* 2015; POWO 2023) and species differ considerably in their distributions, with some species being endemic or having very narrow distribution areas, and others occurring across entire continents (Swarts and Dixon 2009; McCormick and Jacquemyn 2014; Givnish *et al.* 2015). Of the abiotic factors that make up an orchid's environment, climate, elevation, substrate (bedrock and soil) and land-use have been identified as strong predictors of orchid distribution (Acharya *et al.* 2011; Gaskett and Gallagher 2018; Djordjević and Tsiftsis 2022). Apart from abiotic environmental factors, the distribution and ecological niche of orchids is most likely also determined by biotic interactions. A characteristic feature of all orchids is their tiny seeds which rely on mycorrhizae to obtain carbon to germinate and grow into seedlings (Taylor *et al.* 2003; Rasmussen 2008; Smith and Read 2010; Tedersoo *et al.* 2020) and some orchid species continue to be mycoheterotrophic into adulthood (Gebauer and Meyer 2003; Selosse and Roy 2009; Jacquemyn and Merckx 2019). The high dispersal capabilities of these seeds creates the potential for large species ranges, while the dependence on the occurrence of mycorrhizal fungi in a substrate may limit local orchid distribution (Hollick *et al.* 2007; McCormick and Jacquemyn 2014; McCormick *et al.* 2018). On the other hand, mycorrhizal interactions can facilitate range size expansion of orchids in the same way as in other plants (Downing *et al.* 2020), the paradox of which complicates predicting the distribution of orchids.

Several studies have suggested that orchid mycorrhizal fungi are widespread and, at least on a large scale, their availability does not necessarily predict orchid distributions (McCormick and Jacquemyn 2014; Jacquemyn, Duffy, *et al.* 2017). However, like orchid distributions, fungal communities are patchily distributed and can differ between sites with different soil chemistry and presence of ectomycorrhizal hosts (McCormick *et al.* 2012; Bunch *et al.* 2013; Ferlian *et al.* 2021), and therefore differences in fungal community structure likely contribute to patterns of orchid distribution, particularly for habitat specialists (but see (Waud *et al.* 2017). Considering the relationship between range size and niche breadth, investigating the effect of mycorrhizal associations on range

size in the context of environmental niche breadth may shed light on the variety of distribution patterns found in the Orchidaceae. On the one hand, associating with many fungi may increase the area that an orchid can colonise (Swarts *et al.* 2010; Downing *et al.* 2020). Associating with only a few, specific fungal taxa may limit the distribution of the orchid species if these taxa also have a limited distribution, but can improve the carbon or nutrient transfer, which may influence plant fitness and therefore population persistence (Bonnardeaux *et al.* 2007; van der Heijden *et al.* 2015). This reflects the different advantages related to niche breadth, where habitat specialists are well adapted to fewer habitats, while habitat generalists can colonise a larger range of habitat types. However, the interplay between spatial distribution, ecological niche and fungal community structure is not yet well-defined for orchids.

In this study, we tested the hypothesis that niche breadth and overlap are related to mycorrhizal diversity and community structure in the orchid genus *Epipactis*. Previous research has shown that differences in mating system did not explain variation in niche breadth and differentiation (Evans and Jacquemyn 2020), suggesting that other factors such as mycorrhizal specificity may be more important drivers of range size and niche breadth in *Epipactis*. In particular, we hypothesize that 1) species that associate with a low diversity of mycorrhizal fungi have a narrower niche breadth than species that associate with a large diversity of fungi, and 2) species that associate with similar mycorrhizal communities tend to have strong overlap in distribution and environmental niche. To test these hypotheses, we used ecological niche modelling to define the abiotic environmental niche of nine *Epipactis* species in Europe. Mycorrhizal fungal taxa associating with *Epipactis* populations were identified using high-throughput sequencing and metabarcoding. Finally, we related fungal diversity indices to niche breadth and assessed whether similarities in mycorrhizal community composition between orchids were related to niche overlap.

Materials and methods

Study species

The terrestrial orchid genus *Epipactis* is taxonomically complex, with a multitude of species described in publications (Delforge 2006), which are still widely used despite recent taxonomic revisions (Sramkó *et al.* 2019; Bateman 2020). We investigated nine *Epipactis* species which have sufficient evidence to suggest valid species distinction according to the phylogenies of Sramkó *et al.* (2019) and Bateman (2020) and information from Crespo *et al.* (2001), Bernardos *et al.* (2004), Hollingsworth *et al.* (2006) and Zhou and Jin (2018): *E. atrorubens*, *helleborine*, *kleinii*, *leptochila*, *microphylla*, *muelleri*, *palustris*, *purpurata* and *tremolsii*.

Epipactis species can be found in a variety of habitats and their occurrence is highest on limestone bedrock, coniferous and broad-leaf forests, moderate temperatures and low-nutrient soils (Hemrová *et al.* 2019; Evans and Jacquemyn 2020; Djordjević and Tsiftsis 2022) but some species also occur in coastal dunes, forest, and wet meadows at a broad range of elevations. As with other orchids, the seeds of *Epipactis* species lack a nourishing endosperm and are therefore reliant on mycorrhizal fungi to germinate (Bidartondo and Read 2008; Jacquemyn *et al.* 2018). Most *Epipactis* species are partially mycoheterotrophic as adults that rely on fungi to obtain a part of their carbon (Selosse *et al.* 2004; Hynson *et al.* 2016; Schiebold *et al.* 2017; Jacquemyn *et al.* 2021). They associate with a wide diversity of mycorrhizal fungi, particularly the Agaricomycete genera *Tomentella*, *Sebacina*, *Cortinarius* and *Russula*, as well as Pezizomycete genera such as *Tuber*, *Peziza* and *Wilcoxina* (Bidartondo *et al.* 2004; Jacquemyn *et al.* 2016, 2021; Suetsugu *et al.* 2017). These fungal taxa are mainly from the Basidiomycota but also some, namely ectomycorrhizal, from the Ascomycota (Selosse *et al.* 2004; Ogura-Tsujita and Yukawa 2008; Suetsugu *et al.* 2017).

Sampling and genetic sequencing

For each species, populations were sampled in France and Spain in the summer of 2022. Between one and three populations were sampled per species (**Fig. 1**), and between three and five flowering individuals per population. Three lateral roots with evidence of orchid

mycorrhizal fungus (OMF) colonization (opaque, yellowish colouration) were collected per individual and immediately placed in a portable refrigerator and then subsequently frozen at -20 °C until processing.

Roots were surface sterilized with a 1 % sodium hypochlorite solution for 30 seconds, followed by two 30 second rinses with distilled water. DNA was extracted from 1 mg root material per individual with the UltraClean Plant DNA Isolation Kit following the instruction of the manufacturer (MoBio Laboratories Inc., Solana Beach, CA, USA). The extracted DNA sequences were amplified with PCR using fungal-specific primers ITS86F (5'-GTGAATCATCGAATCTTTGAA-3′) (Turenne *et al.* 1999) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.* 1990) following the method of (Jacquemyn *et al.* 2021). Two replicate PCR reactions were performed using a dual-indexing strategy (Kozich et al., 2013) and combined for each sample in reaction volumes of 25 µl with a 1x Titanium Taq PCR buffer, 150 µM of each dNTP, 0.5 µM of each primer, 1× Titanium Taq DNA polymerase (Clontech, Saint-Germain-en- Laye, France), and 2 µl 10-times diluted DNA. Denaturation was initiated at 94 °C for 120 seconds, followed by 30 cycles of 45 s each of denaturation at 94°C, annealing at 59 °C, elongation at 72 °C, and a final elongation at 72 °C for 10 minutes. Amplicons were purified using Agencourt AMPure XP magnetic beads (Beckman Coulter Genomics GmbH, South Plainfield, UK) and duplicates were combined. Purified products were quantified using a Qubit High Sensitivity Fluorometer kit (Invitrogen, Carlsbad, CA, USA) and amplicons from each sample were combined at equimolar concentrations into an amplicon library. The library was subjected to an ethanol precipitation and loaded on an agarose gel. The band was excised to the expected size of 400 bp and DNA was purified with a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) before the library was diluted to 2 nM. Resulting DNA was sequenced using the Ill umina MiSeq platform Illumina, San Diego, CA, USA) at the KU Leuven Genomics Core facility.

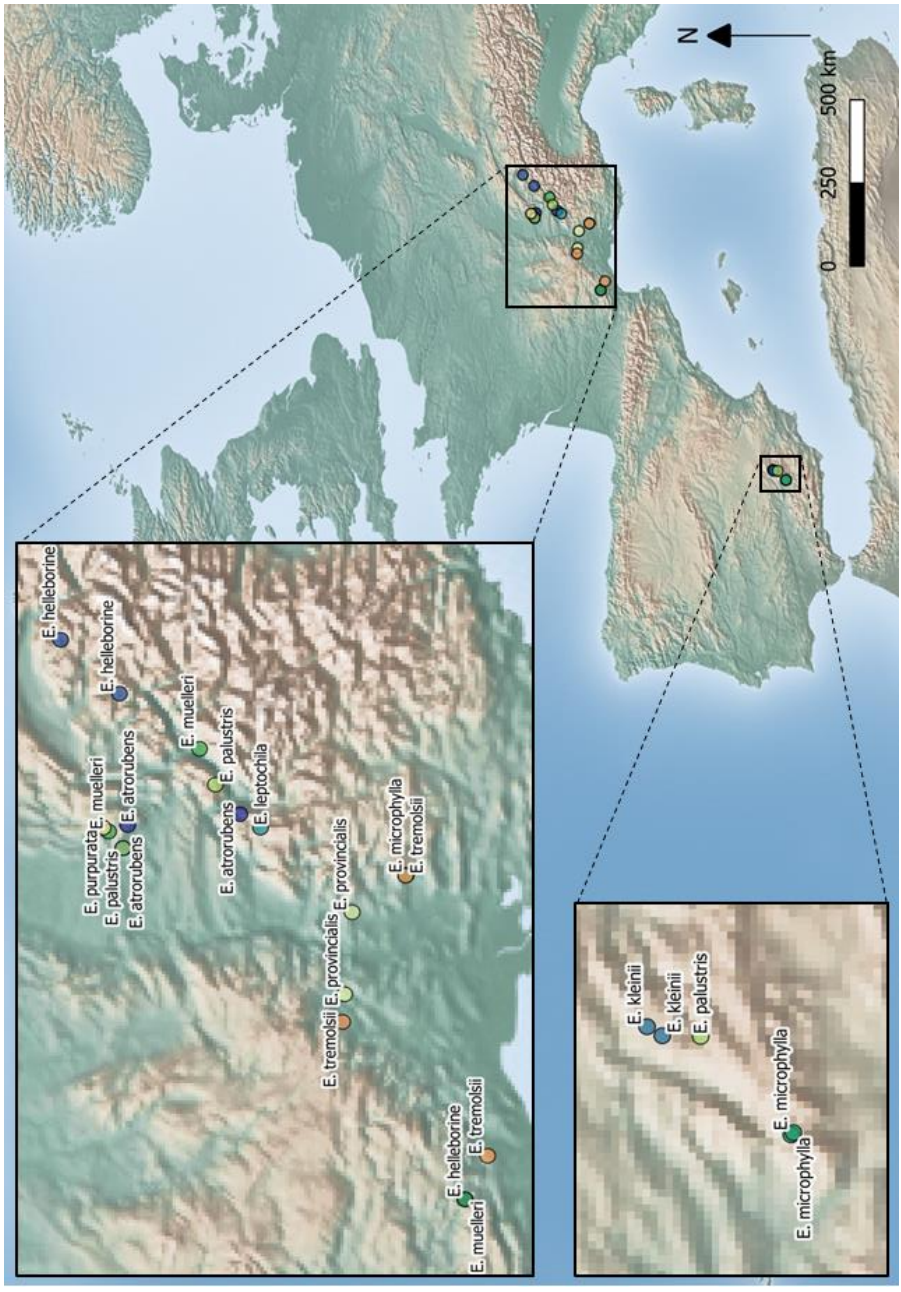


Figure 1. *Epipactis* populations sampled for mycorrhizal fungi.

The resulting forward and reverse read sequences were merged into one consensus sequence per sample and filtered to a maximum of 10 mismatches, a minimum match of 80 % id, 200 base pairs, and no unique sequences using the USEARCH sequence analysis tool (Edgar 2010). USEARCH was further used to assure correct orientation using the UTAX reference database. High-quality sequences were grouped into Operational Taxonomic Units (OTUs) based on 97 % similarity with the UPARSE algorithm, checked for chimeras and assigned taxonomic identities to the highest taxonomic rank possible based on BLAST results of representative sequences using NCBI (Cole *et al.* 2014) and UNITE (10.05.2021) (Kõljalg *et al.* 2013) databases. The OTUs were filtered to retain fungal taxa with a minimum rank of genus, and further filtered using a list of potential OMF taxa found in *Epipactis* compiled from the findings of other studies. The taxa assigned to OTUs corresponded well between the databases (> 95% similarity) and therefore the dataset using the NCBI database was used in further analyses as it yielded more OTUs identified to genus level.

Orchid niche breadth and overlap

For each species niche breadth and overlap were calculated following (Evans and Jacquemyn 2022). Briefly, occurrence data for each species was obtained as spatial coordinates between the years 2000 and 2019 from the online database GBIF (www.GBIF.org; https://doi.org/10.15468/dl.aicdym, 1 Jul 2019). Records with uncertain identification or with a spatial resolution below 100 m were discarded. Occurrences were aggregated into 10 km^2 grid cells and the centre point of each grid cell was extracted and used as the presence point, to reduce issues related to sampling bias and spatial clustering. Environmental data was obtained from satellite imagery for the extent of Europe at a resolution of 1 km for elevation (EarthEnv database) (Amatulli *et al.* 2018), mean annual temperature and annual rainfall (WorldClim v.2) (Fick and Hijmans 2017), and soil characteristics (see **Supplementary material S2** for details; ESDAC, (Hiederer 2013; Ballabio *et al.* 2019), as well as two categorical variables bedrock (ESDAC; Van Liedekerke et al., 2006) and Corine Land Cover (European Environmental Program (Heymann 1994). To quantify the range of environmental conditions in which the orchid species are most likely to occur, we used the ecological niche modelling program Maxent v.3.4 (Phillips *et al.* 2018) to produce a distribution model of suitable habitat for each

species. Maxent was run through the ENMTools v1.0.5 R package (Warren *et al.* 2021) using the occurrence and environmental data with a random seed, 100 bootstrap replicates and 25 % random test points.

ENMTools was used to calculate two measures of Levins' *B2* metric of niche breadth (Levins 1968): niche breadth in environmental space ($B2_{env}$), which quantifies the range of environmental conditions with which a species is associated, and niche breadth in geographic space ($B2_{geo}$), which quantifies the distribution of a species' habitat (Warren *et al.* 2019). The output for both functions is an estimate between 0 and 1 for each species, with a value closer to 0 denoting a more specific range of habitat conditions occupied (narrow niche breadth) and a value closer to 1 for a more generalist habitat (wide niche breadth). ENMTools was further used to calculate Schoener's *D* (Schoener 1970) to quantify the overlap in environment use in environmental space (D_{env}) and habitat distribution in geographic space (D_{geo}) between each pair of species, with values closer to 0 indicating low overlap and values closer to 1 indicating high overlap.

Data analysis

The table of fungal OTU reads per orchid sample was converted into a matrix of presence/absence of each OTU per sample, as well as a matrix of presence/absence of each OTU per species. To quantify fungal diversity we counted the number of unique fungal OTUs detected at least once in an orchid sample (sample OTU count), and at least once in a species (species OTU count). Orchid samples with no OTUs detected were removed, as were OTUs that occurred in fewer than three samples. A Pearson correlation test was performed to test the relationship between OTU count and sample size per species, and populations were removed from species with a large number of samples to reduce the uneven sample size between species. This resulted in three populations for each species except for *E. kleinii* (two populations) and *E. purpurata* (one population) (**Table 1**).

To summarise and visualise variation in fungal communities among orchid species, we performed a nonmetric multi-dimensional scaling (NMDS) analysis on OTU presence/absence per species. To test whether mycorrhizal community differed significantly between species and site, a PERMANOVA was run for 1000 permutations

on OTU presence/absence per sample, with OTU presence as the dependent variable and species and site as independent variables, allowing for an interaction between species and site.

To test the hypothesis that niche breadth was related to fungal diversity, we used linear regression to assess the relationship between log-transformed species OTU count and niche breadth in environmental ($B2_{env}$) and geographic ($B2_{geo}$) space. A Jaccard similarity value (Jaccard 1908) was calculated with the R package igraph (Csardi 2013) for each pair of orchid populations and each pair of orchid species using the presence/absence of each OTU per population and species, respectively. To test whether orchid species with similar ecological requirements (D_{env}) and overlapping habitat ranges (D_{geo}) had similar mycorrhizal fungus communities, Mantel tests were run using the species Jaccard similarity matrix and the Schoener's *D* niche overlap matrices using the R vegan package (Oksanen *et al.* 2019).

All data analyses were performed in R v4.0.2 (R Core Team 2021) and plots were visualised with the ggplot2 package (Wickham 2011).

Results

Fungal diversity and community composition

Genetic sequencing generated 4,779,196 sequences (2339 OTUs). After filtering, 282 unique OTUs corresponding with orchid mycorrhizal fungus (OMF) genera from 111 orchid samples were retained, covering 1,304,033 sequences (27 %). The taxa assigned to OTUs corresponded well between the NCBI and UNITE databases (> 95% similarity) and subsequent analyses yielded similar results and therefore the dataset using the NCBI database was used in further analyses as it yielded more OTUs identified to genus level. In total, 31 different genera were identified, with the greatest number of OTUs from *Tomentella* (53), *Sebacina* (37), *Tuber* (22) and *Inocybe* (21) (**Fig. 2**; **Appendix 1**). 194 of the OTUs (69 %) were identified from the division Basidiomycota and the remaining 88 OTUS (31 %) from the Ascomycota.

Fungal OTU count per species varied between 26 for *E. kleinii* and 86 for *E. helleborine* (**Table 1**). Fungal OTU count per species was not correlated with number of individuals sampled ($t_7 = 1.58$, p-value = 0.16). An NMDS indicated that *E. atrorubens*, *E. palustris* and *E. leptochila* associated with similar fungal communities, while *Epipactis muelleri* and *E. microphylla* were closer to the mean fungal community structure (**Fig. 3(a)**). *Epipactis kleinii* and *E. tremolsii* were separated from the other species, as was *E. helleborine*, indicating more distinct fungal communities. While most taxa associated with high proportions of *Tomentella*, *Sebacina* and *Tuber* OTUs, *E. kleinii* and *E. helleborine* associated with fungal taxa in more even proportions (**Fig. 3(b)**). A PERMANOVA detected a significant difference in community structure between species ($F_8 = 2.93$, $R^2 = 0.17$, p-value < 0.001) and between sites ($F_{15} = 1.91$, $R^2 = 0.21$, p-value < 0.001). A post-hoc pairwise PERMANOVA detected significant differences in fungal community between many of the *Epipactis* species (**Supplementary material S3**), both among fungal generalists (e.g. *E. helleborine* vs *E. atrorubens*) and fungal specialists (e.g. *E. tremolsii* vs *E. kleinii*), as well as between fungal generalists and specialists (e.g. *E. helleborine* vs *E. kleinii*).

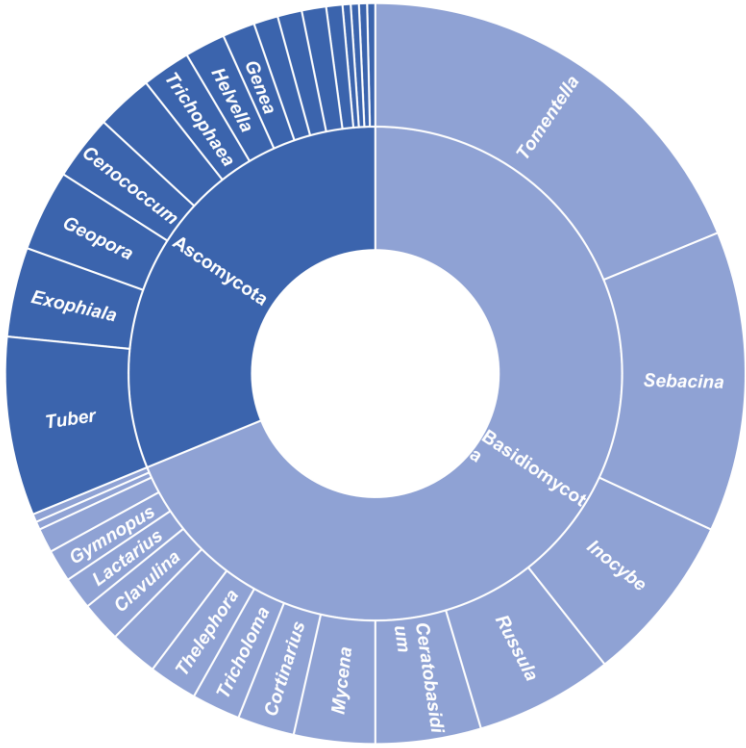


Figure 2. Proportion of fungal OTUs (Operational Taxonomic Units) identified for *Epipactis* species belonging to each fungal division (inner circle) and genus (outer circle). Proportions shown represent number of unique OTUs per taxonomic division.

Table 1. Niche breadth (in geographic space ($B2_{geo}$) and environmental space ($B2_{env}$)) and counts of unique fungal OTUs detected for each *Epipactis* species and corresponding number of samples and populations.

Species	Samples	Populations	OTU count	*B2geo*	*B2env*
E. atrorubens	15	3	70	0.8482	0.6887
E. helleborine	14	3	86	0.8524	0.7676
E. kleinii	10	2	26	0.6998	0.2792
E. leptochila	12	3	68	0.5831	0.6154
E. microphylla	14	3	55	0.8050	0.5386
E. muelleri	11	3	71	0.7267	0.6163
E. palustris	15	3	74	0.8535	0.6581
E. purpurata	5	1	55	0.6662	0.5072
E. tremolsii	14	3	82	0.7190	0.3547

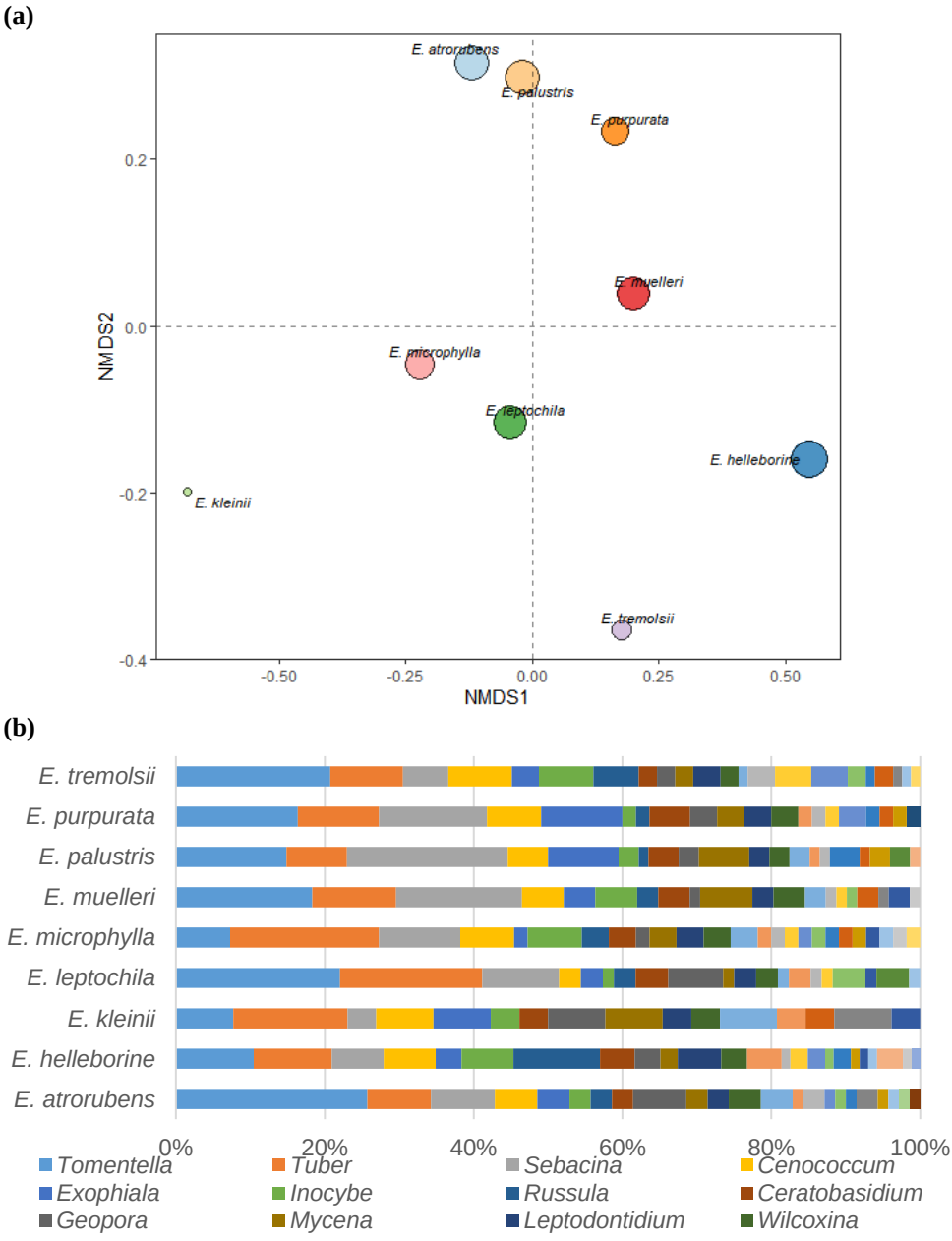


Figure 3. NMDS (Nonmetric Multidimensional Scaling) plot **(a)** and bar plot **(b)** showing differences in fungal community composition between nine *Epipactis* species. Point size on the NMDS represents relative niche breadth in environmental space (Levins' $B2_{env}$) and bars represent proportions of OTUs belonging to fungal genera.

Relationship between niche metrics and fungal communities

Niche breadth varied substantially among *Epipactis* species. $B2_{env}$ ranged from 0.279 (*E. kleinii*) to 0.768 (*E. helleborine*) and $B2_{geo}$ from 0.583 (*E. leptochila*) to 0.854 (*E. helleborine*; **Table 1**). Ecological niche breadth in environmental space ($B2_{env}$) was higher in *Epipactis* species with higher OTU counts (**Fig. 4**; $R^2 = 0.46$, $F_{1,7} = 5.95$, p-value = 0.044). Niche overlap in environmental space (**Supplementary material S4**) varied between 0.033 (*E. kleinii* and *muelleri*) and 0.81 (*E. helleborine* and *palustris*), and niche overlap in geographic space (**Supplementary material S5**) between 0.21 (*E. purpurata* and *tremolsii*) and 0.84 (*E. helleborine* and *palustris*). Jaccard similarity in fungal communities between species (**Supplementary material S6**) varied between 0.077 (*E. helleborine* and *kleinii*) and 0.24 (*E. atrorubens* and *palustris*). A Mantel test indicated that *Epipactis* species with overlapping ecological niches had similar mycorrhizal communities, both for niche overlap in environmental space ($R = 0.45$, p-value = 0.044) and geographic space ($R = 0.47$ p-value = 0.032) (**Fig. 5**).

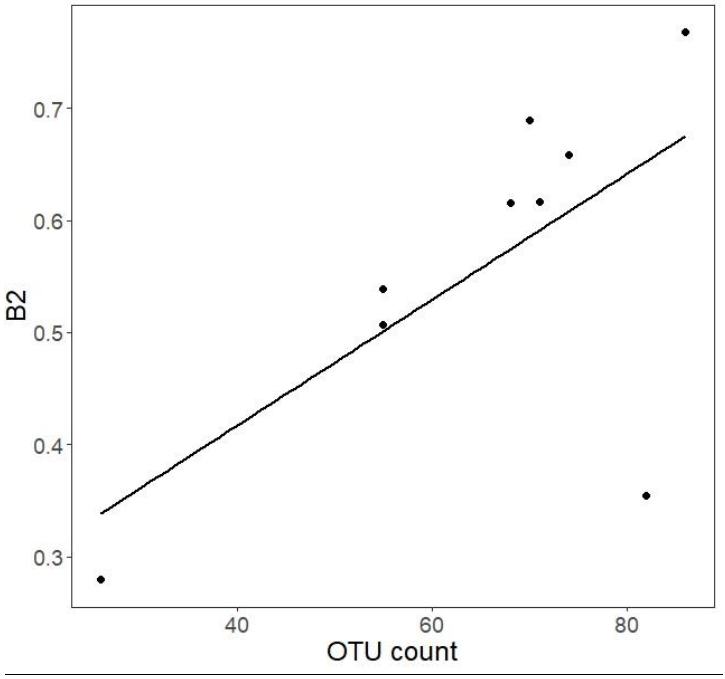
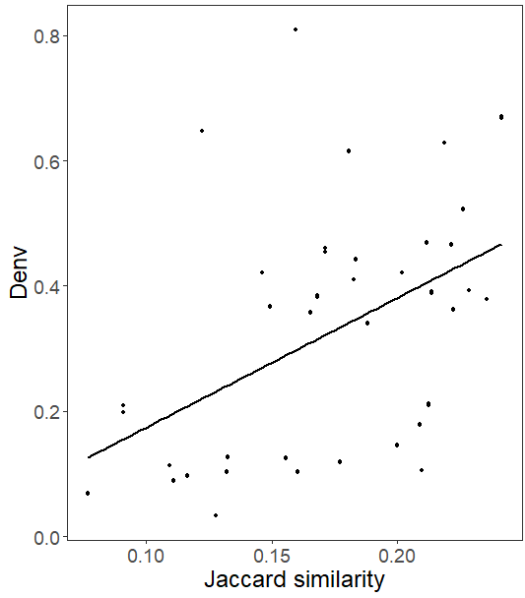


Figure 4. Relationship between count of unique fungal OTUs detected per *Epipactis* species and ecological niche breadth (Levins' B2) in environmental space.

(a)



(b)

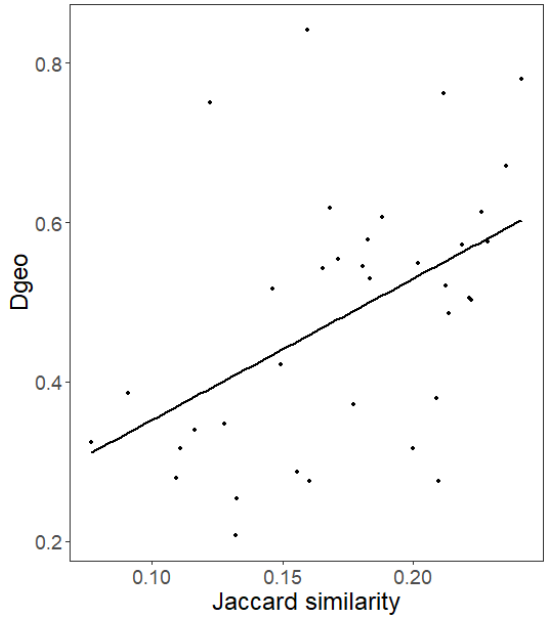


Figure 5. Mantel tests showing the relationship between fungal community similarity and niche overlap (Schoener's *D*) in environmental space **(a)** and geographic space **(b)** for nine *Epipactis* species.

Discussion

Fungal diversity and community composition in Epipactis

The investigated *Epipactis* species associated with a large diversity of mycorrhizal fungi – in total, 282 different OTUs from 31 fungal genera were observed. These results are largely in agreement with previous studies that have investigated mycorrhizal communities in various *Epipactis* species and have shown that most *Epipactis* species are mycorrhizal generalists that associate with a wide variety of fungi (Bidartondo *et al.* 2004; Selosse *et al.* 2004; Ogura-Tsujita and Yukawa 2008; Jacquemyn *et al.* 2016, 2021; Wang *et al.* 2022). This is also consistent with previous findings that have shown that partially mycoheterotrophic orchid species, such as the *Epipactis* species investigated here, tend to associate with a large number of mycorrhizal fungi, while autotrophic orchid species or fully mycoheterotrophic species usually specialize in a limited number of fungal taxa (Jacquemyn and Merckx 2019; Wang *et al.* 2022). The proportions of Basidiomycota (~70%) and Ascomycota (~30%) were also similar to reports for the Orchidaceae as a whole (Wang *et al.* 2022), suggesting that either these fungal taxa are available in *Epipactis* habitats in similar proportions as for Orchidaceae globally, or that the variety of roles that different mycorrhizal taxa play in orchid ecology is conserved in *Epipactis*.

Associating with a large number of fungi may be beneficial when the different partners differ in quality or functionality. Fungal taxa differ in nutrient and carbon transfer capabilities (McCormick *et al.* 2018; Tedersoo and Bahram 2019; Li, Wu, *et al.* 2021) and therefore a more diverse community may be more likely to include the most beneficial partner (sampling effect) or multiple partners may provide different benefits (complementarity effect) (Batstone *et al.* 2018). Partners may also have complementary effects over time if the focal mutualist's needs change across ontogeny, finally resulting in a different ideal partner at different life stages (Batstone *et al.* 2018). Adult plants may also maintain associations with fungi that induced seed germination. Complementarity effects over time have already been demonstrated by (Bidartondo and Read 2008), who showed that the diversity of mycorrhizal fungi associating with seedlings and adult plants increased from the seedling stage to the adult stage and that plants gradually accumulated

fungi during their development. Given that all *Epipactis* species investigated here are partially mycoheterotrophic, it is reasonable to assume that their adult fungal diversity represents both fungi that have the capacity to induce seed germination and growth, as well as fungi that can supply sufficient amounts of carbon and nitrogen to provide fitness benefits at other life stages such as increased seedling growth and below-ground survival of adults (Gonneau *et al.* 2014).

Fungal specificity, or the number of fungal taxa with which an orchid species associates, has evolved both to be less specific (more taxa) and more specific (fewer taxa) in the Orchidaceae (Shefferson *et al.* 2007). Although *Epipactis* species may be considered fungal generalists by their variety of fungal partners, degrees of fungal specialisation are reflected in the different number and community composition of fungal taxa associating with each species. *Epipactis kleinii* associated with the fewest fungal taxa (**Table 1**) and had a distinct fungal community with higher proportions of *Pezizales* (**Fig. 3**), which have been found to sometimes form ectomycorrhizal associations in *Epipactis* in other studies (Bidartondo *et al.* 2004; Selosse *et al.* 2004). Rhizoctonia genera such as *Tomentella* and *Sebacina*, which commonly form mycorrhizas with orchids (Dearnaley *et al.* 2012; Wang *et al.* 2022) were dominant in most other *Epipactis* species. *Epipactis muelleri* had fairly high fungal richness and its fungal communities were closer to the mean fungal community composition, indicating fungal generalisation. A low fungal richness and stronger community distinction indicates that *E. kleinii* associated with a small selection of specific fungi in the study area and may be interpreted as a relative degree of fungal specialisation compared to *E. muelleri* (Shefferson *et al.* 2019). Fungal specialisation can improve carbon transfer to orchids in light-limited environments (Bonnardeaux *et al.* 2007) and therefore associating with fewer fungi, including ectomycorrhizal *Pezizales*, may benefit *E. kleinii*, although considering there are more widely available fungi that could perform this function, it is more likely that this species lost the ability to associate with more fungi through stochastic evolutionary processes (Batstone *et al.* 2018). While *Epipactis kleinii* and *E. muelleri* demonstrate clear patterns for different degrees of relative fungal specialisation and generalisation respectively, the remaining species demonstrated more complex patterns in fungal richness and community composition. For example, *E. helleborine* had the highest fungal richness but relatively distinct communities, while *Epipactis microphylla* associated with few fungal taxa but with

indistinct fungal communities. These differences in fungal richness and community structure indicate that it is necessary to consider other species-specific ecological effects such as environmental niche to understand fungal association in orchids.

Fungal diversity and environmental niche breadth

We detected a strong relationship between fungal richness and niche breadth in environmental space (**Fig. 4**), indicating that species associating with more fungal taxa had wider niche breadth, while those associating with few fungal taxa had narrower niche breadth. Species able to associate with a larger variety of mycorrhizal fungi such as *E. helleborine* are able to occupy a greater variety of habitats, demonstrated by the occurrence of *E. helleborine* in habitats from woodland to coastal dunes and roadsides (Delforge 2006; Hemrová *et al.* 2019; Evans and Jacquemyn 2020). High selectivity, on the other hand, indicates that orchids specialize on a few fungal partners and if these are lacking, the fitness of the orchid decreases and the species cannot maintain viable populations in the long term. Considering the distribution of fungal taxa is heterogeneous (Pickles 2010), if the specific fungal taxa with which a fungal specialist associates is not present in a habitat, the specialist will be unlikely to occur there (McCormick and Jacquemyn 2014). In this case, high selectivity restricts its distribution to places where beneficial partners are present. For example, the small number of fungi that *E. kleinii* and *microphylla* associate with may restrict the variety of habitats that they are able to occupy and likely also contributes to the relatively small distribution ranges that have been recorded for these species (Delforge 2006; Evans and Jacquemyn 2020). In this way, orchid mycorrhizal fungus (OMF) distribution and diversity are more likely to limit the distribution of fungal specialist orchids than generalists (McCormick and Jacquemyn 2014; McCormick *et al.* 2018; Vogt-Schilb *et al.* 2020).

However, it remains unclear whether the wide distribution of a habitat generalist orchid is the result of its ability to associate with a wide number of fungi, or if a wide variety of fungal partners is more available in a wide range of environments. In both cases, we expect large spatial variation in mycorrhizal community composition and the ability of orchids to switch mycorrhizal partners between habitats. Previous research in *Epipactis helleborine* has already shown large spatial variation in mycorrhizal communities, with

most of the variation due to partner turnover (Xing *et al.* 2020). *Epipactis helleborine* is quite opportunistic in its fungal partners and can easily switch partners depending on local availability (Xing *et al.* 2020). Given that this species only weakly relies on mycorrhizal fungi for its nitrogen and carbon supply and adults can survive without mycorrhizal association (May *et al.* 2020), this may explain its flexibility in partner choice and hence its capability of occupying a large number of habitats. Furthermore, a species that can associate with many mutualistic partners may be more likely to cope with partner turnover over time (Batstone *et al.* 2018), which may have supported the long-term persistence of *E. helleborine* in multiple areas despite temporal changes in fungal communities (Ventre Lespiaucq *et al.* 2021). Hence, pronounced spatial variation in mycorrhizal communities and the ability to easily switch partners may explain why this species has managed to occupy a large distribution range and environmental niche.

Although *E. helleborine* may occur in many environments due to association with many fungal taxa, seed introduction experiments have shown that introducing seeds of *E. helleborine* into wet dune slacks does not result in successful germination (Jacquemyn *et al.* 2018), suggesting that its distinct mycorrhizal communities limit its distribution to particular habitats. *Epipactis helleborine* is also moderately limited in its abiotic niche by temperature and its populations exhibit strong genetic signals for adaptation to local elevation and temperature (Evans and Jacquemyn 2022; Evans *et al.* 2023). This pattern of wide niche breadth but distinct fungal communities is also demonstrated in *E. palustris* (**Table 1**) which occurs in high moisture environments such as marshes and dune slacks (Jacquemyn, Brys, and Hutchings 2014). Both species associate with high numbers of fungal taxa but with distinctive fungal communities (see also Jacquemyn *et al.* 2016); while *E. helleborine* had relatively even representation of fungal genera, it associated with a higher proportion of *Russula* than other *Epipactis*, while *Sebacina* was dominant in *E. palustris* (**Fig. 3**). Association with these commonly-occurring fungal taxa (Wang *et al.* 2022) increases the chance of encountering suitable fungi in a habitat. On the other hand, while the high fungal richness of *E. tremolsii* creates the potential for it to occupy many habitats, it has a narrow niche breadth. Similar to *E. palustris*, the distribution of *E. tremolsii* is strongly linked to land cover (Evans and Jacquemyn 2020) but its low niche breadth values indicate that this habitat type may be less common in the landscape (Warren *et al.* 2019). In the case of *E. tremolsii*, a high fungal richness does not ensure a

wide niche breadth, likely because the species (and perhaps its fungal associates) is limited to a specific abiotic environment that is not widely distributed.

Niche overlap and mycorrhizal similarity

Our results showed a significant relationship between mycorrhizal similarity and environmental niche overlap (**Fig. 5(a)**), suggesting that orchid species associating with similar mycorrhizal communities tend to occur in similar habitats. Similarly, significant variation in mycorrhizal community composition was observed between *Dactylorhiza* species inhabiting different ecological habitats (Jacquemyn *et al.* 2016). The greater similarity in fungal communities between *Epipactis* species with overlapping habitat ranges (**Fig. 5(b)**) is also consistent with other findings (Jacquemyn *et al.* 2016; Xing *et al.* 2020) and is likely in part due to habitats that are closer together having more similar environments. This indicates that the distribution and availability of mycorrhizal taxa plays a role in the type of fungal taxa with which *Epipactis* interacts, which further shapes the distributions of *Epipactis* species. This is supported by previous findings that the distributions of *Epipactis* species were linked to the spatial distribution of ectomycorrhizal fungi (Ogura-Tsujita and Yukawa 2008; Hemrová *et al.* 2019).

Although it remains unclear what drives fungal presence, it is reasonable to assume that it is to some extent dependent on local growth conditions (Toljander *et al.* 2006) particularly because fungal taxa can differ between sites with different soil conditions and tree presence (Bunch *et al.* 2013; Ferlian *et al.* 2021). This particularly applies to fungal taxa that enable the transfer of carbon from trees to light-restricted adult orchids (Taylor and Bruns 1997; Bidartondo *et al.* 2004). Mycorrhizal communities were similar in *E. atrorubens*, *E. palustris* and *E. purpurata*, with dominant rhizoctonia *Tomentella* and *Sebacina* presence (**Fig. 4**). These species can all occur in low-light conditions and therefore may all similarly benefit from the carbon transfer opportunities offered by these genera (Taylor *et al.* 2003; Dearnaley *et al.* 2012; Wang *et al.* 2022). *Epipactis microphylla*, *E. leptochila* and *E. muelleri* also associated with these fungal types that could provide carbon in their similarly low-light conditions, but they had different mycorrhizal community composition. Considering these latter species depend on fungi for carbon and nitrogen more than *E. atrorubens*, *E. palustris*, and *E. purpurata* (Schiebold *et*

al. 2017), these community differences may be due to different ecological needs. However other studies have concluded that mycorrhizal diversity and community differences are not necessarily dependent on orchid photosynthetic capacity or trophic mode (McCormick *et al.* 2004; Jacquemyn, Waud, *et al.* 2017) and therefore it is more likely that, as with fungal richness, the differences in community structure between habitat generalists and specialists reflect the larger range of habitat challenges and fungal availability that generalists encounter across their ranges (Těšitelová *et al.* 2012).

Conclusion

Our results indicate that associating with a lower number of fungal taxa can limit the niche breadth of orchid species. Additionally, we demonstrate how orchid species that occupy a large range of habitats can associate with distinct fungal communities, even when fungal richness is high. However, a species with many fungal associates can still be limited in its niche breadth if its distribution is strongly linked to a habitat that does not occur frequently in a landscape. These results demonstrate a relationship between fungal specialisation and habitat specialisation, and how a complex interplay between environmental structure and fungal associations can contribute to niche expansion in some orchids and limit niche breadth in others. Further investigations involving functional roles of mycorrhizae in *Epipactis* species with different niche breadths may contribute to our understanding of this relationship. Additionally, incorporating more detailed niche quantification may shed light on how fungal interactions shape the tolerance of orchid species to different components of the abiotic niche.

Appendix 1. Count of unique fungal OTUs (Operational Taxonomic Units) with taxonomic identity identified in the full sample of *Epipactis* species.

Division	Order	Family	Genus	Count of OTU
Ascomycota	Chaetothyriales	Herpotrichiellaceae	*Exophiala*	11
	Helotiales	Leptodontidiaceae	*Leptodontidium*	7
	Mytilinidiales	Gloniaceae	*Cenococcum*	8
	Pezizales	Discinaceae	*Hydnotrya*	1
			Hymenogaster	3
		Helvellaceae	*Helvella*	5
		Morchellaceae	*Morchella*	1
		Pezizaceae	*Peziza*	3
		Psathyrellaceae	*Psathyrella*	1
		Pyronemataceae	*Genea*	4
			Geopora	10
			Geopyxis	1
			Tricharina	2
			Trichophaea	6
			Wilcoxina	3
		Tuberaceae	*Tuber*	22
Basidiomycota	Agaricales	Cortinariaceae	*Cortinarius*	7
		Hymenogastraceae	*Hebeloma*	3
		Inocybaceae	*Inocybe*	21
		Mycenaceae	*Mycena*	10
		Omphalotaceae	*Gymnopus*	4
		Tricholomataceae	*Tricholoma*	6
	Boletales	Suillaceae	*Suillus*	1
	Cantharellales	Ceratobasidiaceae	*Ceratobasidium*	13
		Clavulinaceae	*Clavulina*	5
	Polyporales	Meruliaceae	*Resinicium*	1
	Russulales	Russulaceae	*Lactarius*	4
			Russula	17
	Sebacinales	Sebacinaceae	*Sebacina*	37
	Thelephorales	Thelephoraceae	*Pseudotomentella*	6
			Thelephora	6
			Tomentella	53

Chapter 4

Range size and niche breadth as predictors of climate-induced habitat change in *Epipactis* (Orchidaceae)

Adapted from *Evans, A. and Jacquemyn, H., 2022. Range size and niche breadth as predictors of climate-induced habitat change in Epipactis (Orchidaceae). Frontiers in Ecology and Evolution, 10.*

Abstract

Background and aims While there is mounting evidence that ongoing changes in the climate system are shifting species ranges poleward and to higher altitudes, responses to climate change vary considerably between species. In general, it can be expected that species responses to climate change largely depend on how broad their ecological niches are, but evidence is still scant. In this study, we investigated the effects of predicted future climate change on the availability of suitable habitat for 14 *Epipactis* (Orchidaceae) species, and tested whether habitat specialists would experience greater changes in the extent of their habitats than habitat generalists.

Methods We used Maxent to model the ecological niche of each species in terms of climate, soil, elevation and land-use and projected it onto climate scenarios predicted for 2061-2080. To test the hypothesis that temperate terrestrial orchid species with small ranges or small niche breadths may be at greater risk under climate change than species with wide ranges or large niche breadths, we related niche breadth in both geographic and environmental space to changes in size and location of suitable habitat.

Key Results and Conclusions The habitat distributions of half of the species shifted northwards in future projections. The area of suitable habitat increased for eight species but decreased for the remaining six species. If expansion at the leading edge of the distribution was not possible, the area of suitable habitat decreased for twelve species. Species with wide niche breadth in geographic space experienced greater northwards expansions and higher habitat suitability scores than species with small niche breadth. Niche breadth in environmental space was not significantly related to change in habitat distribution. Overall, these results indicate that terrestrial orchid species with a wide distribution will be more capable of shifting their distributions under climate change than species with a limited distribution, but only if they are fully able to expand into habitats at the leading edge of their distributions.

Introduction

Climate plays an important role in the distribution of plant and animal species and in light of the global climate crisis, the effects of changing climate on plant species distributions is a prominent topic in ecology (Chen *et al.* 2011; Tayleur *et al.* 2015; Lehikoinen and Virkkala 2016). In order to survive climate change, species must either shift their range limits to environments that are able to support them or adapt to the new conditions in their current environments (Thuiller 2007; Kelly and Goulden 2008; Scheffers *et al.* 2016; Ash *et al.* 2017). Predicting how a species' suitable habitat alters due to climate change is necessary when planning its long-term conservation, but can be difficult because of the wide variety of habitat needs and tolerances among species.

Species differ in their responses to climate change based on how broad their ecological niches are (Thuiller *et al.* 2005). Previous research has already shown that species within a genus can vary considerably in habitat preferences and distributions (Brown *et al.* 1996; Grossenbacher and Whittall 2011; Anacker and Strauss 2014; Duffy and Jacquemyn 2019). Habitat generalists tend to have wider ranges of conditions where they can survive, grow and reproduce and are therefore assumed to be more adaptable to environmental change (Marvier *et al.* 2004; Thuiller *et al.* 2005). Specialist species, on the other hand, tend to have more specific environmental requirements and therefore can only occupy a narrow ecological niche. It is expected that species which have narrow temperature or precipitation tolerances are the most likely to be affected by climate change (Slatyer *et al.* 2013). However, empirical evidence is still limited (Shay *et al.* 2021) and for many species we do not know the factors that limit their distributions, whether leading edge expansions are sustainable, or how these species respond to climate change. Gaining a better understanding of the physical factors underlying the distribution of organisms is crucial to predict how species will respond to climate change (Hagsater *et al.* 1996; Tsiftsis *et al.* 2008).

Although orchids are generally considered rare and have small population sizes (Tremblay *et al.* 2005; Otero and Flanagan 2006; Shefferson *et al.* 2020), there is often large variation in range size and environmental tolerance between species, both within and among orchid genera (McCormick and Jacquemyn 2014; Evans and Jacquemyn

2020). What drives variation in orchid species range size is not well known, but is likely a combination of factors including niche breadth, species age, niche availability and range position (Sheth *et al.* 2020). Previous research has shown that orchid species vary in their dependence on specific abiotic environmental conditions, with some species being limited primarily by temperature and precipitation (McCormick *et al.* 2009; Vladan Djordjević *et al.* 2016; Evans *et al.* 2020) and others being limited more by local growth conditions related to bedrock and soil (Bowles *et al.* 2005; Tsiftsis *et al.* 2008; Bunch *et al.* 2013). Consequently, specialist orchid species are often associated with the habitat types that arise from the specific combinations of these abiotic characteristics, from coastal dunes to temperate forests, and the spatial extent of these habitats therefore can limit the range of the species they support (McCormick and Jacquemyn 2014; Djordjević and Tsiftsis 2022). Species traits related to growth and reproduction in a habitat, such as root system and pollination, can affect spatial distribution. For example, wide spatial distributions of orchids in the Czech Republic were associated with a rhizomatous root system (Štípková *et al.* 2021), and the wide variety of pollinators utilised by the terrestrial orchid *Epipactis helleborine* is likely an important contributor to its large range and ability to colonise various habitats (Rewicz *et al.* 2017).

Recently, it has become clear that weather conditions can have a strong impact on orchid population dynamics, suggesting that changing climatic conditions have the potential to affect the geographic distribution of orchids. For example, climatic changes during the last three decades have been shown to have a positive effect on the survival of the terrestrial orchid *Himantoglossum hircinum* at the northern edge of its population in the UK (van der Meer *et al.* 2016) and warmer winter weather conditions have also been shown to be beneficial to German populations of this species (Pfeifer *et al.* 2006). Williams *et al.* (2015) demonstrated that the population dynamics, vital rates and reproduction of the lady orchid (*Orchis purpurea*) at the northern edge of its distribution were affected by seasonal temperature and precipitation and, specifically, that milder winters and wetter springs were beneficial for its population growth. These results suggest that a warmer climate will generally benefit orchids at the northern edges of their distributions. A recent modelling study has indeed shown that predicted changes in climatic conditions increased habitat suitability available to three *Orchis* species by 2050

at the northern edge of their distribution (Evans *et al.* 2020). However, given that these species showed very similar distribution areas and often co-occur, such a generalisation may not be appropriate and it remains unclear how differences in range size or environmental niche breadth predict vulnerability under global change (Shay *et al.* 2021).

In this study, we tested the hypothesis that orchid species with small ranges or small niche breadths may be at greater risk under climate change than species with wide ranges or large niche breadths. We used the orchid genus *Epipactis* as a study system. *Epipactis* is a widespread genus occurring throughout the European and Asian continents with 37 species according to the ("The Euro+Med Plantbase Project"), although the results of phylogenetic research in recent years has brought into question the status of many species (Sramkó *et al.* 2019; Bateman 2020). Previous research has shown that among fourteen European *Epipactis* species, range size differed by more than three orders of magnitude between species with the smallest and largest ranges (Evans and Jacquemyn 2020). The distribution of small-range species was strongly associated with local habitat conditions and landscape structure, while that of large-range species was more associated with climatic conditions (Evans and Jacquemyn 2020). However, whether the habitat distributions of generalist species are more strongly affected by climate change than small-range, specialist species, is yet unknown. Specifically, we investigated how the habitat of the same fourteen *Epipactis* species would be affected by changes in temperature and precipitation in Europe predicted for 2061-2080, and assessed whether species with small ranges or narrow ecological niches would suffer greater changes in size and latitudinal position of habitat than species with large ranges.

Materials and methods

Study species and occurrence data

The genus *Epipactis* contains a large number of terrestrial orchids which vary greatly in distribution area and habitat type (Sramkó *et al.* 2019; Evans and Jacquemyn 2020). Some species (e.g. *E. dunensis* and *E. albensis*) have very localized distributions and are restricted to particular habitats such as coastal dunes and beech forests, whereas others (e.g. *E. helleborine*, *E. atrorubens*) are widespread and can tolerate a relatively wide range of habitat conditions. There are several ecotypes of *E. helleborine* that can be found in specific habitats such as coastal dunes and forests (Jacquemyn *et al.* 2018). Species are autogamous, allogamous, or facultative allogamous (Claessens and Kleynen 2011; Brys and Jacquemyn 2016). The numerous seeds produced by *Epipactis* species are very small, dispersed by wind, and rely on the presence of mycorrhizal fungi in the soil to germinate and establish (Bidartondo and Read 2008; Smith and Read 2010; McCormick and Jacquemyn 2014; Jacquemyn *et al.* 2018; Xing *et al.* 2020). Differences in mycorrhizal communities between localities may contribute to reproductive isolation and spatial distribution of *Epipactis* species and populations (Ogura-Tsujita and Yukawa 2008; Jacquemyn *et al.* 2016, 2018 but see Těšitelová *et al.* 2012).

Records of fourteen species' occurrences from 2000 to 2020 on the continent of Europe were obtained from the online database GBIF (www.GBIF.org; **Supplementary material S1**). We discarded records with missing GIS coordinates, ambiguous species identification or with coordinates with a spatial resolution lower than 100m, as well as those outside of the extent of the ecogeographic variables (i.e. the European Union, see below). This resulted in between 31 (*E. lusitanica*) and 45 354 (*E. helleborine*) occurrences per species. Records for each species were aggregated into 10 km^2 grid cells to reduce the effects of spatial clustering resulting from sampling bias, by extracting the centre coordinates of each grid cell in which the species was recorded (**Supplementary material S1**). Processing of occurrence data was performed in QGIS v3.4.9 (QGIS Development Team 2019).

Ecogeographic variables

Previous studies have shown that land cover, bedrock, precipitation and temperature are important variables predicting the distributions of some *Epipactis* species (Tsiftsis *et al.* 2008; Vladan Djordjević *et al.* 2016; Evans and Jacquemyn 2020). We therefore used nine raster-format predictor variables with < 0.5 correlation with one other. Two of the 19 bioclimatic variables available at the WorldClim v2 online database (Fick and Hijmans, 2017; https://www.worldclim.org/data/worldclim21.html) were used in our model, mean annual temperature and annual precipitation, projected for the near-present climate (1970-2000). These two variables were chosen because they are the most representative of the mean climate of an area, and are therefore appropriate for a continent-wide distribution study such as this. We also obtained the mean annual temperature and annual precipitation rasters predicted for the years 2061-2080 predicted by two Shared Socio-economic Pathways (SSPs), SSP 2-4.5 and SSP 5-8.5 from WorldClim (https://www.worldclim.org/data/cmip6/cmip6climate.html). SSP 2-4.5 models the climate in a scenario where greenhouse gas emissions are at their highest (~44 GT CO_2) in 2040 and then decrease to 9.6 GT in 2100, while in SSP 5-8.5, emissions increase steeply until the year 2080 (~130 GT) before starting to stabilise and decrease (Riahi *et al.* 2017). Maps of the distribution of temperature and precipitation values in Europe were created by calculating the mean temperature for each cell of a $50km^2$ cell grid of Europe and summarising the values in QGIS.

The other seven variables used were the same as those used to model *Epipactis* species in Evans and Jacquemyn (2020). These include the first two components of two PCAs run on two topsoil datasets (physical and biochemical measures; **Supplementary material S2**) acquired through the European Soil Data Centre (ESDAC) (Hiederer 2013; Ballabio *et al.* 2019), dominant bedrock from the ESDAC database (Van Liedekerke *et al.* 2006), Corine Land Cover (CLC) from the Copernicus program of the European Environmental Program (Heymann, 1994) and elevation (Amatulli et al., 2018). All raster processing was performed in RStudio v4.0.2 (R Core Team 2021).

Ecological niche modelling

Defining and quantitatively comparing plant niches can be achieved using ecological niche models (ENMs). Ecological niche modelling has been applied successfully to numerous species to investigate ecological niches and to assess the impacts of climate change and land use on species ranges (Guisan and Thuiller 2005). We used the program Maxent v3.4.1. (Phillips *et al.* 2017) to model the effects of predicted climate change on species' habitats. Maxent is a popular ENM tool that uses species occurrence data and environmental rasters to calculate a Gibbs value for each pixel of the study area, or the probability that the pixel has suitable habitat conditions for the species (Phillips 2005) and performs well in comparison to other modelling methods (Elith *et al.* 2006; Phillips and Dudík 2008; Valavi *et al.* 2021). Maxent creates habitat suitability maps over the study area from these data, as well as a table of the contribution of each predictor variable to the distribution of suitable habitat for each species. The choice of Maxent settings was informed by (Barbet-Massin *et al.* 2012) and (Merow *et al.* 2013). Each model was run using a random seed and 100 bootstrap replicates with 75% of the data used to train the model and 25% to test it. The rest of the settings were left as the default (convergence threshold of 0.00001, regularisation threshold of 1 and a maximum of 10000 background points) and allowed for linear, quadratic, product and hinge features to be chosen automatically, producing a cloglog output. The models were run for the current climatic features and projected onto the SSP climate data to produce separate environmental niche map outputs for the current and future climate conditions.

Data analysis

For each habitat suitability map, the mean Gibbs value with standard error was calculated for every latitudinal interval of 0.5 decimal degree of the study area (Europe) using the Zonal Statistics tool in QGIS. We ran a Wilcoxon signed rank test on these mean Gibbs values multiplied by their corresponding latitudes to test whether the suitable habitat of each species will shift in latitude in future climate scenarios. Before running the Wilcoxon tests, the set of Gibbs values for each climate scenario was centred by dividing by the mean to eliminate the influence of different mean Gibbs values between climate scenarios and test only for shifts in latitude.

The continuous probabilistic maps produced by Maxent were converted into binary presence maps using the Maximum Test Sensitivity plus Specificity (MTSS) value of each species as a threshold. The pixels with a Gibbs value of greater than the MTSS were extracted and plotted as a new map for each species, with each pixel representing the species being present at that location. The total numbers of pixels occupied by these habitat distribution maps were compared between current and future climate scenarios. The overlapping pixels between the current and future distributions (i.e. pixels for which occurrence equalled one for both maps) of each species were extracted and counted to provide a measurement of the area suitable for a species if it were unable to expand into any newly available areas created by future climate change.

Mean species occurrence per climate scenario and per species, for both the continuous Gibbs values and the number of pixels occupied of the binary maps, were compared between the climate scenarios using Kruskal-Wallis and Dunn tests with a Holm correction or ANOVA and Tukey tests if the data were normally-distributed.
We calculated Levins' *B2* values of niche breadth in geographic ($B2_{geo}$) and environmental space ($B2_{env}$) for each species using the functions raster.breadth and env.breadth respectively in the ENMTools 1.0.5 R package (Warren *et al.* 2021). *B2* ranges from 0 to 1, with values closer to 0 representing narrow (specialised) niche breadth and values closer to 1 representing wide (generalised) niche breadth. Finally, we investigated whether species niche breadth predicts changes in distribution in response to climate change by dividing the range change of a species from current to future scenarios, converting to the proportional change for each species, and comparing these values to each species' *B2* value using ordinary least-squares regression. Ordinary least-squares regression was also used to compare the Levins' *B2* values between geographic and environmental space. The difference in niche breadth between species with positive range changes and negative range changes in response to habitat change was investigated using Kruskal Wallis tests. The effect of mating system on response to climate change was tested by comparing the mean changes in latitudinal habitat distribution and proportional range size between autogamous, allogamous and facultative autogamous species, using Kruskal Wallis tests. All analyses were performed in RStudio v4.0.2 (R Core Team 2021).

Results

The mean temperature in continental Europe will increase from a near-current mean of 9.21 ± 0.10 °C (standard error) to 12.16 ± 0.09 °C for SSP 2-4.5 and to 13.42 ± 0.09 °C for SSP 5-8.5 (**Fig. 1**) predicted for the years 2061-2080. The mean annual precipitation will increase slightly in the future projections, with a current mean of 742.74 ± 5.86 mm^3 to 761.95 ± 5.86 mm^3 for SSP 2-4.5 and 761.43 ± 5.93 mm^3 for SSP 5-8.5.

The mean current habitat suitability or probability of occurrence (Gibbs p value) predicted by the Maxent model ranged from 0.0026 ± 0.0006 for *Epipactis lusitanica* to 0.29 ± 0.022 for *E. helleborine*. When the model was projected for the climate of 2061-2080, there was no significant difference in mean habitat suitability between the current climatic conditions and either of the two future climate scenarios ($\chi^2 = 0.18$, *p*-value = 0.91). Although the mean species' habitat suitability did not change, when species were tested individually, the habitat suitability of *E. helleborine*, *lusitanica*, *phyllanthes* and *tremolsii* significantly increased under both SSP scenarios, and *E. albensis* increased significantly for SSP 5-8.5 (**Table 1**; **Supplementary material S7**). Seven species (*E. albensis, fageticola, kleinii, leptochila, microphylla, muelleri* and *tremolsii*) demonstrated significant northwards shifts in their habitat distributions in both future climate scenarios (**Table 2**; **Supplementary material S9** for illustration of individual range shifts).

The area of suitable habitat available (pixels where the Gibbs p value was above the species' MTSS threshold) increased for eight species (*E. albensis*, *dunensis, fageticola helleborine*, *lusitanica*, *microphylla*, *phyllanthes* and *tremolsii*) in the future scenarios, but decreased for the remaining six species (*E. atrorubens*, *kleinii*, *leptochila*, *muelleri*, *palustris* and *purpurata*; **Table 3**; **Supplementary material S8**). For species that responded positively to the climatic changes, the increase in habitat ranged between 5% and 1000% (*E. dunensis* and *E. lusitanica*), while for those that responded negatively, decrease in habitat area ranged between 5% (*E. kleinii, muelleri* and *palustris*) and 88% (*E. purpurata*).

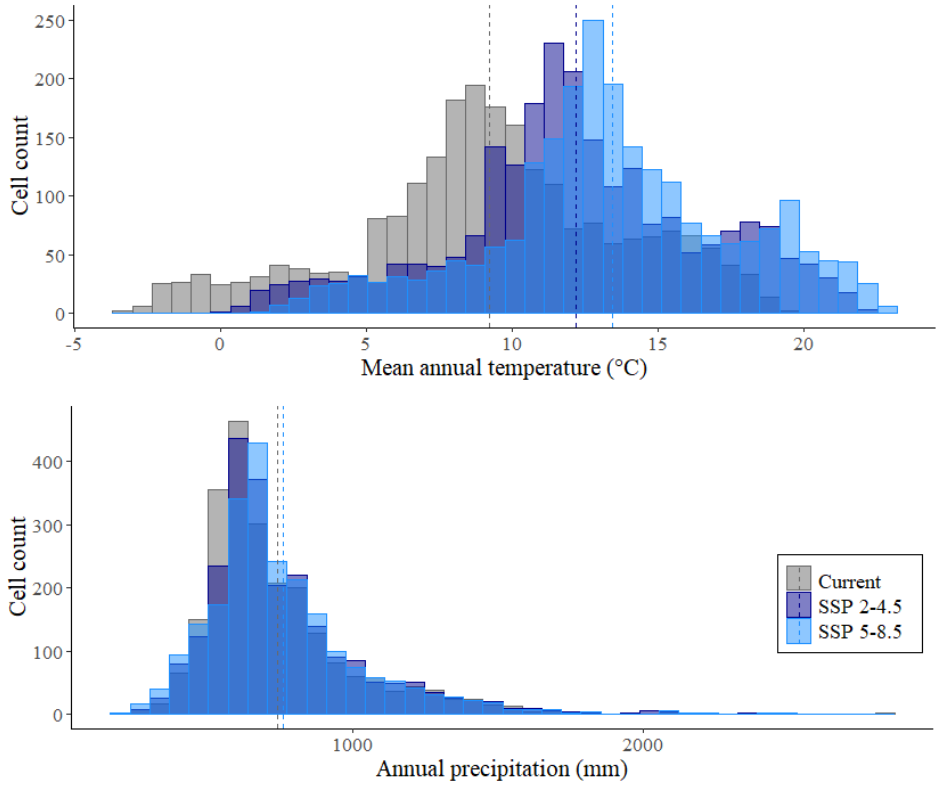


Figure 1. Distribution of mean annual temperature and annual precipitation in Europe predicted for the current climate, and projected to occur in the years 2061-2080 under two Shared Socio-economic Pathways (SSP2-4.5 & SSP5-8.5). Bars represent the number of 50km grid squares of Europe with a corresponding mean temperature or precipitation value, and dotted lines represent the mean values.

Overlap in habitat distribution areas between current and future climate scenarios was fairly high, ranging from 57-100% for SSP 2-4.5 and 33-100% for SSP 5-8.5, except for *E. purpurata* which showed notably low overlap (16 and 4% for the two scenarios respectively; **Supplementary material S8**). The change in habitat area experienced by species if they would not be able to track the climate in the future decreased by up to 95% (*E. purpurata*) for all except two species, *E. lusitanica* and *E. phyllanthes*, which showed no decrease in distribution area (100% overlap, only expansion).

Table 1. Change in mean Gibbs values (habitat suitability) for *Epipactis* species from current to future (2061-2080) climate scenarios (SSP 2-4.5 & SSP 5-8.5) and results of Dunn tests comparing current and future mean Gibbs p values (showing only results for significant differences, in bold, and marginally significant differences, in italics).

Species	Change mean Gibbs p		Current – SSP 2-4.5		Current – SSP 5-8.5	
	SSP 2-4.5	SSP 5-8.5	*Z*	*p*	*Z*	*p*
E. albensis	0.0025	0.0037	-2.1573	*0.0620*	-3.1508	**0.0049**
E. atrorubens	-0.0196	-0.0378				
E. dunensis	0.0007	0.0059	-0.3820	0.7024	-2.3288	*0.0596*
E. fageticola	0.0003	0.0002				
E. helleborine	0.0645	0.0791	-2.2945	**0.0435**	-2.5807	**0.0296**
E. kleinii	-0.0002	-0.0008				
E. leptochila	-0.0038	-0.0060				
E. lusitanica	0.0064	0.0102	-3.4435	**0.0011**	-4.9054	**<0.0001**
E. microphylla	0.0024	0.0008				
E. muelleri	-0.0014	-0.0065				
E. palustris	0.0193	0.0136				
E. phyllanthes	0.0056	0.0084	-2.7392	**0.0123**	-3.8179	**0.0004**
E. purpurata	-0.0113	-0.0182				
E. tremolsii	0.0044	0.0065	-2.5263	**0.0231**	-3.7593	**0.0005**

The niche breadth values of Levins' $B2_{geo}$ (in geographic space) ranged from 0.39 for *E. fageticola* to 0.85 for *E. palustris*, while $B2_{env}$ ranged from 0.16 for *E. dunensis* to 0.90 for *E. lusitanica* (**Supplementary material S7**). *B2* values in geographic and environmental spaces were not correlated with each another (*p*-value = 0.74). The means Gibbs value of habitat suitability was positively correlated with $B2_{geo}$ for both current (R^2 = 0.33, $F_{1,12}$ = 7.52, *p*-value = 0.012) and future (SSP 2-4.5: R^2 = 0.50, $F_{1,12}$ = 13.81, *p*-value = 0.0029; SSP 5-8.5: R^2 = 0.53, $F_{1,12}$ = 15.81, *p*-value = 0.0020) climate projections (**Fig. 2(a)**). Similarly, $B2_{geo}$ had a positive relationship with range size for current (R^2 = 0.32, $F_{1,12}$ = 7.11, *p*-value = 0.021) and future (SSP 2-4.5: R^2 = 0.53, $F_{1,12}$ = 15.41, *p*-value = 0.0020; SSP 5-8.5: R^2 = 0.52, $F_{1,12}$ = 13.19, *p*-value = 0.0034) climate projections (**Fig. 2(b)**). Species with higher $B2_{geo}$ values also experienced greater changes in Gibbs values between current and future climate scenarios (**Fig. 2(c)**; SSP 2-4.5: R^2 = 0.68, $F_{1,12}$ = 17.93, *p*-value = 0.0039; SSP 5-8.5: R^2 = 0.43, $F_{1,12}$ = 7.09, *p*-value = 0.032). There was a positive relationship between $B2_{geo}$ and the change in mean Gibbs value between current

climate and SSP 2-4.5 multiplied by latitude (**Fig. 2(d)**; R^2 = 0.24, $F_{1,12}$ = 4.99, *p*-value = 0.045), indicating that species with higher $B2_{geo}$ values would experience a greater northwards shift in suitable habitat than those with low $B2_{geo}$ values, if they were able to track the suitable climate. This was also marginally significant for SSP 5-8.5 (R^2 = 0.18, $F_{1,12}$ = 3.77, *p*-value = 0.076). No comparisons involving $B2_{env}$ were significant at α = 0.05, but marginally significant positive relationships were detected between $B2_{env}$ and proportional range change (proportional to the species' current range) from current to future climate scenarios (SSP 2-4.5: R^2 = 0.31, $F_{1,12}$ = 4.20, *p*-value = 0.086; SSP 5-8.5: R^2 = 0.17, $F_{1,12}$ = 3.64, *p*-value = 0.081). Species with higher $B2_{env}$ also showed some evidence for experiencing a greater northwards shift in suitable habitat from current to SSP 5-8.5 climate (R^2 = 0.16, $F_{1,12}$ = 3.77, *p*-value = 0.076). The mean proportional range change (with and without tracking) and change in latitudinal habitat distribution in response to climate change in either SSP scenario were not significantly different between mating systems (*p*-value > 0.05).

Table 2. Latitudinal shifts in habitat distribution from current to future climate scenarios and results of Wilcoxon tests of differences in habitat distributions (showing only results for significant differences)

Species	Change in mean Gibbs p*latitude		Current – SSP 2-4.5		Current – SSP 5-8.5	
	SSP 2-4.5	SSP 5-8.5	*W*	*p*	*W*	*p*
E. albensis	86792.3	179258.2	1016	**0.0091**	918	**0.0019**
E. atrorubens	370209.9	558240.5				
E. dunensis	7321.7	3531.9				
E. fageticola	45463.3	112859.5	986	**0.0058**	985	**0.0057**
E. helleborine	319826.6	459016.5				
E. kleinii	49895.4	165549.1	998	**0.0069**	904	**0.0015**
E. leptochila	155493.8	204799.6	1121	**0.0369**	1087	**0.0241**
E. lusitanica	105798.9	199849.8				
E. microphylla	243820.9	385056.3	1076	**0.0208**	1110	**0.0322**
E. muelleri	246742.1	397285.9	1063	**0.0175**	1081	**0.0222**
E. palustris	332171.1	506155.0				
E. phyllanthes	91731.5	164969.6				
E. purpurata	433625.6	748751.2				
E. tremolsii	253596.0	412612.4	802	**0.0002**	829	**0.0004**

Table 3. Changes in area of suitable habitat above the Maximum Test Sensitivity and Specificity threshold of each species from the current climate conditions to future climate scenarios (SSP 2-4.5 & SSP 5-8.5), as well as changes if species are unable to expand their ranges into the climatic envelope of future scenarios (climate tracking). Range changes are reported as proportional to the current range.

Species	Proportional range change		Proportional range change without tracking	
	SSP 2-4.5	SSP 5-8.5	SSP 2-4.5	SSP 5-8.5
E. albensis	1.9094	3.4189	-0.0025	-0.0214
E. atrorubens	-0.3250	-0.5481	-0.4259	-0.6711
E. dunensis	0.0554	0.5265	-0.0019	-0.0136
E. fageticola	0.2203	0.5554	-0.0059	-0.2330
E. helleborine	0.2001	0.1391	-0.1960	-0.2970
E. kleinii	-0.0476	-0.3597	-0.1228	-0.4138
E. leptochila	-0.3257	-0.4843	-0.3862	-0.5520
E. lusitanica	5.9117	10.8262	<0.0001	<0.0001
E. microphylla	0.3052	0.2582	-0.2469	-0.4614
E. muelleri	-0.0467	-0.2476	-0.3005	-0.5037
E. palustris	-0.0479	-0.1842	-0.2636	-0.4495
E. phyllanthes	1.5237	2.3245	<0.0001	<0.0001
E. purpurata	-0.6798	-0.8866	-0.8357	-0.9555
E. tremolsii	0.6798	1.0058	-0.1054	-0.1888

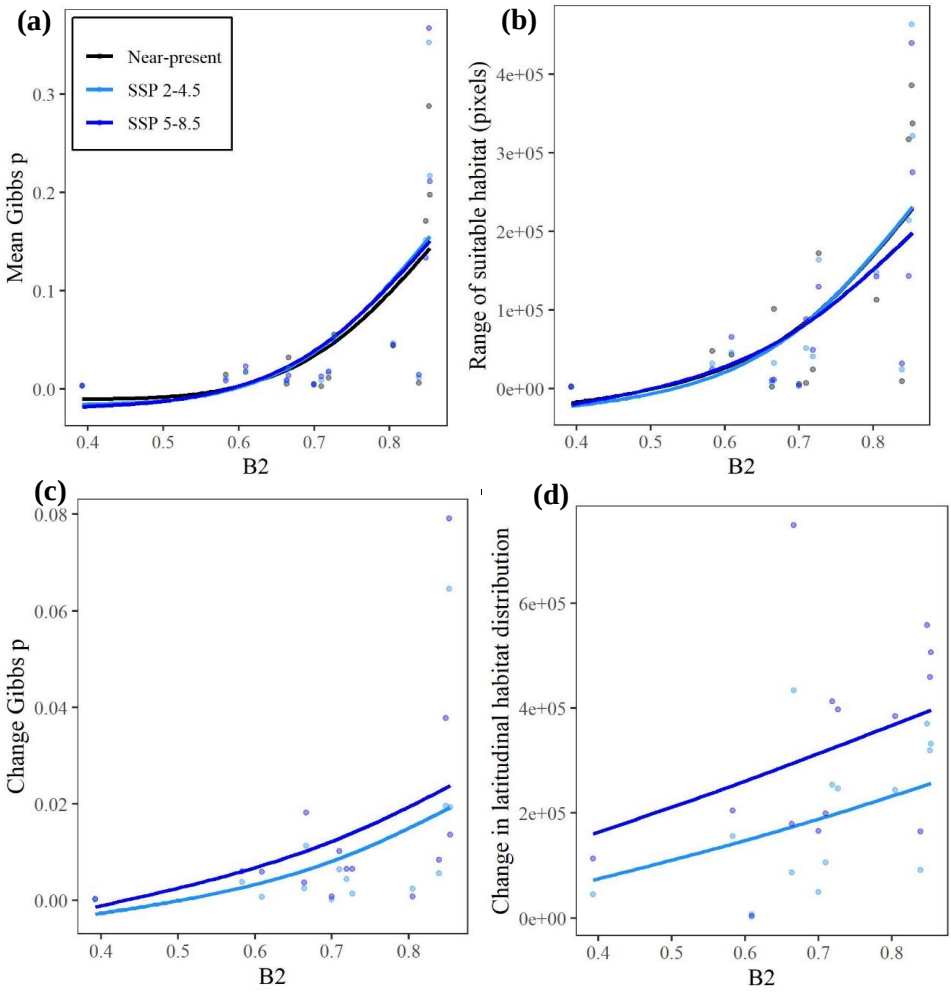


Figure 2. Relationships between Levins' *B2* measure for niche breadth in geographic space and (a) the mean Gibbs p value (habitat suitability), (b) the spatial range of suitable habitat, (c) change in mean Gibbs p from current to future climate scenarios, and (d) change in latitudinal habitat distribution of 14 *Epipactis* species. Two climate scenarios were used, SSP 2-4.5 and SSP 5-8.5 to predict the climate for 2061-2080.

Discussion

In this study we investigated how the distribution of suitable habitat of *Epipactis* species would be affected by predicted climate change and whether species with small ranges or narrow niche breadths are at greater risk from climate change than species with wide ranges or large niche breadths. Our results showed that the habitat available increased on the leading (northern) edge of the distribution for half of the species but decreased for the remaining species, and decreased for all but two species if climate tracking was not possible. Levins' *B2* metric for niche breadth in geographic space was highly correlated with the spatial extent and mean Gibbs value (habitat suitability) of species habitat distributions and species with a higher *B2* value were predicted to experience a greater northwards expansion in response to climate change. We did not detect significant effects of Levins' *B2* in environmental space, although there was marginally significant patterns similar to those of *B2* in geographic space.

Impact of climate change on the distribution of Epipactis in Europe

Although there was no change in the mean Gibbs value of the 14 species between current and future climate scenarios, the Gibbs values for the majority of the species individually was predicted to increase in 2061-2080. The area of suitable habitat increased into the north for some species and decreased in the south for most species in the future, resulting in a mean northern shift in habitat. When expansion into the north (climate tracking) was restricted, the area of habitat decreased by up to 95% for all except two small-range species. Despite the expectation that species with narrow environmental tolerances are most threatened by climate change, in the case of *Epipactis*, the habitats of most of the small-range localised species that we investigated were predicted to increase with future climate change. Some northern hemisphere herbaceous species benefit from increased temperatures at the northern edge of their distribution through increased population growth, which in turn can lead to an increase in geographic range at this edge (Bremer and Jongejans 2009). This includes orchids such as *Himantoglossum hircinum* where climatic changes in the UK were shown to be partially responsible for the species' expansion between 1991 and 2001, as well as for projected future scenarios (van der Meer *et al.* 2016). Similarly, Ongaro *et al.* (2018) predicted that the habitat range for nine

orchid species will increase by 2070 on the island of Sardinia, although the probability of presence in the newly colonised habitats was not predicted to increase. However, Vogt-Schilb *et al.* (2015) found that the distributions of many orchids in western Europe have declined in the last two decades due to land-use change, particularly in the northern parts of their distributions. If land-use continues to change in more northern latitudes, this could limit the areas into which *Epipactis* can move in response to climate change. The results of our study provide further support for the potential for orchid ranges to increase at their leading edges in response to climate change, but go further to demonstrate that this does not necessarily mean an increase in available habitat, particularly if they cannot move into the northern habitats in time.

Testing the range size vulnerability hypothesis

A widely-supported paradigm is that the maximum range limits of a species coincide with its ecological niche limits and that, given the opportunity to disperse, range limits will shift to match the geographical extent of the niche under climate change (Reed *et al.* 2021; Shay *et al.* 2021). The pattern of species with wider niche breadths demonstrating greater latitudinal shifts in response to climate change has been documented in a number of terrestrial plant taxa (Thuiller *et al.* 2005; Alarcón and Cavieres 2018). This was also demonstrated in orchids by Geppert *et al.* (2020) where the distributions of generalist orchid species and those inhabiting forests and semi-natural grasslands tended to be less affected than the more specialised and rare species in subalpine, natural grassland and wetland habitats, whose rear and leading edges shifted upwards. This corresponds with our finding that *Epipactis* species with wider niche breadths (generalists) experience greater change in habitat area in response to changing climate than specialists. If we were to assign species to the groups of specialist and generalist based on current spatial ranges and values of *B2* in geographic space, *E. fageticola, albensis, kleinii* and *lusitanica* would be considered the most specialist (relative to the other species in this study), followed by *E. tremolsii, dunensis* and *leptochilla* as moderately specialist. *E. muelleri, microphylla* and *purpurata* are moderately generalist, while *E. helleborine, palustris* and *atrorubens* could be considered generalists. However, *E. purpurata* had a low *B2* value but a fairly large spatial distribution and *E. phyllanthes* a high *B2* value and small range, which is in

contrast to this pattern. *E. purpurata* was predicted to experience a significant decrease in suitable habitat under climate change which may indicate that species with relatively large current ranges may still have fairly narrow niches which are nonetheless currently common in the environment, but are under threat from changing climate.

The distributions of all investigated species, even generalists, tended to lag behind climate warming, without being able to fully track the upwards shift in suitable climate resulting in a range contraction, in both our study and Geppert *et al.* (2020). Plant species inhabiting forests may be somewhat buffered from the effects of climate warming (De Frenne *et al.* 2013; Zellweger *et al.* 2020) and those in grasslands tend to have high thermal ranges because of the lack of this buffering (Geppert *et al.* 2020). Similarly, Vogt-Schilb *et al.* (2015) found higher rates of disappearances in wetland orchid species in Western Europe than those in grassland, and more appearances in forest. There did not seem to be any clear pattern in response to climate change and habitat-use in our species (other than with niche breadth), with woodland species such as *E. muelleri* decreasing in suitable habitat and *E. microphylla* increasing. However our study used a broad-scale specification of land cover, while more may be revealed at a finer resolution that captures microclimate gradients.

An important caveat to consider when carrying out niche breadth studies is that the metric used to describe niche breadth can greatly affect the results. Levins' *B2* is the reciprocal of Simpson's diversity index (Levins 1968) and has been a popular metric of niche breadth for more than fifty years. However, it has been noted that the traditional calculation of this metric is in geographic space (see Peterson and Soberón (2012) for more on geographic and environmental space) and more accurately represents the 'flatness' of the geographic distribution of suitable habitat (Warren *et al.* 2019), which may be a useful measure of spatial habitat-use but is not niche breadth in terms of specificity of resource-use. This is demonstrated clearly in our results, where *B2* in geographic space was consistently correlated with measures of the size of the habitat distribution and the mean Gibbs value. *B2* in environmental space as proposed by Warren *et al.* (2019) and developed in Warren *et al.* (2021) filters the geographic habitat suitability distribution through the set of environmental variables that was used to create

the Maxent model, resulting in a *B2* value that is closer to the concept of niche breadth as being the specificity in environmental conditions of a species' habitat. It is important to note, however, that although closer to what we understand to be niche breadth, *B2* in environmental space is still dependent on the availability of habitats in geographic space (Petraitis 1979; Warren *et al.* 2019). Although the values of *B2* in geographic and environmental space were not correlated, *B2* in environmental space showed some evidence for having the same relationship with habitat changes as *B2* in geographic space. This indicates that *B2* in environmental space has the potential to be a useful representation of niche breadth for *Epipactis* in Europe, but further study is required to conclude this.

Other factors contributing to range shifts

Although the abiotic characteristics discussed here are important for predicting orchid ranges, biotic interactions and species-specific characteristics are also essential contributors to the realised niche, and including these interactions can improve the accuracy and performance of niche models (Flores-Tolentino *et al.* 2020; Phillips *et al.* 2020). Orchids rely on insect pollinators and mycorrhizal fungi to reproduce and germinate (Rasmussen 2008; McCormick and Jacquemyn 2014). As with other pollinator-reliant plants, allogamous *Epipactis* will only persist and track shifting climate if their pollinators are also able to disperse (Benning and Moeller 2019; Shay *et al.* 2021), such as has been predicted for a Neotropical orchid bee which is predicted to persist and increase its habitat range under future climate change (Silva *et al.* 2015). Mating system was not significantly associated with changes in habitat distribution in response to climate change, indicating that in the specific case of these species, autogamous and allogamous species did differ in response to predicted climate change. This is not surprising, considering that mating system was not significantly associated with niche breadth or range size for *Epipactis* species in previous studies (Evans and Jacquemyn 2020) and niche breadth in geographic space is directly linked to range size. The presence of soil microbes has also been linked to the ability of plants to expand into newly available habitats (David *et al.* 2019; Bueno de Mesquita *et al.* 2020; Benning and Moeller 2021; Shay *et al.* 2021). The diversity of mycorrhizal fungi is linked to latitudinal gradients for some orchid species (Duffy *et al.* 2019), but it is unclear whether the northern shifts in

orchid distributions will be supported by the lower diversity of fungi in more northern latitudes. Our understanding and predictions of orchid distribution changes in response to climate change would be greatly improved with the addition of pollinator and fungal symbiont distributional data.

Implications for conservation

Studies that model the ecological niches of species are useful for conservation planning, particularly for identifying newly accessible areas available to plants (more so than predicting range contractions) and assessing the risk faced by populations as a consequence of their range size (Schwartz 2012; Shay *et al.* 2021). This study provides a useful estimate of new areas into which *Epipactis* can expand, which in conjunction with more information on predicted land change in these areas, could be used in conservation schemes to allow the genus to flourish under climate change. However, it is important to assess the results in light of individual patterns in addition to drawing general conclusions. This is demonstrated in the contrast between mean change in Gibbs value (no change) and the change in Gibbs value for individual species, where a number of species were predicted to increase in the future and, the increase in habitat area for some species and the decrease for other. This disparity between general vs individual species patterns has also been demonstrated in Geppert *et al.* (2020), who showed high interspecific variation among orchids grouped by habitat preference. We also show how some species with large areas of habitat such as *E. purpurata* should not be considered immune to the detrimental effects of future climate change as they may suffer considerable range reductions if they are not able to sufficiently disperse northwards.

Chapter 5

Historical biogeography and local adaptation explain population genetic structure in a widespread terrestrial orchid

Adapted from *Evans, A., de Kort, H., Brys, R., Duffy, K.J., Jersáková, J., Kull, T., Selosse, M.A., Tsiftsis, S., Minasiewicz, J. and Jacquemyn, H., 2023. Historical biogeography and local adaptation explain population genetic structure in a widespread terrestrial orchid. Annals of Botany, p.mcad010.*

Abstract

Background and Aims Historical changes in environmental conditions and colonization-extinction dynamics have a direct impact on the genetic structure of plant populations. However, understanding how past environmental conditions influenced the evolution of species with high gene flow is challenging when signals for genetic isolation and adaptation are swamped by gene flow. We investigated the spatial distribution and genetic structure of the widespread terrestrial orchid *Epipactis helleborine* to identify glacial refugia, characterise postglacial population dynamics and assess its adaptive potential.

Methods Ecological niche modelling was used to locate possible glacial refugia and postglacial recolonization opportunities of *E. helleborine*. A large single-nucleotide polymorphism (SNP) dataset obtained through genotyping-by-sequencing was used to define population genetic diversity and structure and to identify sources of postglacial gene flow. Outlier analyses were used to elucidate how adaptation to local environment contributed to population divergence.

Key Results and Conclusions The distribution of climatically suitable areas was restricted during the Last Glacial Maximum to the Mediterranean, south-western Europe and small areas in the Alps and Carpathians. Within-population genetic diversity was high in *E. helleborine* (mean expected heterozygosity: 0.373 ± 0.006, observed heterozygosity: 0.571 ± 0.012, allelic richness: 1.387 ± 0.007). Italy and central Europe likely acted as important genetic sources during postglacial recolonization. Adaptive SNPs were associated with temperature, elevation and precipitation. Forests in the Mediterranean and Carpathians likely acted as glacial refugia for *Epipactis helleborine*. Postglacial migration northwards and to higher elevations resulted in the dispersal and diversification of *E. helleborine* in central Europe and Italy, as well as geographic isolation and divergent adaptation in Greek and Italian populations. Distinguishing adaptive from neutral genetic diversity allowed us to conclude that *E. helleborine* has a high adaptive potential to climate change and demonstrates that signals of adaptation and historic isolation can be identified even in species with high gene flow.

Key words: environment association analysis, environmental niche modelling, *Epipactis helleborine*, genotype-environment associations, genotyping by sequencing, landscape genomics, local adaptation, natural selection

Introduction

Historical and ecological factors can have substantial effects on the distribution and evolution of plant and animal species and are often reflected in the large-scale spatial genetic structure of species (Comes and Kadereit 1998; Hewitt 2000; Lafontaine *et al.* 2018). Populations that survived in glacial refugia can still show signatures of allele frequencies and diversities reflecting long term persistence and periods of genetic isolation (Tribsch 2004; Keppel *et al.* 2012; Reisch and Rosbakh 2021). However, characterising postglacial dispersal patterns for wind-dispersed, herbaceous species such as orchids is complicated because their high dispersal potential but specialised habitat requirements (McCormick and Jacquemyn 2014) result in contrasting gene flow mechanisms (Tremblay *et al.* 2005; Givnish *et al.* 2016). This has resulted in gaps in our understanding of the effects of climatic changes on the genetic structure, spatial distribution, and evolution of ecologically specialised plants (Peakall 2007; Givnish *et al.* 2016).

Glacial periods resulted in the fragmentation of continuous distributions of plant species when large areas became inhospitable, with isolated populations surviving only in the more stable habitats of glacial refugia (Ashcroft 2010; Keppel *et al.* 2012). In Europe, glacial macrorefugia for plant species have been identified mainly around the Mediterranean basin, an area that was relatively sheltered from the permafrost covering more northern areas during this time (Bhagwat and Willis 2008), but also in smaller isolated areas further north, with the microhabitats of these areas acting as buffered shelters (Stewart and Lister 2001), such as in the Alps (Tribsch 2004; Schönswetter *et al.* 2005; Parisod 2022) and the Carpathians (Kolář *et al.* 2016). Populations that expanded from glacial refugia into the newly suitable conditions of post-glacial Europe can carry genetic signatures of their isolated source populations, but the evidence for patterns of refugial and non-refugial populations is inconsistent within the literature. In a study of 179 European plant species, populations of alpine species tended to be more genetically distinct but had lower genetic diversity than non-alpine species, presumably in part due to alpine populations being genetically isolated during glacial periods (Reisch and Rosbakh 2021). In other cases, refugial populations tend to have higher genetic diversity than

offshoot populations due to founder effects (Hewitt 2004) and more recently established populations may show stronger spatial genetic structure than older populations (Ilves *et al.* 2015). This disparity can occur when gene flow between populations is re-established during postglacial expansion, and genetic structure that has arisen in isolated refugial populations is homogenized (Slatkin 1987; Hutchison and Templeton 1999; Ellstrand 2014; García-Navas *et al.* 2015; Fitzpatrick and Reid 2019).

The erasure of refugial genetic structure by postglacial gene flow is more likely to occur in species with high gene dispersal capabilities (Loveless and Hamrick 1984; Givnish 2010). This has been demonstrated in orchids that, through their easily-dispersed dust seeds, have a high potential for gene flow and thus population admixture (Phillips *et al.* 2012). However, several studies have found substantial adaptive differentiation in plant species with high dispersal capabilities (De Kort *et al.* 2014; Muir *et al.* 2014; Hämälä and Savolainen 2019). These studies demonstrated that landscape features promoting gene flow do not necessarily hamper adaptive evolution and suggest that plants even with high dispersal capabilities may exhibit genetic signals for adaptation to local environments. In orchids, this may be a result of most seeds landing near the mother plant which would reduce gene flow despite the theoretically high potential for gene dispersal (Jersáková and Malinová 2007), as well as specific ecological requirements that dictate where orchids can successfully colonise and persist. Terrestrial orchid distributions in Europe are linked to substrate, elevation and climate (Djordjević and Tsiftsis 2022) and often the presence of pollinators and mycorrhizal fungi on which they rely to germinate (McCormick *et al.* 2018). Differences in environmental pressures and consequent divergent selection of populations can further affect the genetic structure of plant species (Stinchcombe and Hoekstra 2008; Nosil *et al.* 2009; Orsini *et al.* 2013). The habitat requirements of orchids create a potential for strong signals for adaptation, but these signals can be swamped by high gene flow resulting from a high potential for seed dispersal which complicates inferring the evolutionary history of orchids.

To shed light on the historical and ecological processes that have led to contemporary patterns of distribution and genetic structure of orchids, we investigated the historical distribution and genetic structure of the widespread terrestrial orchid *Epipactis helleborine*. Ecological niche modelling was used to identify possible locations of glacial

refugia and postglacial recolonization opportunities. Genetic structure and diversity were calculated from a large single-nucleotide polymorphism (SNP) dataset to distinguish reproductively isolated from admixing populations, and to identify outlier loci associated with environmental characteristics to assess whether local adaptation contributed to population divergence. Finally, we explored the relationship between genetic diversity and the change in habitat suitability from the Last Glacial Maximum (LGM) to the near-present and predicted locations of diversification sources to assess how post-glacial environmental change affected genetic diversity. We test the hypothesis that populations located in glacial refugia exhibit some signals of historic isolation such as lower genetic diversity and presence of adaptive loci, but that postglacial colonisation and increased gene flow resulted in reduced population differentiation.

Materials and methods

Study species

Epipactis helleborine is a widespread terrestrial orchid that occurs across Europe and Asia and has been naturalised in America. Although *E. helleborine* is a habitat-generalist in comparison to other orchids, its occurrence is still limited to some extent by bedrock, deciduous tree cover, soil and climate (Hemrová *et al.* 2019; Evans and Jacquemyn 2020). Reproduction in *E. helleborine* can be either sexual (primarily allogamous, with pollination occurring via insects) or asexual (vegetative via rhizomes) (Delforge 2006). The seeds produced are very small, wind-dispersed 'dust seeds' that depend on the presence of mycorrhizal fungi in the soil for successful germination and establishment (Bidartondo and Read 2008; Jacquemyn *et al.* 2018). The high seed-dispersal capability of *E. helleborine*, combined with its ability to colonise a relatively broad array of habitats, is thought to have given rise to a large number of subspecies or variants through temporal, ecological or geographical isolation (Delforge, 2006), but the validity of their taxonomic status warrants more detailed research (Sramkó *et al.* 2019; Bateman 2020).

Ecological niche model

To define the spatial distribution of suitable habitat of *E. helleborine*, we used Maxent v.3.4 (Phillips *et al.* 2018) to create ecological niche models (ENMs) for *E. helleborine* distributed in Europe. Maxent is limited like other presence-only modelling methods and the use of pseudo-presences can lead to incorrect interpretation of the output (Elith *et al.* 2011). However, when the appropriate parameter settings are used, Maxent performs well (Merow *et al.* 2013). Occurrence data for *E. helleborine* in Europe from 2000-2019 was obtained from the online database GBIF (www.GBIF.org; https://doi.org/10.15468/dl.aicdym, 1 Jul 2019). Records with a resolution coarser than 100 m were discarded, as were those with uncertain species identification. Using QGIS v.3.4.9 (QGIS Development Team 2019), the data were aggregated to reduce the effects of spatial clustering by creating a grid of 10 km^2 pixels and extracting the midpoint of each pixel containing at least one species record. This resulted in 6221 midpoints, which were used as the occurrence data in the model.

For the environmental variables, we used elevation from the EarthEnv database (Amatulli *et al.* 2018), bedrock (dominant parent material) from the European Soil Database (ESDAC; Van Liedekerke et al., 2006), and mean annual temperature and annual precipitation from WorldClim v.2.1 (Fick and Hijmans 2017). Mean annual temperature and annual precipitation projected by the MIROC-ESM climate models were also obtained for the Last Interglacial period (LI), Last Glacial Maximum (LGM) and Mid-Holocene (MH), from WorldClim v.1.4 (Hijmans et al., 2005; Otto-Bliesner et al., 2006). We also investigated the CC projection but as mean precipitation or temperature for our study area did not differ significantly from the MIROC model, we continued with only the MIROC data. All environmental variables were downloaded as rasters with a spatial resolution of 1 km, except for elevation (100 m) which was resampled to 1 km. Rasters were tested for correlation using Pearson correlation coefficients and were excluded if highly correlated (correlation > 0.5). All raster processing (resampling, reprojection and cropping) was performed in R v.4 (R Core Team 2021) using the 'raster' v.3.3 (Hijmans et al., 2015) and 'RStoolbox' v.0.2.6 (Leutner *et al.* 2017) packages.

We used the occurrence points and environmental data to run Maxent with 100 replicates, with 75% of the occurrences used for model training and 25% for model testing, a random seed, and the other settings at default (including a cloglog output). AUC (area under curve) and TSS (test skill statistic) values were used to assess model performance. The model was projected onto the three past climate scenarios to generate maps of habitat suitability for *E. helleborine* during the LI, LGM and MH, which were used to infer the locations of habitat that could have acted as refugia for the species during unfavourable climatic conditions.

To quantify the change in habitat of *E. helleborine* resulting from climatic changes, the mean Gibbs value (habitat suitability) of each Maxent map was calculated using QGIS v.3.4.9 (QGIS Development Team 2019) and compared between climate scenarios with an ANOVA. Gibbs values were also extracted from a 10km buffer around the centre point of each population that was sampled for genetic data, from which the mean habitat suitability score per population was calculated and used in regressions with genetic diversity (see below). To visualise change in suitable habitat between climate scenarios,

pixels above the Equal training sensitivity and specificity Cloglog threshold value of the model (0.53) were extracted and plotted to represent 'highly suitable' habitat.

Sampling and genotyping by sequencing

We sampled 20 individuals from each of 23 *E. helleborine* populations in 11 European countries (see **Fig. 1** for population locations) in June-August 2019. For the three populations in Italy, only five individuals per population were sampled. One leaf from each plant was collected and stored in silica gel before DNA extraction (**Supplementary material S10**). A total of 288 leaves were genotyped using the LGC Genomics genotyping by sequencing (GBS) protocol (LGC Genomics, Teddington, UK; **Supplementary material S10**). GBS is a next generation genetic screening method for Single Nucleotide Polymorphism (SNP) discovery, which uses restriction enzymes to enable genotyping of a large number of samples to produce comparative results without the need of a reference genome (Wickland *et al.* 2017). After removing individual samples and SNPs that did not meet missing data requirements (**Supplementary material S10**), the remaining dataset consisted of 893 SNP loci and 206 individuals from 23 populations (9-11 individuals per population, except for Italian populations which each retained the original five individuals). All genomic data can be accessed at the NCBI Sequence Read Archive with the accession number SAMN19104601 or through http://www.ncbi.nlm.nih.gov/bioproject/728837.

Genetic diversity and population genetic structure

To investigate gene flow among populations, we calculated genetic diversity (allelic richness *AR* and observed and expected heterozygosity H_o and H_e) on a rarefied sample of SNPs using the R package 'hierfstat' v.0.5-9 (Goudet 2005) to account for differences in sample sizes among populations. Ordinary least-squares regressions were performed to test the relationship between mean genetic diversity and habitat suitability (Gibbs value of the near-present climate) per population. To test whether post-glacial environmental change may have affected genetic diversity, we also performed regressions to explore the relationships between genetic diversity and the change in habitat suitability for each population from the LGM to the near-present (the mean near-present Gibbs value minus the mean LGM Gibbs value).

GenAlEx v.6.503 (Peakall and Smouse 2012) was used to perform an AMOVA to analyse partitioning of genetic variation within and between populations and to calculate pairwise F_{st} values (genetic distance) between populations. A Mantel test was performed to test whether F_{st} values were correlated with log-transformed geographic distances between populations, using the 'vegan' package v.2.5 (Oksanen *et al.* 2019) in R v.4 (R Core Team 2021) with 9999 iterations.

To further assess gene flow and reproductive isolation of populations, genetic structure was characterised with STRUCTURE v.2.3.4 (Pritchard *et al.* 2000) using 100 000 iterations, with 50 000 as burn-in, and 25 replicates for values of K = 1-24. We used an admixture model with population location included as a prior, which allows for the possibility that individuals have recent ancestors from more than one population, under the assumption of no isolation by distance. The multiple runs were consolidated using CLUMPP v.1.1.2 (Jakobsson and Rosenberg 2007). We then implemented the Evanno method (Evanno *et al.* 2005) to determine the most likely number of genetic clusters that exist in the dataset using the R package 'pophelper' v.2.3.0 (Francis 2017). The membership coefficient of each sampled population to each genetic cluster was visualised using 'pophelper' and code from François (2016).

Genetic outlier detection and analyses

To distinguish signals for adaptive potential (outliers) from diversification through genetic drift (neutral SNPs), we used two outlier detection methods and retained SNPs that were detected by both methods. The first method involved Bayescan v.2.1 (Foll and Gaggiotti 2008), which uses a Bayesian approach to detect outliers based on genetic signatures of diversifying selection. We ran the SNP allele frequency data for 20 pilot runs of 5 000 iterations, followed by 100 000 iterations with 50 000 being used as burn-in and a thinning interval of 10. The F_{st} results obtained were plotted in R and outliers were identified using a false discovery rate of 0.01. For the second outlier detection method we used the R package 'pcadapt' v.4.3.3 (Luu *et al.* 2017). This method used a Principal Component Analysis to summarise the variation in SNP data, with the number of clusters detected by STRUCTURE used as the number of components, after which outlier SNPs were identified based on their estimated deviation from the mean population structure. A q-value threshold of 0.01 was again used for the proportion of false positives, below

which outlier SNPs were identified. Neutral SNPs were defined as those above the *q*-value. The mean genetic diversity (H_e, H_o and *AR*) was calculated for neutral and outlier datasets separately and compared using Student t-tests or Kruskal-Wallis tests (the choice of test being informed by a Shapiro-Wilk test of normality). To test for signals for adaptation to post-glacial environmental change, we performed ordinary least-squares regressions between change in habitat suitability from the LGM to the near-present and genetic diversity of outlier and neutral datasets separately.

To detect SNPs associated specifically with the environmental variables used in the ecological niche analyses, we performed a redundancy analysis (RDA) using the 'vegan' package in R (Oksanen *et al.* 2019). For each continuous environmental variable raster (temperature, precipitation and elevation), values were extracted for each pixel in a 10 km radius buffer around each population sampled. The mean value of each environmental variable for each population was calculated and multicollinearity was checked using variance inflation factor values and variables with values below 10 were retained. Allele frequencies per population were calculated by dividing counts of the major allele per population by the total major allele count. The redundancy analysis was fit using the environmental variables as predictors and the allele frequencies of each population as dependent variables, following the method of Forester (2019). SNPs under selection were identified from the loadings of the significant RDA (constrained) axes as those with values ± 1 standard deviation ($p = 0.05$) from the mean loading.

Geogenetic analyses

To identify sources of diversification and to visualise the postglacial movement of genetic material between populations, we used the package 'spacemix' v.0.13 (Bradburd *et al.* 2016). This software uses allele frequency covariance and sample coordinates to model gene flow among populations. The resulting 'geogenetic' maps aid in visualising genetic distances between populations in a spatial context and inferring long-distance dispersal events and sources of admixture. We used count data in a 'source-and-target' model (with admixture) which was run for 10 million MCMC generations with a sample frequency (thinning rate) of 100 after a burn-in of 10 repetitions of 100 000 generations. Chain mixing and model adequacy were visually assessed using trace plots and plots of sample covariance vs parametric covariance as described in the package vignette.

Results

Ecological niche models

The mean area under the curve (AUC) value ± SE for the Maxent runs was 0.80 ± 0.021 and the true skill statistic (TSS) was 0.51, indicating decent model fit for a widespread species with a large background area. Gibbs values (relative habitat suitability scores) for the near-present climate ranged from 0.208 ± 0.011 (population IT-A) to 0.729 ± 0.007 (population BE-A) with a mean of 0.457 ± 0.010 (**Supplementary material S11**). Precipitation contributed 31.5 % to the mean ecological niche of *E. helleborine*, followed by temperature (27.3 %), bedrock (25.0 %) and elevation (16.2 %).

The suitable habitat of *E. helleborine* in the Last Interglacial (LI) and the current distribution was widely distributed across Europe, with the most suitable habitat situated in the north-west (**Fig. 1**, **Appendix 1**). During the Last Glacial Maximum (LGM), the suitability of habitat was reduced significantly (Δ mean Gibbs value = -0.292, Z = -4.160, $p < 0.001$), with most of northern and eastern Europe becoming unsuitable (**Fig. 1**). Glacial refugia, or areas of suitable habitat during the LGM, were located on the Mediterranean coast (particularly Greek and Italian peninsulas and southern France), the western coasts of France and the Iberian peninsula, and small isolated areas in the Alps and central European Carpathians. Between the LGM and near-current climate, habitat returned to a similar mean suitability and distribution as in the LI period (**Fig. 1**; Δ mean Gibbs value = 0.334, Z = 4.990, $p < 0.001$).

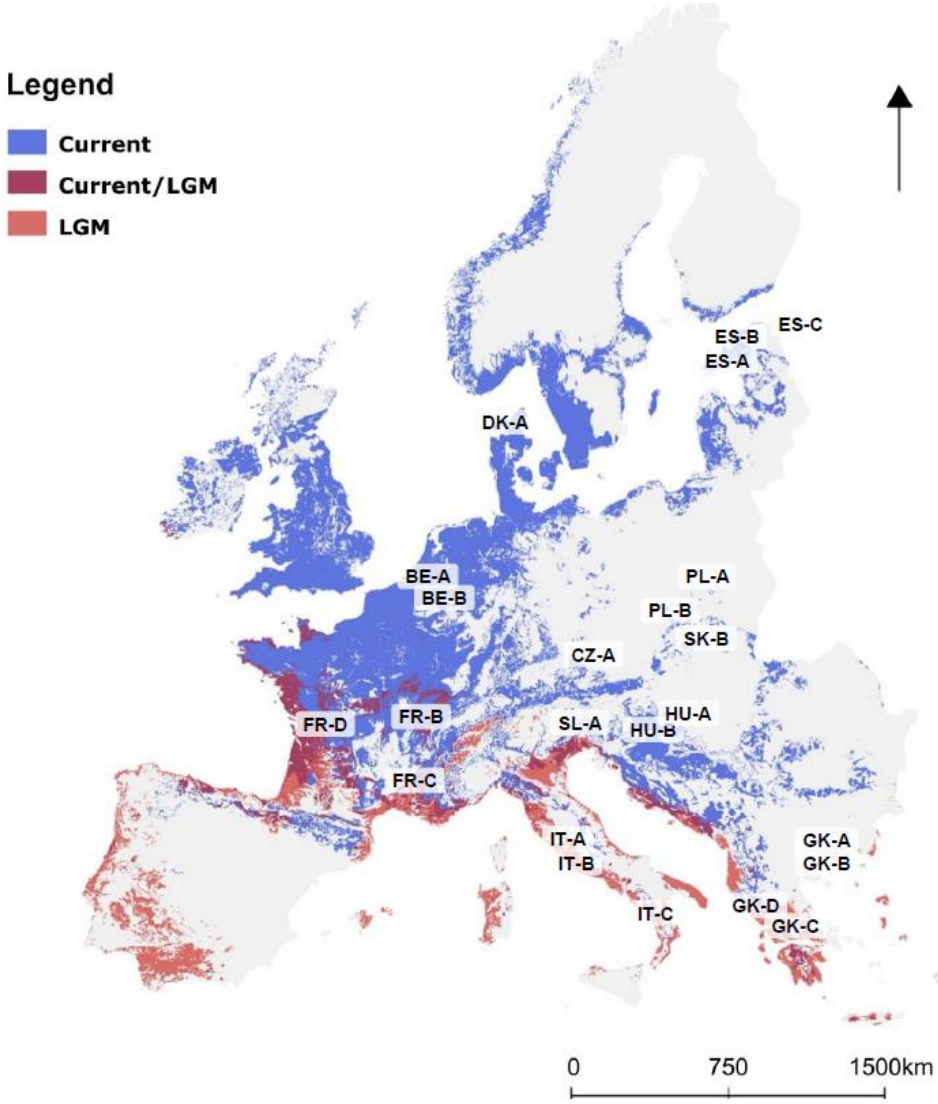


Figure 1. European distributions of suitable habitat of *Epipactis helleborine* predicted for the current climate and the climate of the Last Glacial Maximum (LGM), showing areas of overlap between current and past climate. The continuous range of habitat probabilities of the Maxent output were converted to binary presence/absence maps by extracting and plotting the pixels above the Equal training sensitivity and specificity Cloglog threshold value (0.53).

Genetic diversity and population genetic structure

Allelic richness (*AR*) ranged from 1.353 ± 0.008 SE (GK-C) to 1.403 ± 0.008 (IT-B) (**Supplementary material S11**). Observed heterozygosity (H_o) values were between 0.551 ± 0.012 (IT-C) and 0.592 ± 0.012 (FR-B) and expected heterozygosity (H_e) between 0.340 ± 0.007 (GK-C) and 0.386 ± 0.006 (ES-C). Greek, Italian and Danish populations tended to have measures of within-population genetic diversity below the mean for observed and expected heterozygosity, while Belgian, Polish and Estonian populations had higher diversity than the mean. However, Italian populations had high allele richness. Mean habitat suitability was not correlated with an increase in genetic diversity. However, populations located in areas that experienced a greater change in habitat suitability from the LGM to near-present demonstrated higher expected heterozygosity ($R^2 = 0.45$, $F_{(1,21)} = 17.17$, $p = 0.0005$) and observed heterozygosity ($R^2 = 0.52$, $F_{(1,21)} = 22.64$, $p = 0.0001$).

The AMOVA indicated that most of the genetic variation observed (95%) occurs within populations, and the remaining 5% occurs between populations. The mean F_{st} (the mean of the pairwise distance values between populations) was 0.089 ± 0.003. A Mantel test indicated that genetic distance (F_{st}) was not a function of geographic distance between populations ($r_M = 0.014$, $p = 0.45$) (**Supplementary material S12**).

Thirteen genetic clusters were detected in the STRUCTURE analyses (**Supplementary material S13**), with substantial admixture among most sampled populations (**Fig. 2**). The Greek populations showed clear membership to a genetic cluster. The Slovak, Slovenian, and Danish populations, as well as one French and one Hungarian population, showed strong genetic structure.

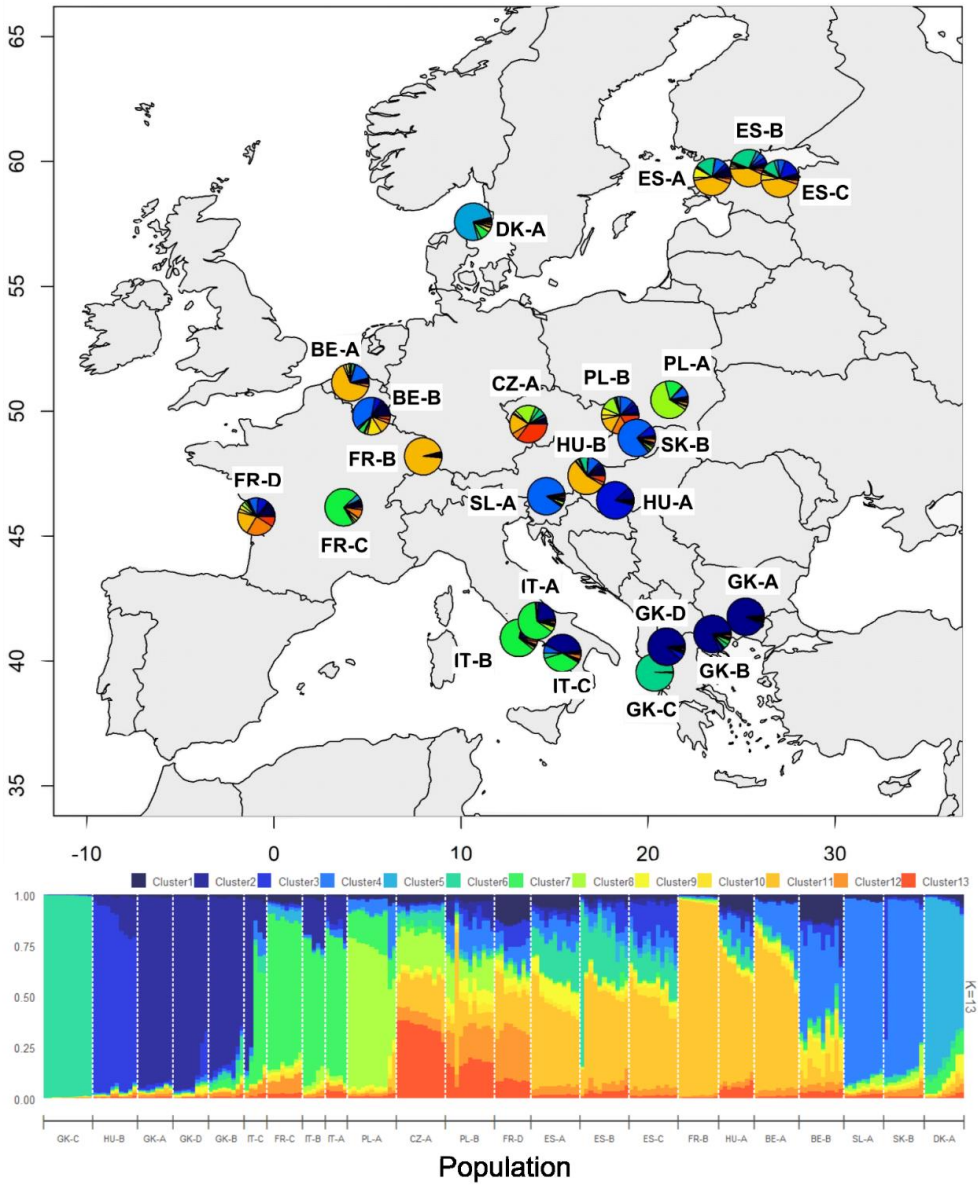


Figure 2. Genetic clusters detected among the samples from 23 sampling locations (populations) in Europe of *Epipactis helleborine*. The membership coefficient of each sampled population is represented as a proportion of each colour-coded genetic cluster.

Outlier detection and analyses

There were 117 outlier SNP loci detected by Bayescan and 101 by PCAdapt, with 76 outlier loci and 181 neutral loci shared by the two methods, of the 893 total loci (**Supplementary material S14**). The mean observed heterozygosity was significantly higher for outliers (0.59 ± 0.007 SE) than neutral SNPS (0.52 ± 0.007) (t_{21} = 10.62, p < 0.0001), but there was no significant difference between SNP types detected for expected heterozygosity or allele richness. All genetic diversity indices had significant positive relationships with an increase in habitat suitability for the outlier dataset (**Table 1**), indicating adaptive potential in these measures with regards to change in suitable habitat. For the neutral SNP dataset, no diversity indices were significantly associated with the change in habitat suitability.

The first axis of the redundancy analysis (RDA) was significant (F = 2.01, p = 0.025) and from the loadings of this axis we detected 91 outlier SNPs associated with temperature, 52 with elevation, and 28 with precipitation. The RDA indicated that Greek and Italian populations were distinct from the rest of the populations (**Fig. 3**). Greek populations were strongly associated with higher elevations and Italian populations with higher temperatures while the remaining populations were moderately associated with lower rainfall.

Table 1. Results of regressions testing the effects of change in habitat suitability from the Last Glacial Maximum to the near-present on genetic diversity, for outlier and neutral markers separately. Genetic diversity measures tested were observed and expected heterozygosity and allele richness (H_o, H_e and AR) of a rarefied sample. Significant p-values are in bold.

Diversity measure	Outlier SNPs			Neutral SNPs		
	Adjusted R-squared	F-statistic	p-value	Adjusted R-squared	F-statistic	p-value
H_o	0.141	4.459	**0.048**	-0.045	0.092	0.765
H_e	0.149	4.666	**0.043**	-0.018	0.636	0.435
AR	0.152	4.77	**0.041**	-0.007	0.857	0.366

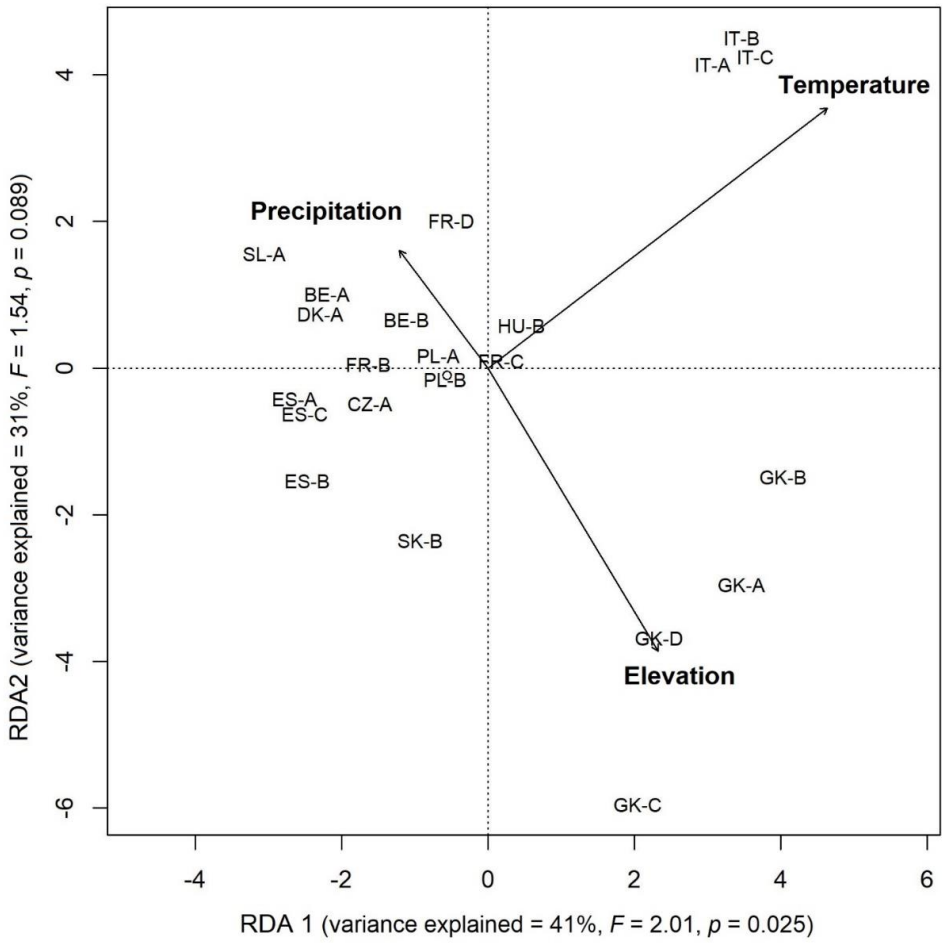


Figure 3. Redundancy analysis (RDA) of *Epipactis helleborine* SNPs, showing the effects of annual precipitation, mean annual temperature and elevation on genetic structure.

Geogenetic analysis

The geogenetic analyses indicated sources of genetic enrichment (i.e., the predicted historical spatial location of genetic source populations that contributed to the current gene pool of the sampled populations) mainly in Italy and central Europe, with a few sources scattered in northern Europe and one in Albania (**Fig. 4**). The genetic sources in Italy dispersed throughout Europe, mainly northeast towards central and northern Europe including Denmark and Estonia, while the sources in central Europe contributed to

western populations in France and Italy. The central European sources also contributed to populations within central Europe, such as the Slovak and Czech populations.

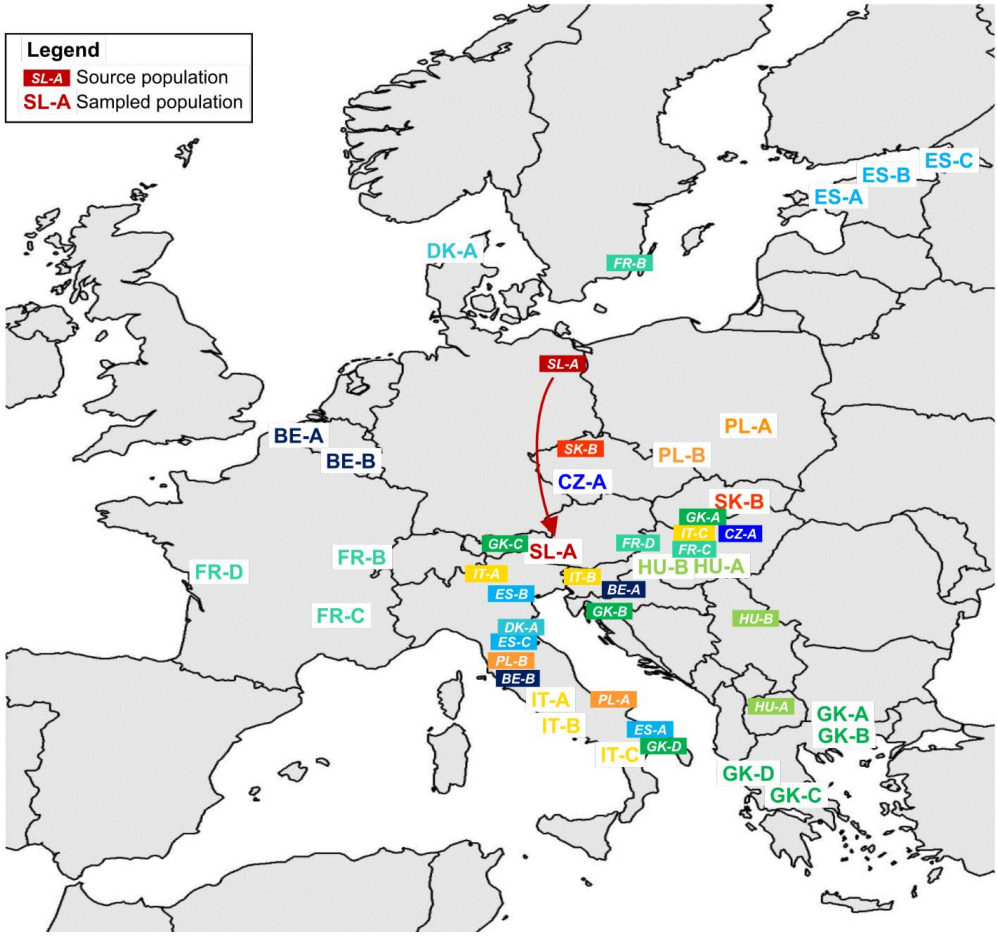


Figure 4. Locations of main sources of genetic enrichment (coloured blocks with white text) predicted by spacemix for each sampled population (coloured text) of *E. helleborine* in Europe. An example is shown by an arrow of the potential movement of genetic material from a hypothetical historical source population to the current sampled population SL-A.

Discussion

Refugia and genetic isolation

Considering that dispersal of *E. helleborine* is most likely not distance-limited, genetic signals for restricted gene flow in these populations are likely due to geographical barriers and historic isolation. The wide northern European distribution of suitable habitat of *E. helleborine* during the Last Interglacial shrank and shifted to areas around the Mediterranean and Aegean, the western coasts of France and the Iberian Peninsula, and small areas of the Alps and Carpathians during the Last Glacial Maximum (LGM) (**Fig. 1**). Previous research has indicated that broad-leafed forests were able to persist through the LGM in glacial refugia (Bhagwat and Willis 2008), notably in mountainous areas on the Greek and Italian peninsulas and central European Carpathians (Tzedakis 2004; Giesecke 2013). These habitats may have provided buffered microclimates to *Epipactis helleborine* which, being herbaceous, small-seeded, and dependent on mycorrhizal fungi, would not otherwise have survived glaciation (Keppel *et al.* 2012).

The steep environmental gradients present in mountainous areas limit the exchange of genetic material between populations through physical barriers to pollination and seed dispersal (Riebesell 1982; Steinbauer *et al.* 2016; Wu *et al.* 2020) and a mismatch in flowering times due to temperature differences at different elevations (Gustafsson and Lönn 2003; Gaudinier and Blackman 2020). Populations of *E. helleborine* in Greece demonstrated low heterozygosity and strong genetic structure (**Fig. 2**), which may be indicative of geographic isolation (Habel and Schmitt 2012; Carvalho *et al.* 2019) as well as elevation-associated adaptive alleles (**Fig. 3**). Populations that survived in mountain glacial refugia in Greece likely tracked the suitable climate up to the high elevations they occur at today, following the migration of boreal forests (Hewitt 1999), as surrounding conditions became too warm during the climate amelioration (**Fig. 1**). This geographic isolation created a genetic bottleneck and resulted in relatively lower genetic diversity and strong genetic structure (Nei *et al.* 1975). Although heterozygosity was low relative to the other populations, it was not as low as may be expected in geographically isolated populations of other species, possibly due to the role of vegetative reproduction in reducing the loss of genetic diversity through genetic drift (Gargiulo *et al.* 2019).

The importance of Mediterranean refugia has been widely recognised in the literature (Médail and Diadema 2009), while the presence of central European microrefugia is becoming more apparent (see Schmitt & Varga, 2012). Although the spatial resolution we used did not allow us to detect microrefugia, some evidence of suitable habitat during the LGM can be seen in the vicinity of Slovakia and Hungary (**Fig. 1**) and glacial refugia have been identified here for species with which *E. helleborine* commonly co-occurs, such as beech (Delforge 2006; Giesecke 2013). Additionally, these areas contain populations that are genetically distinct (**Fig. 2**). Populations in the French Alps and Denmark also demonstrated signs of geographic isolation which may be a result of geographic barriers, but their genetic distinctness was not linked to adaptation to the environmental variables we tested. Considering that these areas were glaciated in the LGM, it is likely that these populations originated from more southern refugia and became isolated after following the migration of cold-adapted forest trees north and towards higher elevations (Hewitt 1999; Parisod 2022).

Postglacial radiation and gene flow

As the distribution of suitable habitat expanded northwards following the LGM, Italy and central Europe acted as centres of genetic enrichment for other populations in Europe during postglacial recolonization of the continent (**Fig. 4**). The dispersal of genetic material from source populations of the species was possible due to the high dispersal capabilities of orchid 'dust seeds' and despite most seeds landing near the mother plant, the small proportion of seeds that disperses from the parental population is likely enough to allow for gene flow across populations and the colonisation of new habitats (Arditti and Ghani 2000; Jersáková and Malinová 2007; Kotilínek *et al.* 2020). This is reflected in the relatively high levels of genetic diversity and low structuring in non-refugial populations, which is in agreement with previous studies on the genetic structure of other European *E. helleborine* populations (Tranchida-Lombardo *et al.* 2011; Jacquemyn *et al.* 2018, 2020) and species capable of long-distance dispersal (Phillips *et al.* 2012; Helsen *et al.* 2015). Populations that were situated in areas that experienced a great increase in suitability of habitat between the LGM and near-present (i.e. glaciated to habitable) had the highest measures of heterozygosity (**Table 1**). This may be due to increased gene flow that came with improved survival and establishment and therefore greater opportunities

for reproduction and population growth, which increased the gene pool and chance of admixture.

The value of using multiple methods to investigate genetic structure to deduce gene flow is evident in the population FR-D, which shows membership to many genetic clusters evident in other populations (Fig. 2) but does not show notably high genetic diversity (Supplementary material S11). As it is located in a glacial refugium and holds much of the diversity seen in other populations, it may have acted as a genetic source during postglacial expansion. The high genetic diversity in this population might reflect the original genetic diversity, while the fewer numbers of genetic clusters present in other populations may reflect the subset of original genetic diversity that is found in offshoot populations created by a long distance dispersal effect. However, the spacemix analysis (Fig. 4) did not indicate genetic sources in western France, and it is possible that FR-D is a more recently established population that has a high diversity due to gene flow from other populations. This is supported by the spacemix analysis which indicates that the main source of genetic diversity of FR-D originates in central Europe, so the population currently occurring in western France may have originated from a secondary dispersal event from other highly diverse populations in that area such as CZ-A, PL-B and HU-B (Fig. 2), or from the refugial ancestors of these populations. Further investigation into the relative time of divergence of these populations would be required to identify which process was more likely to have contributed to the genetic structure of current populations.

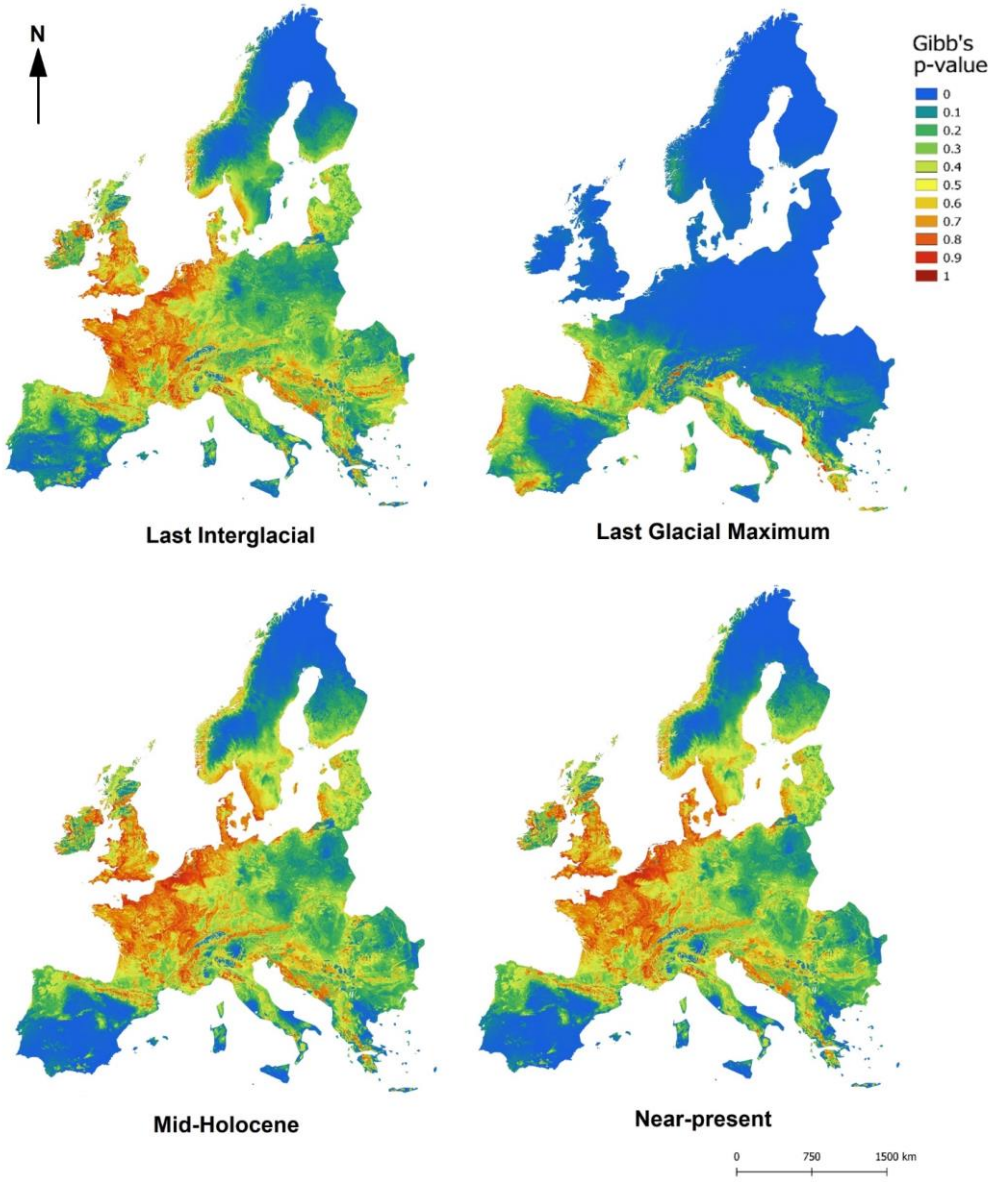
Implications for adaptive potential

The observed patterns of genomic divergence support previous claims that the genus *Epipactis* is currently undergoing an evolutionary radiation driven by a wide spectrum of genotypic, phenotypic and environmental factors and that *E. helleborine* may serve as a source of local variants from which new ecotypes have arisen (Hollingsworth *et al.* 2006; Tranchida-Lombardo *et al.* 2011; Jacquemyn *et al.* 2020). However, the observed levels of genomic divergence are most likely not strong enough to have led to complete reproductive isolation between populations and local variants can at best be described as varieties or ecotypes (Jacquemyn *et al.* 2018; Sramkó *et al.* 2019).

When adaptive markers are analysed separately from neutral markers, one can disentangle the effects of natural selection and neutral evolution on genetic diversity, and identify the potential of a species to adapt to environmental change (Kirk and Freeland 2011). The higher genetic diversity in adaptive markers that was observed in populations located in areas that underwent a greater change in habitat suitability supports the potential of this species for adapting to and surviving changes in climate. This is especially apparent in the high allelic richness of Italian populations, indicating adaptive potential (Vilas *et al.* 2015), as well as the evidence of adaptive alleles associated with higher temperatures. In contrast to the climate tracking of Greek populations, the genetic structure of Italian populations may have arisen more recently through adaptation to increasing postglacial temperatures (**Fig. 3**), resulting in ecological isolation reflected by similarly low heterozygosity and moderate genetic structure. However, the Greek populations will become more isolated if they continue to track suitable climate up to higher elevations, and their lower neutral genetic diversity reduces the chance of adaptive alleles arising (Luikart *et al.* 2003; Kirk and Freeland 2011). Unless outlier loci are directly linked to a phenotypic trait that demonstrates adaptation, linking outlier loci to environmental gradients should be assessed with caution because genetic drift can cause populations to diverge at neutral loci through reproductive isolation (Stinchcombe and Hoekstra 2008; Kirk and Freeland 2011). Considering that there was no significant relationship between genetic diversity in neutral SNPs and habitat suitability, we believe that adaptation to local environments contributed more to population structure than genetic drift.

Conclusion

The observed spatial genetic structure of *Epipactis helleborine* results from a complex interplay between changes in habitat availability since the Last Glacial Maximum, gene flow, and adaptation to local environmental conditions. Our results support the occurrence of central European glacial refugia as well as Mediterranean refugia, and their importance in contributing to postglacial recolonization and genetic enrichment of forest species. The assumption that refugial populations have higher genetic diversity than offshoot populations may not be consistent for species with highly-dispersible seeds and complex environmental associations such as *E. helleborine*, where colonising populations are subject to founder effects but can also act as sources of genetic enrichment. Adaptive alleles associated with temperature and elevation contributed to the divergence of Italian and Greek populations, with different implications for the adaptive potential of these populations regarding climate change. This study demonstrates how the evolutionary histories of herbaceous, small-seeded species that are not readily fossilised can be characterised using a combination of ecological niche modelling and genomics, and may serve as a basis for future studies aiming to disentangle the phylogeny of the *E. helleborine* species complex.



Appendix 1. Ecological niche distributions of *Epipactis helleborine* in Europe, projected for three past climatic periods: a) Last Interglacial period, b) Last Glacial Maximum, c) Mid-Holocene and d) the near-present climate (1970-2000).

Chapter 6

General discussion

The diversity and distribution of orchid species result from a complex combination of factors involving seed dispersal, historical environmental changes and relationships with other organisms (Tremblay *et al.* 2005; Otero and Flanagan 2006; Givnish *et al.* 2016; Taylor *et al.* 2021). The corresponding variety of unique morphological and ecological adaptations that have arisen in the family has inspired much fascination with orchids in science and society (Bronstein *et al.* 2014). Orchid species share some aspects of the ecological niche which impose limits on their distribution due to shared morphology and ecology such as herbaceous life form, dust seeds with high dispersal potential but reliance on mycorrhizal fungi to germinate and pollinators for sexual reproduction (McCormick and Jacquemyn 2014; Tsiftsis *et al.* 2019; Djordjević and Tsiftsis 2022). However, these characteristics have also contributed to their diversity and wide global distribution, which is reflected in differences between orchid species in other aspects of the ecological niche. For this reason, the general pattern of abiotic components of the ecological niche is moderate conditions in climate and substrate support the most species of orchids, but the ability of orchids to push the limits of the ecological niche is seen in the occurrence of a significant number of adaptations to extreme conditions (Djordjević and Tsiftsis 2022). The extreme habitat specialisation seen in adult orchids is in contrast with the high dispersal potential of orchid seeds and large distributions of some species (Darwin 1877; Tremblay *et al.* 2005; Otero and Flanagan 2006; Givnish *et al.* 2016) which makes orchids ideal organisms to study the relationship between ecological niche, distribution and divergence.

While the adaptations evident in the life history and morphology of orchids make them model organisms in which to study descent by modification (Darwin 1877), it is complex to disentangle the mechanisms involved in their evolution and distribution due to conflicting pressures (Givnish 2010; Bronstein *et al.* 2014). However, recent advances in methods to quantify distribution, ecological interactions and gene flow have ushered in a new era of orchid research which has shed light on these mechanisms (e.g. Bateman 2020; Wang *et al.* 2022). Additionally, such investigations into the ecological niche and distribution of orchids is highly relevant in light of global change, as orchids are at significant risk of extinction (Swarts and Dixon 2009; Vogt-Schilb *et al.* 2015). This thesis aimed to elucidate the role of the environmental niche in the historic, present and future distributions of *Epipactis*, and its relationship with mating system and mycorrhizal

fungi. The conclusions drawn about the main topics covered in the thesis are discussed below, along with limitations of the thesis and opportunities for addressing remaining knowledge gaps.

The abiotic niche of *Epipactis* – general patterns, notable differences, and why scale and definition matter

Ecological niche models were created in **Chapter 2** with Maxent using distribution and environmental data for the known ranges of *Epipactis* species in Europe, and formed the basis of the habitat analyses in the rest of the thesis. These models indicated that, in general, *Epipactis* distributions are strongly shaped by bedrock type and land-use and moderately by climate and soil properties. In particular, the preference demonstrated for limestone bedrock and broad-leafed and coniferous forests is consistent with our knowledge of the habitats of terrestrial orchids in temperate Europe (Djordjević and Tsiftsis 2022) and with other studies on *Epipactis* specifically (Hemrová *et al.* 2019). Mean range size of *Epipactis* species increased with environmental niche breadth, another pattern that is consistent with plants in general (Slatyer *et al.* 2013). Despite these general patterns, *Epipactis* species differed substantially from one another in niche breadth, habitat extent and relative contribution of environmental characteristics. **Chapters 2** and **4** did not find a clear link between habitat type (forest, dune, etc.) and range size or niche breadth, with both specialists and generalists occurring in each habitat type. This is to be expected because while habitat specialists are limited to a habitat type, the ranges of generalists can, by definition, include multiple habitats in which specialists may occur (Brown *et al.* 1996; Slatyer *et al.* 2013; Sheth *et al.* 2020). For example, both *Epipactis dunensis* and *palustris* can occur in coastal dunes, but while *E. dunensis* is restricted to this habitat, *E. palustris* can also be found in marshes and forests and therefore has a larger spatial range.

The concept of niche breadth is well-explored in the literature, but not consistently defined or quantified (Townsend Peterson and Soberón 2012; Sheth *et al.* 2020). The niche breadth metrics calculated in environmental space (referred to here as environmental niche breadth) and geographic space (geographic habitat distribution) in **Chapter 2** were not significantly correlated with each other on average, although some

species such as *E. helleborine* had high values for both metrics. This means that a habitat generalist species was able to tolerate a wider range of environmental conditions and was able to colonise an area of habitat in a more even distribution across the landscape than a specialist (Warren *et al.* 2019). This can occur in a species with high phenotypic plasticity or when the populations of a species are able to adapt to local conditions (Slatyer *et al.* 2013). *E. helleborine* has been considered a habitat generalist by other sources based on the number of habitats it can be found in (Delforge 2006; Hemrová *et al.* 2019; Evans and Jacquemyn 2020) and its populations exhibit strong genetic signals for adaptation to local elevation and temperature (Evans *et al.* 2023), which may have contributed to its successful persistence in many areas. Conversely, some species such as *E. kleinii* and *E. tremolsii* had narrow environmental niche breadths but moderately high values of geographic habitat distribution which indicates that although these habitats are composed of specific, narrow ranges of conditions, they may occur fairly often over the landscape (Petraitis 1979; Warren *et al.* 2019). Although *E. kleinii* and *E. tremolsii* have moderately high values of geographic habitat distribution, in reality populations tend to be rare (Delforge 2006; GBIF 2020) which corresponds with their low environmental niche breadth values. Results such as these demonstrate the value of calculating niche breadth in both environmental and geographic space, because although the habitat suitability of species such as *E. kleinii* and *E. tremolsii* may be fairly evenly distributed, the specificity of the habitat likely makes it sensitive to change, and the low tolerance of these species to conditions outside of this habitat puts them at risk of extinction (Thuiller *et al.* 2005; Slatyer *et al.* 2013).

The lack of a consistent correlation between the niche breadth metrics also reflects the ongoing evolution of definitions and techniques to quantify niche breadth that can be seen in the literature. The limitations of the traditional calculation of niche breadth in geographic space and the potential of using environmental space became apparent only towards the end of **Chapter 2** and coincided with the development of the newer version and improved documentation of ENMTools v1.2. Levins' *B2* in geographic space is a measure of the flatness of the habitat distribution, a summary of the continuous Gibbs values across space which is calculated in ENMTools from the output habitat distribution map of the Maxent model. While this measure of the spatial distribution of habitat is useful as a proxy for relative range size (seen in the strong correlation between the two

measures in **Chapter 2**), its calculation does not require data that capture the range of values of each environmental variable across a landscape or species' tolerance to these gradients. Niche breadth in environmental space, on the other hand, may more closely reflect the definition of niche breadth (Levins 1968; Dolédec *et al.* 2000; Gaston 2003) in its calculation in the context of a set of environmental rasters, which include data on the gradients of each environmental variable over the landscape, as well as the upper and lower limits of each variable associated with the occurrence of each species. There are limitations to this metric too, as it is still dependent on the availability of habitats in a landscape and for species with very few occurrence points, is highly sensitive to overfitting (Elith *et al.* 2011; Warren *et al.* 2019) which resulted in the exclusion of some rarer taxa from **Chapter 3**. Additionally, reporting this metric of niche breadth should be accompanied by relevant context and details of the species; for example the habitat distributions of some large-range species such as *E. palustris* and *E. purpurata* are strongly influenced by land cover such as forest, while the small-ranged specialist *E. kleinii* and *dunensis* are rather more dependent on bedrock type (**Chapter 2**). This indicates that tolerance to a particular axis of the abiotic niche, as well as its availability in the landscape (**Fig. 1**), may influence distributions to different degrees, but this distinction is not captured in the single value of the niche breadth metric, which represents the average tolerance across niche components (Warren *et al.* 2010).

Range size was correlated with Mean Gibbs p, a proxy for habitat suitability or probability of occurrence (Phillips *et al.* 2018). This indicates that having a larger range increases the probability of highly suitable habitat, while species with small ranges will likely also be rare within their ranges habitat. However, Gibbs p is calculated in the context of the habitat requirements specific to each species, and even species that are rare in a landscape should have a high habitat suitability or probability of occurrence at the exact spatial point where they do occur. Indeed, populations of rare plant species can often be densely populated if they are adapted to their local habitats, even if the spatial range of the species is small (Lesica *et al.* 2006). Therefore this result is most likely an effect of resolution, as a grid square at a coarse resolution may contain a heterogeneous range of habitat suitability values at a finer scale, including highly suitable habitat. The fairly coarse resolution of 10 km^2 used when creating the ecological niche models, although appropriate for a continent-level study, might have resulted in the lack of strong

associations to habitat variables for some small-range, specialist *Epipactis* species such as *E. dunensis* which is expected to be strongly linked to land-use (specifically coastal dunes). In this way, range size and Gibbs p can still be used to characterise habitat specialisation in addition to niche breadth, but future ecological niche research would benefit from scaling the resolution used with species range size, so that fine-scale habitat-use patterns are retained for small range species (Kambach *et al.* 2019). In addition to the environmental variables, the spatial resolution and quality of the occurrence data used is important to consider when interpreting the results of ecological niche models. I aggregated species points into 10km center points to reduce issues related to spatial clustering, but the result of this is that the values of the environmental variables extracted for each point are only accurate to 10km. Furthermore, the accuracy of a dataset obtained from online sources such as GBIF can be gauged to some degree by the precision with which data is recorded, but the true reliability of the data cannot easily be verified. For species with many occurrences, the effects of a few erroneous occurrences will be minimal, but for species with only a few points, this becomes more important. Future research may benefit from verifying the occurrence data of rarer species.

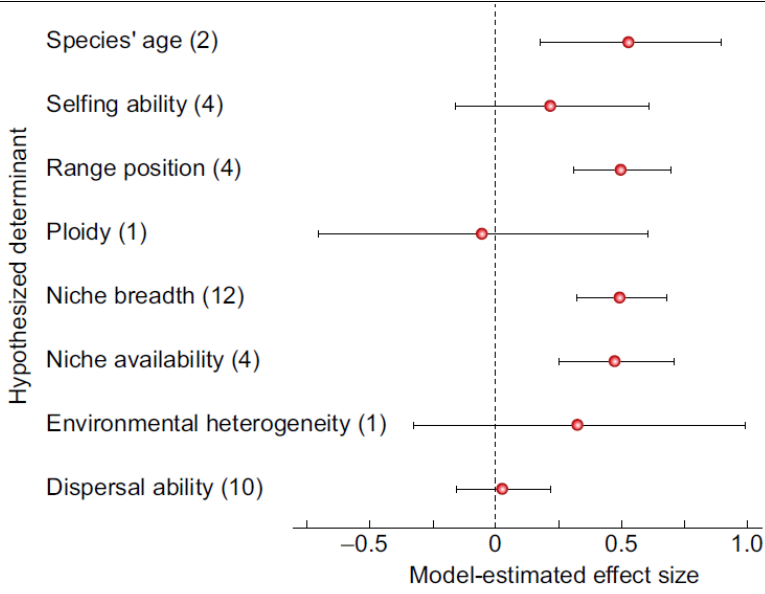


Figure 1. Relative contribution of hypothetical predictors of plant range size (Sheth *et al.* 2020), demonstrating how availability of species-specific suitable habitats in a landscape (niche availability) is of similar importance to range size as niche breadth.

Mating system and mycorrhizal fungi – do life history traits and biotic interactions fill in the gaps?

Considering the high variation in habitat use among *Epipactis* species was not entirely explained by abiotic environment, I considered the contribution of mating system to *Epipactis* niche breadth and distribution. *Epipactis* has a relatively high proportion of selfing species (Claessens and Kleynen 2011), with half of the species tested in **Chapter 2** being either fully autogamous or capable of selfing through facultative autogamy. Mating system has been used to explain the divergence of *Epipactis*, where switching from allogamy to autogamy has resulted in a large number of species (Hollingsworth *et al.* 2006; Tranchida-Lombardo *et al.* 2011; Sramkó *et al.* 2019) and therefore niche differences may also be expected to differ between mating system. Autogamy offers the potential to expand species ranges where colonisation is less limited by the presence of available mates (Baker 1955; Grossenbacher *et al.* 2015), but **Chapter 2** did not detect differences between autogams and allogams in niche breadth or range size, or in climate-induced distribution changes in **Chapter 4**. The transition to autogamy is considered an evolutionary dead-end, and low genetic variation reduces the potential of autogams to adapt to a range of environments (Fisher 1958; Holt and Gaines 1992; Honnay and Jacquemyn 2007) which may account for this. Mating system is important to consider when investigating the distribution of individual species, but it is not a strong predictor of niche breadth for *Epipactis* as a genus, which is a similar result for plants in general (Park *et al.* 2018), possibly due to these the contrasting mechanisms.

Species interactions are important determinants of range size, as the presence of mutualists in an area can promote range expansion, while the absence of mutualist partners limits the distributions of reliant species (Bruno *et al.* 2003; Van der Putten *et al.* 2010; Pigot and Tobias 2013; Alexander *et al.* 2022). For orchids in particular, mycorrhizal fungi are required for seed germination and can also contribute to seedling and adult growth and survival in some taxa such as *Epipactis* (Rasmussen 2008; Li, Wu, *et al.* 2021). Therefore the presence of fungi in an area dictates whether an orchid will colonise a habitat, as well as population persistence and growth through adult fitness, which contribute directly to maintaining and expanding range size (McCormick *et al.* 2018; Sheth *et al.* 2020). However, the germination capabilities of *Epipactis* do not reflect

the limited distribution of adults (Těšitelová *et al.* 2012) and the importance of mycorrhizal associations for adult *Epipactis* is conflicted in the literature (Ogura-Tsujita and Yukawa 2008; May *et al.* 2020), which led to the investigation of **Chapter 3** into the relationship between mycorrhizal diversity and environmental niche breadth in nine *Epipactis* species. Although all of the *Epipactis* species associated with many fungi (**Fig. 2**), those that associated with a higher number of fungal taxa had wider environmental niche breadths. Niche breadth in geographic space was not significantly associated with fungal richness, indicating that the variety of environmental conditions a habitat generalist can tolerate, rather than its larger habitat distribution, is more significant when predicting fungal associations in *Epipactis*.

When defining the mycorrhizal richness of an orchid species, I considered the number of fungal taxa that were detected in all samples at different sites, because this was most relevant to investigate the hypothesis that the ability of a species to associate with fungi at different sites increases the number of habitats it can occupy. A fungal generalist may be considered to have a broad fungal partner breadth (Batstone *et al.* 2018). However, this does not necessarily mean that an individual orchid associates with many mycorrhizae but rather that the range of the whole species includes many partners with which it can associate (Fort *et al.* 2016). It is possible that such a fungal (and habitat) generalist species can include populations that become highly adapted to their local mycorrhizal communities and diverge. This raises the question of whether habitat specialisation may have arisen first, and fungal specialisation followed. The divergence of *Epipactis* is believed to have been driven by adaptation to local conditions after colonisation (Squirrell *et al.* 2002b), and local conditions may have included specific mycorrhizal assemblages.

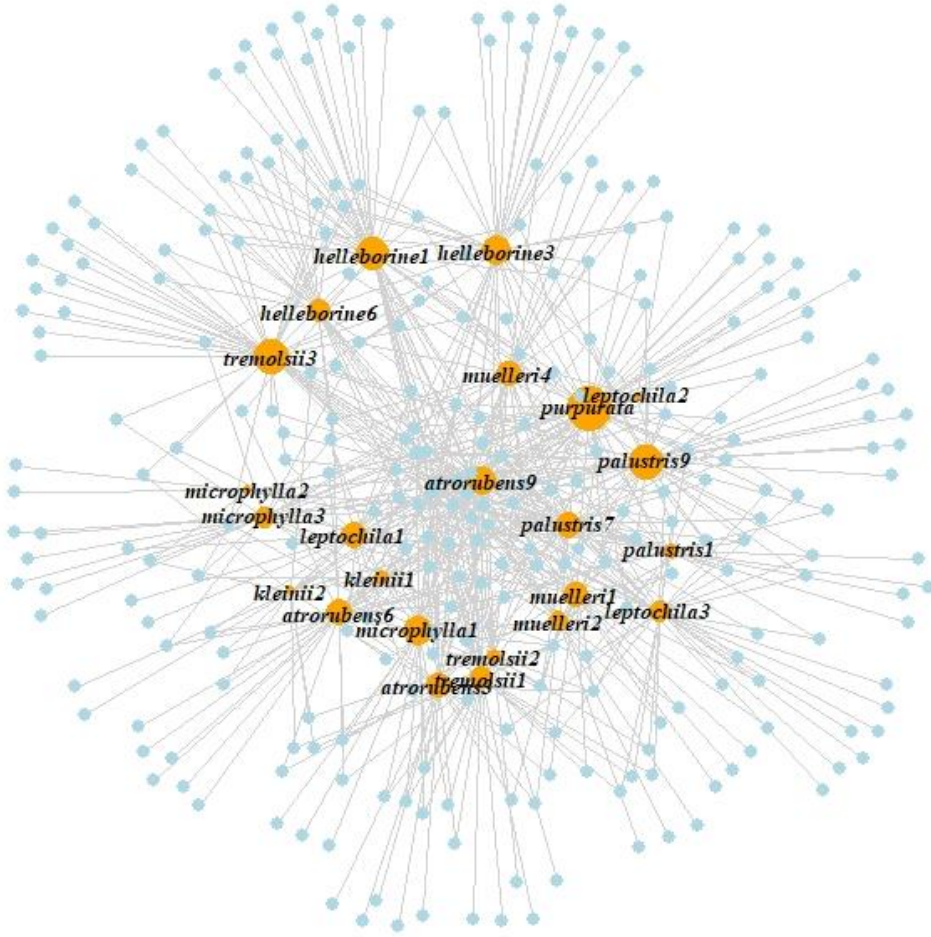


Figure 2. Network of *Epipactis* populations (orange points) and fungal associates (grey points), showing the variety of fungal OTUs shared between, and unique to, populations. Populations within a species at different sites tended to associate with more similar fungal associates (represented by proximity of population points), indicating a degree of partner selection along taxonomic lines. The size of the *Epipactis* points represents the relative number of fungal partners detected in each population. *Epipactis helleborine* associated with the greatest number of fungal taxa, including many common taxa shared with other *Epipactis* species (grey lines connecting to fungal taxa towards the center of the plot) as well as unique taxa (fungal taxa at the periphery of the plot). *Epipactis* species may be considered fungal generalists by their large number of fungal associates, but while many fungal partners are common to species, presumably due to availability across habitats and ability to provide a beneficial ecological role to the orchids, the number of fungal OTUs unique to each orchid population indicates habitat-specific availability or selection of fungal taxa by the orchid population.

However, fungal taxa are widespread and considering co-occurring orchids can differ in mycorrhizal communities, fungal distribution is not likely to limit them in this way (McCormick and Jacquemyn 2014). This is demonstrated in *E. dunensis* which only recently diverged into a habitat specialist through a genetic bottleneck and, within its dune habitat, its presence is not limited by fungal communities (Jacquemyn *et al.* 2020). In addition to the results of **Chapter 3**, this indicates that fungal association apparent in current populations contributes to the different habitat associations between some *Epipactis* species, but that fungal specificity may have followed ecogeographic isolation that drove divergence originally. Our ongoing research into fungal communities of *Epipactis helleborine* populations may yield interesting insights; if populations that were found to be more reproductively isolated and adapted to local conditions in **Chapter 5**, such as the Italian and Greek populations, have more fungal specialised communities, this may give tentative support to the hypothesis that fungal specialisation follows habitat specialisation. It is plausible that fungal specialisation both drove and was driven by habitat specialisation in the evolution of *Epipactis*, and that both processes occurred iteratively to contribute to specialisation and the spatial distributions of the species. To gain an idea of the importance and rates at which these processes occur and contribute to evolution in *Epipactis*, the change in genetic variation and proportion of alleles linked to fungal association in a generalist species such as *E. helleborine* could be observed over generations and compared between samples with different mycorrhizal presence, while the change in number of mycorrhizal associations between orchid generations could be assessed between populations in different environmental conditions.

Whether the first driver of specialisation and divergence of *Epipactis* was fungal or habitat associations depends on whether ancestral populations of *Epipactis* species were more limited by fungal availability or by environmental factors of the locations to which they dispersed from the ancestral *E. helleborine*, and therefore the primary driver could differ between species. In this case, knowing the spatial distribution of fungal partners over time, particularly at key points in the evolutionary history of *Epipactis*, would be useful. *Epipactis* distributions are linked to forest distributions (Delforge 2006; Hemrová *et al.* 2019), as seen in **Chapter 2** and hypothesised in **Chapter 5**, which is presumably in part due to their mycorrhizal partners associated with trees (Jacquemyn, Duffy, *et al.* 2017; Djordjević *et al.* 2020). In the same way that the climatic changes associated with

glaciation affected the distribution of forests, the distribution and community composition of fungi were likely different during the divergence of *Epipactis* to now (Oehl *et al.* 2011; Pärtel *et al.* 2017). A detailed dataset of fungal distribution could be used to model where the fungal taxa with which *Epipactis* associates occurred at key points in the evolution of the genus, which would contribute to our understanding of the link between mycorrhizal fungus and orchid distribution and divergence. Additionally, associating with multiple partners can be beneficial when partner availability varies over time through a strategy of bet-hedging (Batstone *et al.* 2018), which may also explain why *E. helleborine* and *E. palustris* associated with many partners, as the first is ancestral to the genus and the second diverged early on (Ehlers and Pedersen 2000; Hollingsworth *et al.* 2006; Sramkó *et al.* 2019). These species may have survived until now due to the ability to accommodate changes in mutualist availability over time, which can be reflected in the accumulation of partners in extant populations of ancestral species (Heath and Stinchcombe 2014; Batstone *et al.* 2018). Establishing a link between mycorrhizal specialisation, niche breadth and divergence would require further genetic and mycorrhizal data, as well as niche metrics at a finer resolution to accommodate small-ranged taxa such as *E. kleinii*, *E. tremolsii* and *E. albensis*, ideally with a consistent, comparable dataset of fungal counts and phylogeny of each sample. Furthermore, calculating the phylogenetic breadth of fungal partners for each *Epipactis* species and using this as a measure of fungal diversity in the context of a full phylogeny of all *Epipactis* species investigated here may shed light on the relationships between orchid divergence and fungal diversity.

These results and conclusions demonstrate the need for more investigation into the spatial distribution of mycorrhizal fungi. **Chapter 3** found that species with overlapping niches in both environmental and geographic space had similar communities, which means that species with both similar environmental tolerances as well as those whose habitat distributions coincided spatially, associated with similar fungal taxa. Although not investigated directly, our results indicate that fungal distribution does contribute to differences in *Epipactis* niche breadth. Other studies, and unpublished data from **Chapter 3**, found that dune populations of *E. palustris* and *E. helleborine* ss. *neerlandica* differ in their fungal communities from forest populations of *E. helleborine*, indicating that habitat type plays a role in fungal community structure and may have contributed to niche

partitioning and divergence (Jacquemyn *et al.* 2016), but whether these orchids differ in their fungal communities because of differing availability of fungal taxa in their habitats, or the orchids selectively associate with fungal taxa that provide a habitat-specific benefit, is uncertain. It is clear that data on fungal distributions across habitats and over timescales is severely lacking (Jacquemyn, Duffy, *et al.* 2017; Cameron *et al.* 2018; Guerra *et al.* 2020) and distribution models of fungal taxa in Europe would greatly enhance our understanding of whether fungal availability does indeed contribute to environmental niche differences between *Epipactis* species in different habitats. The broad-scale distribution of fungal taxa may not be appropriate for the fine scale at which orchids associate with mycorrhizal fungi in the soil (McCormick and Jacquemyn 2014), but it may still give some insight into which fungi are most likely to be encountered by an orchid in an area. Furthermore, some fungal taxa can form both ectomycorrhizal and orchid mycorrhizal associations depending on the host (Jacquemyn, Duffy, *et al.* 2017), suggesting that the role they perform can differ between orchid species and therefore the function of a fungal species in a mycorrhizal association cannot be entirely predicted based on its taxonomic identity. Additionally, the potential for mycorrhizal association with more unusual or taxonomically revised fungal taxa should be tested, as the practice of filtering out taxa that are not already known to form mycorrhizal associations with Epipactis may have excluded some taxa that do in fact have an ecologically important role. For example, the reclassification of some Sebacinaceae into the new Serendipitaceae (Weiß *et al.* 2016) is reflected in fungal databases used to identify fungal associations in orchids (https://www.ncbi.nlm.nih.gov/datasets/genome/?taxon=358905), but this taxonomic revision is not included in older literature from which filtering lists are obtained, and therefore such taxa are excluded from analyses. Although defining the spatial distribution and functional roles of fungal partners was beyond the scope of this investigation, it has the potential to greatly enhance our understanding of *Epipactis* distributions.

Epipactis distributions and niche breadth in a changing climate – insights from the past and implications for the future

The value of defining ecological niche breadth when comparing orchid species distributions in space is made clear in **Chapters 2** and **3**, particularly in the context of mycorrhizal associations. Considering the effects of large-scale climatic changes on the distributions and evolution of plants (Comes and Kadereit 1998; Hewitt 2004; Lafontaine *et al.* 2018), it is also relevant to consider how historic climatic changes contributed to current distribution patterns of *Epipactis*. Furthermore, investigating niche breadth and responses to past climatic changes can be informative in predicting how species will respond to contemporary climate change (Thuiller *et al.* 2005; Slatyer *et al.* 2013; Bai *et al.* 2018; Cortés *et al.* 2020), but the high variation in niche breadth and range size in *Epipactis* presents challenges (Thuiller *et al.* 2008; Shay *et al.* 2021). **Chapter 4** explored the implications of future climate change for *Epipactis* distributions and **Chapter 5** investigated the potential of *E. helleborine* to respond and adapt to historical climate change.

To gain an understanding of the genetic mechanisms involved in ecological specialisation that contributed to the variation in niche and distribution in *Epipactis*, **Chapter 5** focused on the extant ancestral species of the genus, *Epipactis helleborine*. The ecological niche model used in the previous chapters was projected onto historical climate data and estimated that the habitat distribution of *E. helleborine* was widely distributed through Europe in the Last Interglacial (LI) period, similar to the current distribution. During the Last Glacial Maximum (LGM), the range reduced and became patchily distributed, with suitable habitat restricted to refugia around the Mediterranean, Iberian peninsula and some areas in western France and the Carpathians (**Fig. 3**). Glacial refugia in these locations have also been identified for other plants (Tribsch 2004; Bhagwat and Willis 2008; Kolář *et al.* 2016). As temperatures rose, suitable habitat expanded northward and populations were reconnected, resulting in a high level of genetic admixture between extant populations. This northward expansion of habitat is predicted in **Chapter 4** to continue into 2061-2080 for *E. helleborine* and seven other species (*E. albensis*, *dunensis*, *fageticola*, *lusitanica*, *microphylla*, *phyllanthes* and *tremolsii*) as increasing temperatures in the north make new areas of habitat available, a pattern recorded and predicted for

other European orchid species (van der Meer *et al.* 2016; Ongaro *et al.* 2018). However, this expansion will only be possible if suitable habitats remain available in the north. This will be particularly important for *kleinii*, *leptochila* and *muelleri* that are predicted to experience a northward shift of habitat but a decrease in current habitat, and therefore will rely on these new areas becoming available. The distributions of *E. atrorubens*, *palustris* and *purpurata* will not shift northwards and will decrease in size with future temperature increases, even if current land-use remains the same.

This raises the limitation of using a model based on the distribution of near-present land-use (including forest distribution) to estimate future habitat distributions of *Epipactis*. The future expansion predicted for many *Epipactis* species will only be possible if the forests in their present and new ranges remain intact. The postglacial recolonization of Europe by forests likely made possible the expansion of *E. helleborine* in the past (**Fig. 3**) (Hewitt 2004; Giesecke 2013; Parisod 2022), but such reforestation occurred over thousands of years and therefore is unlikely to happen within a time frame to match current climate change (Giesecke 2013). Even if forests are replanted, it would likely take a considerable amount of time for them to become supportive systems for orchids with the necessary mycorrhizal communities and soil structure, although such studies on host-mycorrhizal dynamics over large time scales are scarce (Oehl *et al.* 2011; Pärtel *et al.* 2017; Guerra *et al.* 2020; Aavik *et al.* 2021; Fei *et al.* 2022). This demonstrates the critical need to conserve the current range of forests, as a reduction in forest distribution will result in range decreases in many *Epipactis* species that are dependent on forests, no matter the potential range expansions that increasing temperatures offer. Similarly, the effects of changing temperatures on soil structure was not accounted for in the ecological niche models in this thesis. Climate change is predicted to change the pH, water retention properties and carbon content of soils (Rengel 2011; Lal 2014) and therefore the presence of soil microbes (Jansson and Hofmockel 2020) that require specific soil types. Ecological niche models including predicted changes in distribution of forests and soil types, as well as the associated fungal distribution changes, may yield more realistic estimates of future *Epipactis* distributio

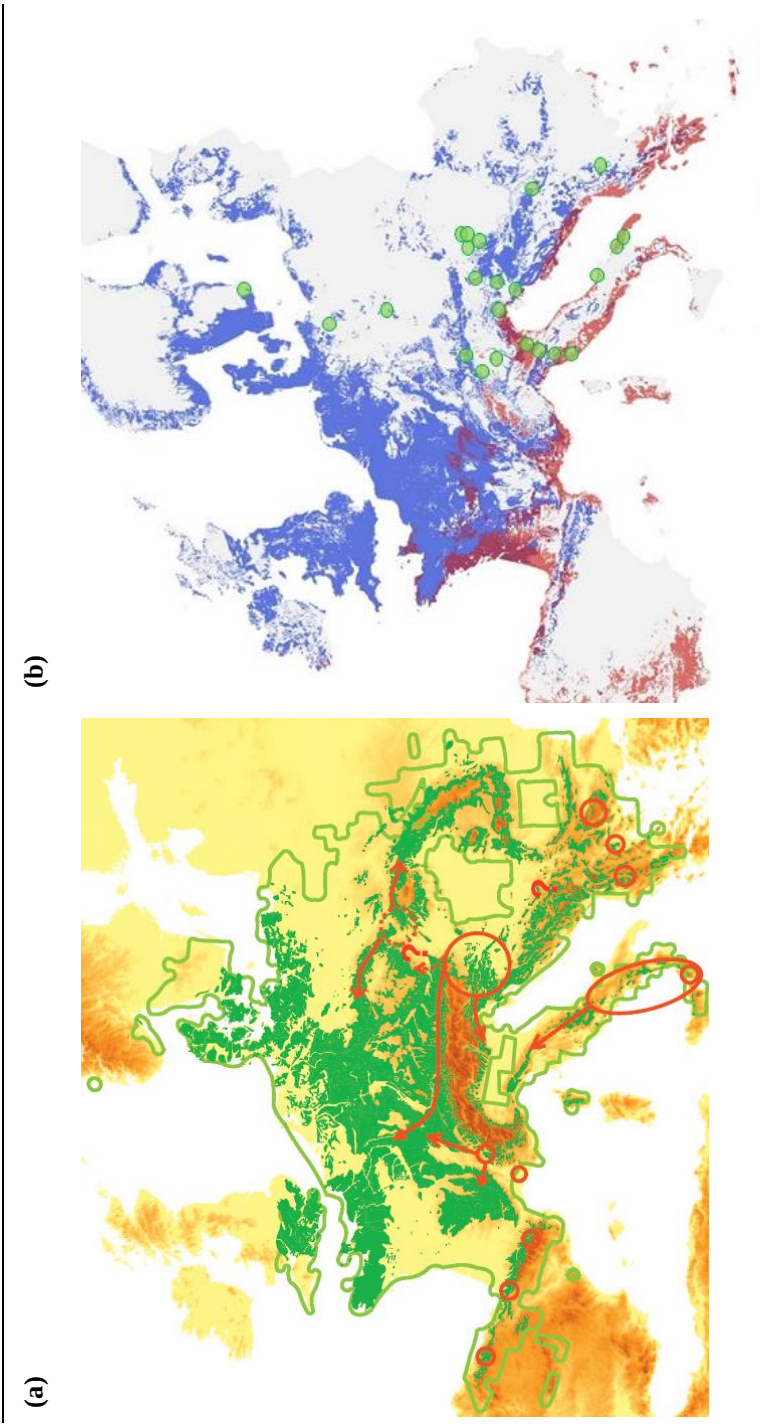


Figure 3. Near current distribution (in green), glacial refugia (red circles) and postglacial recolonization routes (red arrows) of *Fagus sylvatica* (beech) in Europe **(a)** adapted from Giesecke (2013). Postglacial expansion of *Epipactis helleborine* **(b)** from refugia (red areas) and centers of diversification (green points) to populations in its current distribution of suitable habitat (blue area) (**Chapter 5**) likely followed the distribution chan ges of such forests.

The importance of niche breadth in estimating range changes in *Epipactis* is demonstrated in **Chapter 4**, where I detected a general trend that habitat generalists will experience the greatest change in habitat in the future, which is in line with the hypothesis that generalists have a greater capacity for range expansion as they can inhabit a wider range of habitats (Thuiller *et al.* 2005; Alarcón and Cavieres 2018; Geppert *et al.* 2020). However, the generalists *E. palustris*, *purpurata* and *atrorubens* were predicted to experience the greatest negative range changes. This is likely because niche breadth is a measure of average habitat specialisation across variables, but if there is one aspect of a generalist's niche that is limiting, it will be unable to expand into its climatic envelope (Petraitis 1979; Slatyer *et al.* 2013; Warren *et al.* 2019). For example, *E. palustris* is strongly linked to land cover (**Chapter 2**) and while the availability of this habitat allows it to inhabit a large area currently, its range will be limited if its preferred land type is less common in its future climatic envelope (**Fig. 4**). On the other hand, this result means that specialist and small range species appeared to be 'less affected' by climate change. However, it also means that habitat specialists have a reduced potential to expand into new areas like generalists which increases the risk to their current distributions, a result also observed in other orchids (Geppert *et al.* 2020).

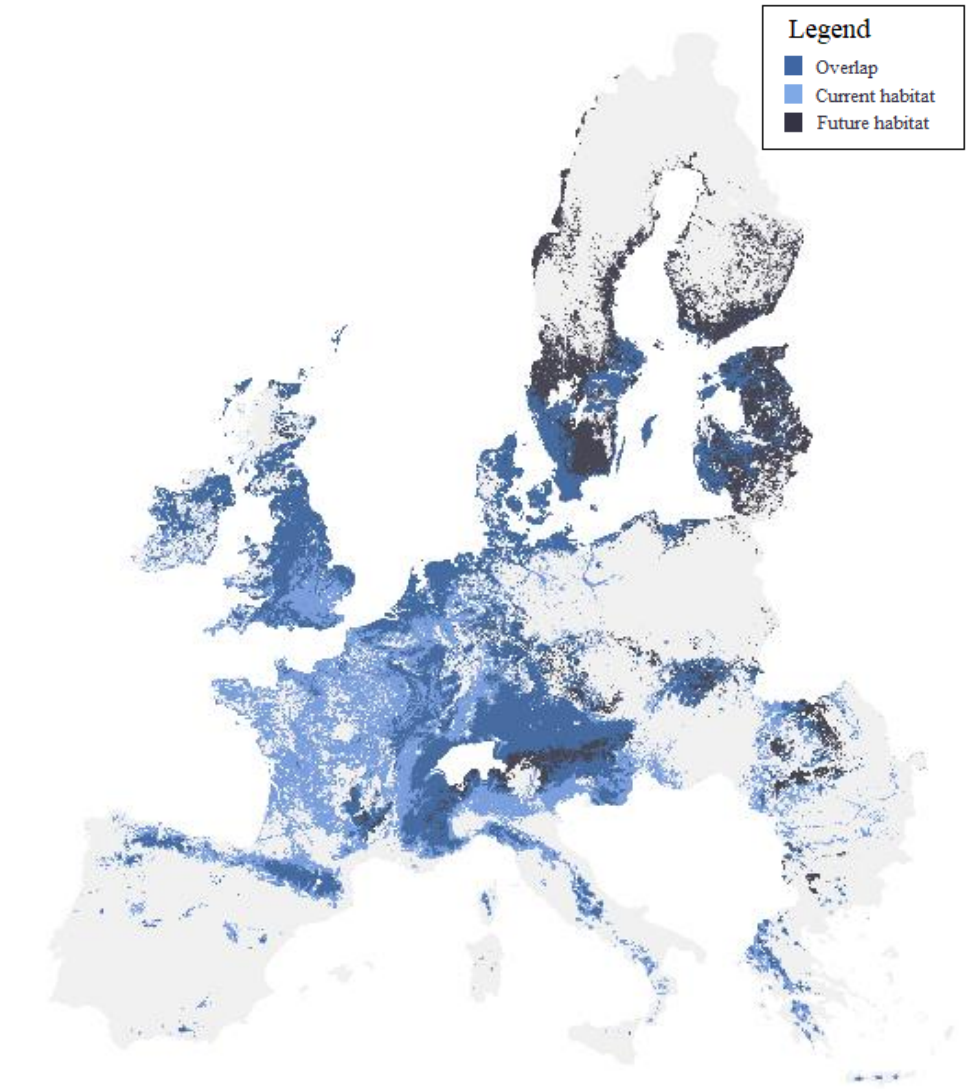


Figure 4. Habitat distribution of *Epipactis palustris* estimated for the current climate (light blue) and 2061-2080 (black) with moderate climate change mitigation modelled by the Shared Socioeconomic Pathway SSP2.45. While some new areas in northern Europe will become available with increased temperatures, the overall range of habitat suitable for the species will significantly reduce in future climate projections.

Epipactis species may also have the option of moving upwards instead of northwards in response to climate change, which may be especially useful for species unable to disperse north (Randin *et al.* 2009; Hampe *et al.* 2013; Shay *et al.* 2021). Genetic analyses in **Chapter 5** indicated that populations of *E. helleborine* tracked cooler habitats to higher elevation in the mountains of Greece during postglacial climate amelioration; as the temperature in southern Europe increases, these cooler mountain habitats act as contemporary climate refugia (Ashcroft 2010; Keppel *et al.* 2012; Hampe *et al.* 2013). However, this climate tracking to mountaintops can result in reproductive isolation (Keppel *et al.* 2012; Reisch and Rosbakh 2021), demonstrated in the genetic differentiation, lower genetic diversity, and adaptive signatures linked to local higher elevations in Greek populations. Furthermore, Italian populations showed similar genetic divergence and adaptation, but to higher local temperatures rather than elevation. Although these populations have not yet diverged enough to form species but rather local variants, similar processes of isolation due to climatic changes may have contributed to the diversification of the *Epipactis* genus, as occurred on a larger scale in the diversification of the Orchidaceae through genetic divergence occurring over elevation gradients (Givnish *et al.* 2015, 2016; Tsiftsis *et al.* 2019). Further elevation shifts and adaptation to local conditions in *Epipactis* may result in increased reproductive isolation, but only if suitable habitats occur at higher elevations to support an upward migration. Furthermore, the adaptive potential demonstrated by the Italian *E. helleborine* populations demonstrates the potential of the species not only to track a climate envelope but to adapt to higher temperatures, which would allow greater range expansion in an increasing climate (Cortés *et al.* 2020). However, considering this adaptation occurred over thousands of years during the Holocene, it is uncertain whether this adaptive potential will be relevant at the current rate of climate change.

Implications for conservation

Considering most of the *Epipactis* species studied in this thesis can occur in or on the periphery of forests, a strategy to conserve these orchids would be to conserve the forests in which they occur. Using mixed broad-leaf and coniferous forests as an umbrella for the conservation of other species may be efficient because an established forest already contains all the components necessary to sustain the species that already occur there, and it is more practical to quantify and communicate the conservation of an area rather than the many different species and processes it contains (Caro and O'Doherty 1999; Hall *et al.* 2011). As demonstrated in Chapters 4 and 5, ecological niche modelling can be used to identify suitable forests in northern Europe that will become available to orchids as increasing temperatures make habitats in the south intolerable (Pfeifer *et al.* 2006; van der Meer *et al.* 2016; Evans *et al.* 2020), although more data on the availability of mycorrhizal fungi and pollinators in these areas would be required to correctly assess whether these habitats would support *Epipactis* colonisation. Conserving these forests would provide areas for future colonisation by orchids with shrinking southern ranges through normal seed dispersal or via assisted migration, the latter of which may be necessary with the rate of climate change and deforestation (Swarts and Dixon 2009).

However, not all *Epipactis* species occur in forests, and indeed some of the most vulnerable species that are restricted to small ranges such as *E. dunensis*, would be excluded from this conservation strategy. Chapter 4 in particular indicated that *Epipactis* species can have contrasting responses to increased temperatures, even among forest species, and applying one generalised conservation tactic to the *Epipactis* genus would not be appropriate to cover the variety of distribution patterns and environmental associations among its species. While habitat generalists that are able to tolerate a wider range of habitats may be able to expand their ranges into areas that are now warm enough, even species that are relatively warm-adapted or tolerant of a range of temperatures may be constrained in other axes of their ecological niche. Furthermore, the effects of the extinction of *Epipactis* species on pollinators may differ between species depending on the degree to which pollinators rely on them, for example the extinction of the allogamous *E. atrorubens* would reduce the nectar available to its wasp pollinators, while autogamous *E. muelleri* does not have reliant pollinators (Claessens and Kleynen 2011). Therefore a

one-size-fits-all approach would not accommodate some of the more specialist species such as *E. dunensis* or *E. tremolsii*, because catering only to the most common ecologies can exclude those with more unusual niches which are often more vulnerable to extinction (Thuiller *et al.* 2005). Therefore, the conservation of *Epipactis* involves two common choices faced in conservation: protecting more species, including more generalists which would have a greater chance of success, by protecting the current and future ranges of forests, or protecting more ecologically or evolutionarily distinct species, which would require a more species-tailored approach. For these specialist species, the ideal situation would be to protect their current habitats and reduce climate change to either stay within their current climate tolerance or slowing it down to a rate to which they could adapt, as their strong associations with specific local habitats reduces the relocation success of specialists compared to generalists (Thuiller *et al.* 2005; Swarts and Dixon 2009).

Conclusion

The paradox of orchid ecology is reflected in the orchid genus *Epipactis* in the variety of niche breadths, distributions, environmental associations and mycorrhizal partners demonstrated by its species in this investigation. This was likely supported by the high seed dispersal capabilities of the genus, and shaped by reliance on mycorrhizal fungi and pollinators, periods of reproductive isolation, and adaptation to local habitat conditions. A spectrum of habitat association is reflected in the genus, from the dune specialist *E. dunensis* to habitat generalist *E. helleborine*. The distributions of habitat specialists are likely limited by smaller numbers of mycorrhizal fungal partners, while the variety of environments occupied by habitat generalists is likely promoted by association with a higher number of widespread fungal partners. However, the complexity of species-environment associations has led to interesting patterns of restriction in habitat generalists on certain niche axes and distinct fungal communities in fungal generalists. The relevance of this is most apparent when distribution changes over time are assessed, as is demonstrated in the predicted future decrease in suitable habitat in the currently widespread species *E. palustris*. Such results raise the importance of understanding how niche and distribution metrics are defined, and factors such as habitat availability in the landscape, sampling effort and spatial resolution must be taken into account when interpreting and reporting results. Future research including functional roles of fungal

partners in the ecology of adult *Epipactis* species and using different spatial resolutions and biotic interactions when modelling habitat distributions will greatly enhance our understanding of *Epipactis* distribution and divergence.

References

Aavik T, Träger S, Zobel M, *et al.* 2021. The joint effect of host plant genetic diversity and arbuscular mycorrhizal fungal communities on restoration success. *Functional Ecology* **35**: 2621–2634.

Acharya KP, Vetaas OR, Birks HJB. **2011**. Orchid species richness along Himalayan elevational gradients. *Journal of Biogeography* **38**: 1821–1833.

Afkhami ME, McIntyre PJ, Strauss SY. **2014**. Mutualist-mediated effects on species' range limits across large geographic scales. *Ecology Letters* **17**: 1265–1273.

Alarcón D, Cavieres LA. **2018**. Relationships between ecological niche and expected shifts in elevation and latitude due to climate change in South American temperate forest plants. *Journal of Biogeography* **45**: 2272–2287.

Alexander JM, Atwater DZ, Colautti RI, Hargreaves AL. **2022**. Effects of species interactions on the potential for evolution at species' range limits. *Philosophical Transactions of the Royal Society B: Biological Sciences* **377**: 20210020.

Amatulli G, Domisch S, Tuanmu M-N, *et al.* 2018. A suite of global, cross-scale topographic variables for environmental and biodiversity modeling. *Scientific Data* **5**: 1–15.

Anacker BL, Strauss SY. **2014**. The geography and ecology of plant speciation: range overlap and niche divergence in sister species. *Proceedings of the Royal Society B: Biological Sciences* **281**: 20132980.

Arditti J, Ghani AKA. **2000**. Tansley Review No. 110. Numerical and physical properties of orchid seeds and their biological implications. *The New Phytologist* **145**: 367–421.

Ash JD, Givnish TJ, Waller DM. **2017**. Tracking lags in historical plant species' shifts in relation to regional climate change. *Global Change Biology* **23**: 1305–1315.

Ashcroft MB. **2010**. Identifying refugia from climate change. *Journal of Biogeography* **37**: 1407–1413.

Bai Y, Wei X, Li X. **2018**. Distributional dynamics of a vulnerable species in response to past and future climate change: a window for conservation prospects. *PeerJ* **6**: e4287.

Baker HG. **1955**. Self-compatibility and establishment after '"long-distance" dispersal. *Evolution* **9**: 347–349.

Ballabio C, Lugato E, Fernández-Ugalde O, *et al.* 2019. Mapping LUCAS topsoil chemical properties at European scale using Gaussian process regression. *Geoderma* **355**: 113912.

Barbet-Massin M, Jiguet F, Albert CH, Thuiller W. **2012**. Selecting pseudo-absences for species distribution models: how, where and how many? *Methods in ecology and evolution* **3**: 327–338.

Barthlott W, Große-Veldmann B, Korotkova N. **2014**. *Orchid seed diversity: A scanning electron microscopy survey*.

Bateman RM. **2020**. Implications of next-generation sequencing for the systematics and evolution of the terrestrial orchid genus *Epipactis*, with particular reference to the British Isles. *Kew Bulletin* **75**: 1–22.

Batstone RT, Carscadden KA, Afkhami ME, Frederickson ME. **2018**. Using niche breadth theory to explain generalization in mutualisms. *Ecology* **99**: 1039–1050.

Benning JW, Moeller DA. **2019**. Maladaptation beyond a geographic range limit driven by antagonistic and mutualistic biotic interactions across an abiotic gradient. *Evolution* **73**: 2044–2059.

Benning JW, Moeller DA. **2021**. Microbes, mutualism, and range margins: testing the fitness consequences of soil microbial communities across and beyond a native plant's range. *New Phytologist* **229**: 2886–2900.

Bernardos S, Tyteca D, Revuelta JL, Amich F. **2004**. A new endemic species of *Epipactis* (Orchidaceae) from north-east Portugal. *Botanical Journal of the Linnean Society* **145**: 239–249.

Bhagwat SA, Willis KJ. **2008**. Species persistence in northerly glacial refugia of Europe: a matter of chance or biogeographical traits? *Journal of Biogeography* **35**: 464–482.

Bidartondo MI, Burghardt B, Gebauer G, Bruns TD, Read DJ. **2004**. Changing partners in the dark: isotopic and molecular evidence of ectomycorrhizal liaisons between forest orchids and trees. *Proceedings of the Royal Society of London. Series B: Biological Sciences* **271**: 1799–1806.

Bidartondo MI, Read DJ. **2008**. Fungal specificity bottlenecks during orchid germination and development. *Molecular Ecology* **17**: 3707–3716.

Blinova I. **2008**. Populations of orchids at the northern limit of their distribution (Murmansk Oblast): Effect of climate. *Russian Journal of Ecology* **39**: 26.

Bonnardeaux Y, Brundrett M, Batty A, Dixon K, Koch J, Sivasithamparam K. **2007**. Diversity of mycorrhizal fungi of terrestrial orchids: compatibility webs, brief encounters, lasting relationships and alien invasions. *Mycological research* **111**: 51–61.

Boria RA, Olson LE, Goodman SM, Anderson RP. **2014**. Spatial filtering to reduce sampling bias can improve the performance of ecological niche models. *Ecological Modelling* **275**: 73–77.

Boulangeat I, Gravel D, Thuiller W. **2012**. Accounting for dispersal and biotic interactions to disentangle the drivers of species distributions and their abundances. *Ecology Letters* **15**: 584–593.

Bowles M, Zettler L, Bell T, Kelsey P. **2005**. Relationships between soil characteristics, distribution and restoration potential of the federal threatened eastern prairie fringed orchid, *Platanthera leucophaea* (Nutt.) Lindl. *The American Midland Naturalist* **154**: 273–286.

Bradburd GS, Ralph PL, Coop GM. **2016**. A spatial framework for understanding population structure and admixture. *PLoS genetics* **12**: e1005703.

Bradbury PJ, Zhang Z, Kroon DE, Casstevens TM, Ramdoss Y, Buckler ES. **2007**. TASSEL: software for association mapping of complex traits in diverse samples. *Bioinformatics* **23**: 2633–2635.

Bradley BA, Blumenthal DM, Wilcove DS, Ziska LH. **2010**. Predicting plant invasions in an era of global change. *Trends in Ecology & Evolution* **25**: 310–318.

Bremer P, Jongejans E. **2009**. Frost and forest stand effects on the population dynamics of *Asplenium scolopendrium*. *Population Ecology* **52**: 211.

Broennimann O, Thuiller W, Hughes G, Midgley GF, Alkemade JMRobert, Guisan A. **2006**. Do geographic distribution, niche property and life form explain plants' vulnerability to global change? *Global Change Biology* **12**: 1079–1093.

Bronstein JL, Armbruster WS, Thompson JN. **2014**. Understanding evolution and the complexity of species interactions using orchids as a model system. *The New Phytologist* **202**: 373–375.

Brown JH, Stevens GC, Kaufman DM. **1996**. The geographic range: size, shape, boundaries, and internal structure. *Annual Review of Ecology and Systematics* **27**: 597–623.

Bruno JF, Stachowicz JJ, Bertness MD. **2003**. Inclusion of facilitation into ecological theory. *Trends in ecology & evolution* **18**: 119–125.

Brys R, Broeck AV, Mergeay J, Jacquemyn H. **2014**. The contribution of mating system variation to reproductive isolation in two closely related *Centaurium* species (Gentianaceae) with a generalized flower morphology. *Evolution* **68**: 1281–1293.

Brys R, Jacquemyn H. **2016**. Severe outbreeding and inbreeding depression maintain mating system differentiation in *Epipactis* (Orchidaceae). *Journal of evolutionary biology* **29**: 352–359.

Brys R, Van Cauwenberghe J, Jacquemyn H. **2016**. The importance of autonomous selfing in preventing hybridization in three closely related plant species. *Journal of Ecology* **104**: 601–610.

Bueno de Mesquita CP, Sartwell SA, Schmidt SK, Suding KN. **2020**. Growing-season length and soil microbes influence the performance of a generalist bunchgrass beyond its current range. *Ecology* **101**: e03095.

Bunch WD, Cowden CC, Wurzburger N, Shefferson RP. **2013**. Geography and soil chemistry drive the distribution of fungal associations in lady's slipper orchid, *Cypripedium acaule*. *Botany* **91**: 850–856.

Burgman MA, Fox JC. **2003**. Bias in species range estimates from minimum convex polygons: implications for conservation and options for improved planning In: Cambridge University Press, 19–28.

Cameron EK, Martins IS, Lavelle P, *et al.* 2018. Global gaps in soil biodiversity data. *Nature Ecology & Evolution* **2**: 1042–1043.

Caro TM, O'Doherty G. **1999**. On the Use of Surrogate Species in Conservation Biology. *Conservation Biology* **13**: 805–814.

Carvalho YGS, Vitorino LC, Souza UJB de, Bessa LA. **2019**. Recent Trends in Research on the Genetic Diversity of Plants: Implications for Conservation. *Diversity* **11**: 62.

Catling P M. **1990**. Auto-pollination in the Orchidaceae. *Orchid Biology. Review and Perspectives. V.*: 123–158.

Chen I-C, Hill JK, Ohlemuller R, Roy DB, Thomas CD. **2011**. Rapid Range Shifts of Species Associated with High Levels of Climate Warming. *Science* **333**: 1024–1026.

Claessens J, Kleynen J. **2011**. *The flower of the European orchid: form and function*. Jean Claessens & Jacques Kleynen Voerendaal.

Cole JR, Wang Q, Fish JA, *et al.* 2014. Ribosomal Database Project: data and tools for high throughput rRNA analysis. *Nucleic acids research* **42**: D633–D642.

Comes HP, Kadereit JW. **1998**. The effect of Quaternary climatic changes on plant distribution and evolution. *Trends in Plant Science* **3**: 432–438.

Cortés AJ, López-Hernández F, Osorio-Rodriguez D. **2020**. Predicting Thermal Adaptation by Looking Into Populations' Genomic Past. *Frontiers in Genetics* **11**.

Crespo MB, Lowe MR, Piera J. **2001**. *Epipactis kleinii*, a new name to replace the illegitimate *E. parviflora* (A. & C. Niesch.) E. Klein, non (Blume) A.A. Eaton (Orchidaceae, Neottieae). *TAXON* **50**: 853–855.

Csardi MG. **2013**. Package 'igraph.' *Last accessed* **3**: 2013.

Darwin C. **1877**. *The various contrivances by which orchids are fertilised by insects*. John Murray.

David AS, Quintana-Ascencio PF, Menges ES, Thapa-Magar KB, Afkhami ME, Searcy CA. **2019**. Soil microbiomes underlie population persistence of an endangered plant species. *The American Naturalist* **194**: 488–494.

De Frenne P, Rodríguez-Sánchez F, Coomes DA, *et al.* 2013. Microclimate moderates plant responses to macroclimate warming. *Proceedings of the National Academy of Sciences* **110**: 18561–18565.

De Kort H, Vandepitte K, Bruun HH, Closset-Kopp D, Honnay O, Mergeay J. **2014**. Landscape genomics and a common garden trial reveal adaptive differentiation to temperature across Europe in the tree species *Alnus glutinosa*. *Molecular Ecology* **23**: 4709–4721.

Dearnaley JD, Martos F, Selosse M-A. **2012**. Orchid mycorrhizas: molecular ecology, physiology, evolution and conservation aspects In: *Fungal associations*. Springer, 207–230.

Delforge P. **2006**. *Orchids of Europe, North Africa and the Middle East*. Timber Press.

Djordjević V, Tsiftsis S. **2022**. The Role of Ecological Factors in Distribution and Abundance of Terrestrial Orchids In: Mérillon J-M, Kodja H, eds. Reference Series in Phytochemistry. *Orchids Phytochemistry, Biology and Horticulture*. Cham: Springer International Publishing, 3–72.

Djordjević V, Tsiftsis S, Lakušić D, Jovanović S, Jakovljević K, Stevanović V. **2020**. Patterns of distribution, abundance and composition of forest terrestrial orchids. *Biodiversity and Conservation*: 1–24.

Djordjević V, Tsiftsis S, Lakušić D, Jovanović S, Stevanović V. **2016b**. Factors affecting the distribution and abundance of orchids in grasslands and herbaceous wetlands. *Systematics and Biodiversity* **14**: 355–370.

Djordjević V., Tsiftsis S, LakuŠić D, Stevanović V. **2016a**. Niche analysis of orchids of serpentine and non-serpentine areas: Implications for conservation. *Plant Biosystems - An International Journal Dealing with all Aspects of Plant Biology* **150**: 710–719.

Dolédec S, Chessel D, Gimaret-Carpentier C. **2000**. Niche separation in community analysis: a new method. *Ecology* **81**: 2914–2927.

Downing JL, Liu H, McCormick MK, Arce J, Alonso D, Lopez-Perez J. **2020**. Generalized mycorrhizal interactions and fungal enemy release drive range expansion of orchids in southern Florida. *Ecosphere* **11**: e03228.

Duffy KJ, Jacquemyn H. **2019**. Climate change increases ecogeographical isolation between closely related plants. *Journal of Ecology* **107**: 167–177.

Duffy KJ, Waud M, Schatz B, Petanidou T, Jacquemyn H. **2019**. Latitudinal variation in mycorrhizal diversity associated with a European orchid. *Journal of Biogeography* **46**: 968–980.

Edelsbrunner H, Kirkpatrick D, Seidel R. **1983**. On the shape of a set of points in the plane. *IEEE Transactions on Information Theory* **29**: 551–559.

Edgar RC. **2010**. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* **26**: 2460–2461.

Efimov P. **2008**. Notes on *Epipactis condensata*, *E. rechingeri* and *E. purpurata* (Orchidaceae) in the Caucasus and Crimea. *Willdenowia* **38**: 71–80.

Ehlers BK, Pedersen HÆ. **2000**. Genetic variation in three species of *Epipactis* (Orchidaceae): geographic scale and evolutionary inferences. *Biological Journal of the Linnean Society* **69**: 411–430.

Ehrlén J, Eriksson O. **2000**. Dispersal Limitation and Patch Occupancy in Forest Herbs. *Ecology* **81**: 1667–1674.

Elith J, Graham* CH, Anderson RP, *et al.* 2006. Novel methods improve prediction of species' distributions from occurrence data. *Ecography* **29**: 129–151.

Elith J, Phillips SJ, Hastie T, Dudík M, Chee YE, Yates CJ. **2011**. A statistical explanation of MaxEnt for ecologists. *Diversity and distributions* **17**: 43–57.

Ellstrand NC. **2014**. Is gene flow the most important evolutionary force in plants? *American Journal of Botany* **101**: 737–753.

European Environment Agency. **2017**. *Ecosystem type map (aggregated classes) — European Environment Agency*. https://www.eea.europa.eu/data-and-maps/figures/ecosystem-type-map-aggregated-classes. 14 Apr. 2023.

Evanno G, Regnaut S, Goudet J. **2005**. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular ecology* **14**: 2611–2620.

Evans A, Jacquemyn H. **2020**. Impact of mating system on range size and niche breadth in *Epipactis* (Orchidaceae). *Annals of Botany*.

Evans A, Jacquemyn H. **2022**. Range Size and Niche Breadth as Predictors of Climate-Induced Habitat Change in *Epipactis* (Orchidaceae). *Frontiers in Ecology and Evolution* **10**.

Evans A, Janssens S, Jacquemyn H. **2020**. Impact of Climate Change on the Distribution of Four Closely Related Orchis (Orchidaceae) Species. *Diversity* **12**: 312.

Evans A, de Kort H, Brys R, *et al.* 2023. Historical biogeography and local adaptation explain population genetic structure in a widespread terrestrial orchid. *Annals of Botany*: mcad010.

Fei S, Kivlin SN, Domke GM, Jo I, LaRue EA, Phillips RP. **2022**. Coupling of plant and mycorrhizal fungal diversity: its occurrence, relevance, and possible implications under global change. *New Phytologist* **234**: 1960–1966.

Fekete R, Löki V, Urgyán R, *et al.* 2019. Roadside verges and cemeteries: Comparative analysis of anthropogenic orchid habitats in the Eastern Mediterranean. *Ecology and Evolution* **9**: 6655–6664.

Fekete R, Nagy T, Bódis J, *et al.* 2017. Roadside verges as habitats for endangered lizard-orchids (Himantoglossum spp.): Ecological traps or refuges? *Science of the Total Environment* **607**: 1001–1008.

Ferlian O, Goldmann K, Eisenhauer N, Tarkka MT, Buscot F, Heintz-Buschart A. 2021. Distinct effects of host and neighbour tree identity on arbuscular and ectomycorrhizal fungi along a tree diversity gradient. *ISME Communications* **1**: 1–10.

Fick SE, Hijmans RJ. 2017. WorldClim 2: new 1-km spatial resolution climate surfaces for global land areas. *International journal of climatology* **37**: 4302–4315.

Figura T, Tylová E, Jersáková J, Vohník M, Ponert J. 2021. Fungal symbionts may modulate nitrate inhibitory effect on orchid seed germination. *Mycorrhiza* **31**: 231–241.

Fisher RA. 1958. *The genetical theory of natural selection*. Рипол Классик.

Fitzpatrick SW, Reid BN. 2019. Does gene flow aggravate or alleviate maladaptation to environmental stress in small populations? *Evolutionary Applications* **12**: 1402–1416.

Flores-Tolentino M, García-Valdés R, Saénz-Romero C, Ávila-Díaz I, Paz H, Lopez-Toledo L. 2020. Distribution and conservation of species is misestimated if biotic interactions are ignored: the case of the orchid *Laelia speciosa*. *Scientific Reports* **10**: 9542.

Foll M, Gaggiotti O. 2008. A genome-scan method to identify selected loci appropriate for both dominant and codominant markers: a Bayesian perspective. *Genetics* **180**: 977–993.

Forester B. 2019. Detecting multilocus adaptation using Redundancy Analysis (RDA). *Population Genetics in R. Retrieved from https://popgen. nesce nt. org/2018-03-27_RDA_GEA. html*.

Fort H, Vázquez DP, Lan BL. 2016. Abundance and generalisation in mutualistic networks: solving the chicken-and-egg dilemma. *Ecology Letters* **19**: 4–11.

Fowler JC, Donald ML, Bronstein JL, Miller TEX. 2023. The geographic footprint of mutualism: How mutualists influence species' range limits. *Ecological Monographs* **93**: e1558.

Francis RM. 2017. pophelper: an R package and web app to analyse and visualize population structure. *Molecular ecology resources* **17**: 27–32.

François O. 2016. Running structure-like population genetic analyses with R. *R tutorials in population genetics, U. Grenoble-Alpes*: 1–9.

Franklin J, Serra-Diaz JM, Syphard AD, Regan HM. **2016**. Global change and terrestrial plant community dynamics. *Proceedings of the National Academy of Sciences* **113**: 3725–3734.

Gamisch A, Fischer GA, Comes HP. **2015**. Multiple independent origins of auto-pollination in tropical orchids (*Bulbophyllum*) in light of the hypothesis of selfing as an evolutionary dead end. *BMC Evolutionary Biology* **15**: 192.

García-Navas V, Bonnet T, Waldvogel D, Wandeler P, Camenisch G, Postma E. **2015**. Gene flow counteracts the effect of drift in a Swiss population of snow voles fluctuating in size. *Biological Conservation* **191**: 168–177.

Gargiulo R, Pironon S, Zheleznaya E, *et al.* 2019. Phylogeography and post-glacial dynamics in the clonal-sexual orchid *Cypripedium calceolus* L. *Journal of Biogeography* **46**: 526–538.

Garrison E, Marth G. **2012**. Haplotype-based variant detection from short-read sequencing. *arXiv:1207.3907 [q-bio]*.

Gaskett AC, Gallagher RV. **2018**. Orchid diversity: Spatial and climatic patterns from herbarium records. *Ecology and Evolution* **8**: 11235–11245.

Gaston KJ. **2003**. *The structure and dynamics of geographic ranges*. Oxford University Press on Demand.

Gaudinier A, Blackman BK. **2020**. Evolutionary processes from the perspective of flowering time diversity. *New Phytologist* **225**: 1883–1898.

GBIF. **2020**. *GBIF*. https://www.gbif.org/en/. 22 Jun. 2020.

Gebauer G, Meyer M. **2003**. 15N and 13C natural abundance of autotrophic and myco-heterotrophic orchids provides insight into nitrogen and carbon gain from fungal association. *New Phytologist* **160**: 209–223.

Geber MA. **2008**. To the edge: studies of species' range limits. *New Phytologist* **178**: 228–230.

Geppert C, Perazza G, Wilson RJ, *et al.* 2020. Consistent population declines but idiosyncratic range shifts in Alpine orchids under global change. *Nature communications* **11**: 1–11.

Gévaudan A, Lewin J-M, Delforge P. **2001**. Contribution à la connaissance du groupe d'*Epipactis phyllanthes*: délimitation, écologie et distribution d'*Epipactis fageticola*.

Giesecke T. **2013**. Changing Plant Distributions and Abundances In: *Encyclopedia of Quaternary Science - 2nd Edition*. Amsterdam: Elsevier, 854–860.

Giesemann P, Eichenberg D, Stöckel M, Seifert L, Gomes S, Merckx V, Gebauer G. 2020. Dark septate endophytes and arbuscular mycorrhizal fungi (Paris-morphotype) affect

the stable isotope composition of 'classically' non-mycorrhizal plants. *Functional Ecology* **34:** 2453-2466.

Giesemann P, Rasmussen H, Gebauer G. 2021. Partial mycoheterotrophy is common among chlorophyllous plants with Paris-type arbuscular mycorrhiza. *Annals of Botany* **127**: 645–653.

Givnish TJ. **2010**. Ecology of plant speciation. *TAXON* **59**: 1326–1366.

Givnish TJ, Spalink D, Ames M, *et al.* 2015. Orchid phylogenomics and multiple drivers of their extraordinary diversification. *Proceedings of the Royal Society B: Biological Sciences* **282**: 20151553.

Givnish TJ, Spalink D, Ames M, *et al.* 2016. Orchid historical biogeography, diversification, Antarctica and the paradox of orchid dispersal. *Journal of Biogeography* **43**: 1905–1916.

Gonneau C, Jersáková J, de Tredern E, *et al.* 2014. Photosynthesis in perennial mixotrophic *Epipactis* spp. (Orchidaceae) contributes more to shoot and fruit biomass than to hypogeous survival. *Journal of Ecology* **102**: 1183–1194.

González-Chávez Ma del CA, Torres-Cruz TJ, Sánchez SA, Carrillo-González R, Carrillo-López LM, Porras-Alfaro A. **2018**. Microscopic characterization of orchid mycorrhizal fungi: Scleroderma as a putative novel orchid mycorrhizal fungus of *Vanilla* in different crop systems. *Mycorrhiza* **28**: 147–157.

Goodwillie C, Kalisz S, Eckert CG. **2005**. The evolutionary enigma of mixed mating systems in plants: occurrence, theoretical explanations, and empirical evidence. *Annu. Rev. Ecol. Evol. Syst.* **36**: 47–79.

Goudet J. **2005**. Hierfstat, a package for R to compute and test hierarchical F-statistics. *Molecular Ecology Notes* **5**: 184–186.

Grant A, Kalisz S. **2019**. Do selfing species have greater niche breadth? Support from ecological niche modeling. *Evolution*.

Grossenbacher D, Briscoe Runquist R, Goldberg EE, Brandvain Y. **2015**. Geographic range size is predicted by plant mating system. *Ecology letters* **18**: 706–713.

Grossenbacher DL, Whittall JB. **2011**. Increased Floral Divergence in Sympatric Monkeyflowers. *Evolution* **65**: 2712–2718.

Guerra CA, Heintz-Buschart A, Sikorski J, *et al.* 2020. Blind spots in global soil biodiversity and ecosystem function research. *Nature Communications* **11**: 3870.

Guisan A, Thuiller W. **2005**. Predicting species distribution: offering more than simple habitat models. *Ecology Letters* **8**: 993–1009.

Gustafsson S, Lönn M. 2003. Genetic differentiation and habitat preference of flowering-time variants within *Gymnadenia conopsea*. *Heredity* **91**: 284–292.

Habel JC, Schmitt T. 2012. The burden of genetic diversity. *Biological Conservation* **147**: 270–274.

Hagsater E, Dumont V, Pridgeon AM. 1996. *Orchids: status survey and conservation action plan*. IUCN.

Hall CM, James M, Baird T. 2011. Forests and trees as charismatic mega-flora: implications for heritage tourism and conservation. *Journal of Heritage Tourism* **6**: 309–323.

Hämälä T, Savolainen O. 2019. Genomic Patterns of Local Adaptation under Gene Flow in *Arabidopsis lyrata*. *Molecular Biology and Evolution* **36**: 2557–2571.

Hampe A, Rodríguez-Sánchez F, Dobrowski S, Hu FS, Gavin DG. 2013. Climate refugia: from the Last Glacial Maximum to the twenty-first century. *New Phytologist* **197**: 16–18.

Hamrick JL, Godt MW. 1996. Effects of life history traits on genetic diversity in plant species. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences* **351**: 1291–1298.

Heath KD, Stinchcombe JR. 2014. Explaining mutualism variation: a new evolutionary paradox? *Evolution* **68**: 309–317.

van der Heijden MGA, Martin FM, Selosse M-A, Sanders IR. 2015. Mycorrhizal ecology and evolution: the past, the present, and the future. *New Phytologist* **205**: 1406–1423.

Helsen K, Jacquemyn H, Honnay O. 2015. Hidden founder effects: small-scale spatial genetic structure in recently established populations of the grassland specialist plant *Anthyllis vulneraria*. *Molecular Ecology* **24**: 2715–2728.

Hemrová L, Kotilínek M, Konečná M, *et al.* 2019. Identification of drivers of landscape distribution of forest orchids using germination experiment and species distribution models. *Oecologia* **190**: 411–423.

Hewitt GM. 1999. Post-glacial re-colonization of European biota. *Biological journal of the Linnean Society* **68**: 87–112.

Hewitt G. 2000. The genetic legacy of the Quaternary ice ages. *Nature* **405**: 907–913.

Hewitt GM. 2004. Genetic consequences of climatic oscillations in the Quaternary. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences* **359**: 183–195.

Heymann Y. **1994**. *CORINE land cover: Technical guide*. Office for Official Publ. of the Europ. Communities.

Hiederer R. **2013**. Mapping soil properties for Europe—spatial representation of soil database attributes. *Publications Office of the European Union, EUR26082EN Scientific and Technical Research series. Luxembourg*.

Hijmans RJ, Cameron SE, Parra JL, Jones PG, Jarvis A. **2005**. Very high resolution interpolated climate surfaces for global land areas. *International Journal of Climatology: A Journal of the Royal Meteorological Society* **25**: 1965–1978.

Hijmans R, Cameron S, Parra J, Jones P, Jarvis A, Richardson K. **2005**. WorldClim, version 1.3. *University of California, Berkeley*.

Hijmans RJ, Van Etten J, Cheng J, *et al.* 2015. Package 'raster.' *R package*.

Hollick PS, McComb JA, Dixon KW. **2007**. Introduction, growth and persistence in situ of orchid mycorrhizal fungi. *Australian Journal of Botany* **55**: 665–672.

Hollingsworth P, Squirrell J, Hollingsworth M, Richards A, Bateman R. **2006**. Taxonomic complexity, conservation and recurrent origins of self-pollination in *Epipactis* (Orchidaceae). *Current taxonomic research on the British and European flora. London: Botanical Society of the British Isles*: 27–44.

Holt RD, Gaines MS. **1992**. Analysis of adaptation in heterogeneous landscapes: implications for the evolution of fundamental niches. *Evolutionary Ecology* **6**: 433–447.

Honnay O, Jacquemyn H. **2007**. Susceptibility of common and rare plant species to the genetic consequences of habitat fragmentation. *Conservation Biology* **21**: 823–831.

Hrivnák M, Slezák M, Galvánek D, *et al.* 2020. Species Richness, Ecology, and Prediction of Orchids in Central Europe: Local-Scale Study. *Diversity* **12**: 154.

Hurley C, Hurley MC. **2019**. Package 'gclus.' *R package version* **1**: 26.

Hutchison DW, Templeton AR. **1999**. Correlation of pairwise genetic and geographic distance measures: inferring the relative influences of gene flow and drift on the distribution of genetic variability. *Evolution* **53**: 1898–1914.

Hynson NA, Schiebold JM-I, Gebauer G. **2016**. Plant family identity distinguishes patterns of carbon and nitrogen stable isotope abundance and nitrogen concentration in mycoheterotrophic plants associated with ectomycorrhizal fungi. *Annals of Botany* **118**: 467–479.

Ilves A, Metsare M, Tali K, Kull T. **2015**. The impact of recent colonization on the genetic diversity and fine-scale genetic structure in *Orchis militaris* (L.). *Plant Systematics and Evolution* **301**: 1875–1886.

Jaccard P. **1908**. Nouvelles recherches sur la distribution florale. *Bull. Soc. Vaud. Sci. Nat.* **44**: 223–270.

Jacquemyn H, Brys R, Hutchings MJ. **2014**. Biological Flora of the British Isles: *Epipactis palustris*. *Journal of Ecology* **102**: 1341–1355.

Jacquemyn H, Brys R, Merckx VS, Waud M, Lievens B, Wiegand T. **2014**. Coexisting orchid species have distinct mycorrhizal communities and display strong spatial segregation. *New Phytologist* **202**: 616–627.

Jacquemyn H, Brys R, Waud M, Busschaert P, Lievens B. **2015**. Mycorrhizal networks and coexistence in species-rich orchid communities. *New Phytologist* **206**: 1127–1134.

Jacquemyn H, Brys R, Waud M, Evans A, Figura T, Selosse M-A. **2021**. Mycorrhizal Communities and Isotope Signatures in Two Partially Mycoheterotrophic Orchids. *Frontiers in Plant Science* **12**.

Jacquemyn H, De Kort H, Vanden Broeck A, Brys R. **2020**. Low genetic divergence and variation in coastal dune populations of the widespread terrestrial orchid *Epipactis helleborine*. *Botanical Journal of the Linnean Society* **193**: 419–430.

Jacquemyn H, Duffy KJ, Selosse M-A. **2017**. Biogeography of orchid mycorrhizas. *Biogeography of mycorrhizal symbiosis*: 159–177.

Jacquemyn H, Kort HD, Broeck AV, Brys R. **2018**. Immigrant and extrinsic hybrid seed inviability contribute to reproductive isolation between forest and dune ecotypes of *Epipactis helleborine* (Orchidaceae). *Oikos* **127**: 73–84.

Jacquemyn H, Merckx VSFT. **2019**. Mycorrhizal symbioses and the evolution of trophic modes in plants (R Shefferson, Ed.). *Journal of Ecology* **107**: 1567–1581.

Jacquemyn H, Waud M, Brys R, *et al.* 2017. Mycorrhizal Associations and Trophic Modes in Coexisting Orchids: An Ecological Continuum between Auto- and Mixotrophy. *Frontiers in Plant Science* **8**.

Jacquemyn H, Waud M, Lievens B, Brys R. **2016**. Differences in mycorrhizal communities between *Epipactis palustris*, *E. helleborine* and its presumed sister species *E. neerlandica*. *Annals of Botany* **118**: 105–114.

Jakobsson M, Rosenberg NA. **2007**. CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. *Bioinformatics* **23**: 1801–1806.

Jakovljević K, Mišljenović T, Djordjević V, *et al.* 2023. Elemental and ecophysiological profiles of orchid Dactylorhiza sambucina show distinct responses to contrasting geological substrates. *Flora* **303**: 152276.

Janečková P, Wotavová K, Schödelbauerová I, Jersáková J, Kindlmann P. **2006**. Relative effects of management and environmental conditions on performance and survival

of populations of a terrestrial orchid, Dactylorhiza majalis. *Biological conservation* **129**: 40–49.

Jansson J.K, Hofmockel K.S. 2020. Soil microbiomes and climate change. *Nat Rev Microbiol* **18**: 35–46

Jersáková J, Malinová T. **2007**. Spatial aspects of seed dispersal and seedling recruitment in orchids. *New Phytologist* **176**: 237–241.

Johnson AL, Govindarajulu R, Ashman T-L. **2014**. Bioclimatic evaluation of geographical range in *Fragaria* (Rosaceae): consequences of variation in breeding system, ploidy and species age: Bioclimatic Evaluation of Range in *Fragaria*. *Botanical Journal of the Linnean Society* **176**: 99–114.

Jump AS, Mátyás C, Peñuelas J. **2009**. The altitude-for-latitude disparity in the range retractions of woody species. *Trends in Ecology & Evolution* **24**: 694–701.

Kambach S, Lenoir J, Decocq G, *et al.* 2019. Of niches and distributions: range size increases with niche breadth both globally and regionally but regional estimates poorly relate to global estimates. *Ecography* **42**: 467–477.

Kelly AE, Goulden ML. **2008**. Rapid shifts in plant distribution with recent climate change. *Proceedings of the National Academy of Sciences* **105**: 11823–11826.

Keppel G, Niel KPV, Wardell-Johnson GW, *et al.* 2012. Refugia: identifying and understanding safe havens for biodiversity under climate change. *Global Ecology and Biogeography* **21**: 393–404.

Kirk H, Freeland JR. **2011**. Applications and Implications of Neutral versus Non-neutral Markers in Molecular Ecology. *International Journal of Molecular Sciences* **12**: 3966–3988.

Kolář F, Fuxová G, Záveská E, *et al.* 2016. Northern glacial refugia and altitudinal niche divergence shape genome-wide differentiation in the emerging plant model *Arabidopsis arenosa*. *Molecular ecology* **25**: 3929–3949.

Kolde R, Kolde MR. **2015**. Package 'pheatmap.' *R Package* **1**.

Kõljalg U, Nilsson RH, Abarenkov K, *et al.* 2013. Towards a unified paradigm for sequence-based identification of fungi. *Molecular Ecology* **22**: 5271–5277.

Kotilínek M, Těšitelová T, Košnar J, *et al.* 2020. Seed dispersal and realized gene flow of two forest orchids in a fragmented landscape. *Plant Biology* **22**: 522–532.

Kull T, Selgis U, Pecina MV, *et al.* 2016. Factors influencing IUCN threat levels to orchids across Europe on the basis of national red lists. *Ecology and Evolution* **6**: 6245–6265.

Lafontaine G de, Napier JD, Petit RJ, Hu FS. **2018**. Invoking adaptation to decipher the genetic legacy of past climate change. *Ecology* **99**: 1530–1546.

Lal. 2013. Soil carbon management and climate change. *Carbon Management* **4**: 439–462.

Landi M, Frignani F, Lazzeri C, Angiolini C. **2009**. Abundance of orchids on calcareous grasslands in relation to community species, environmental, and vegetational conditions. *Russian Journal of Ecology* **40**: 486–494.

Langmead B, Salzberg SL. **2012**. Fast gapped-read alignment with Bowtie 2. *Nature Methods* **9**: 357–359.

Lehikoinen A, Virkkala R. **2016**. North by north-west: Climate change and directions of density shifts in birds. *Global Change Biology* **22**: 1121–1129.

Lesica P, Yurkewycz R, Crone EE. **2006**. Rare plants are common where you find them. *American Journal of Botany* **93**: 454–459.

Leutner B, Horning N, Schwalb-Willmann J, Hijmans R. **2017**. RStoolbox: tools for remote sensing data analysis. *R package version 0.1* **8**.

Levin DA. **2010**. Environment-enhanced self-fertilization: implications for niche shifts in adjacent populations. *Journal of Ecology* **98**: 1276–1283.

Levins R. **1968**. *Evolution in changing environments: some theoretical explorations*. Princeton University Press.

Li Y-Y, Boeraeve M, Cho Y-H, Jacquemyn H, Lee Y-I. **2022**. Mycorrhizal Switching and the Role of Fungal Abundance in Seed Germination in a Fully Mycoheterotrophic Orchid, *Gastrodia confusoides*. *Frontiers in Plant Science* **12**.

Li T, Wu S, Yang W, Selosse M-A, Gao J. **2021**. How Mycorrhizal Associations Influence Orchid Distribution and Population Dynamics. *Frontiers in Plant Science* **12**.

Li T, Yang W, Wu S, Selosse M-A, Gao J. **2021**. Progress and Prospects of Mycorrhizal Fungal Diversity in Orchids. *Frontiers in Plant Science* **12**.

Light MH, MacConaill M. **1998**. Factors affecting germinable seed yield in *Cypripedium calceolus* var. pubescens (Willd.) Correll and *Epipactis helleborine* (L.) Crantz (Orchidaceae). *Botanical Journal of the Linnean Society* **126**: 3–26.

Lischer HEL, Excoffier L. **2012**. PGDSpider: an automated data conversion tool for connecting population genetics and genomics programs. *Bioinformatics* **28**: 298–299.

Loveless MD, Hamrick JL. **1984**. Ecological Determinants of Genetic Structure in Plant Populations. *Annual Review of Ecology and Systematics* **15**: 33.

Luikart G, England PR, Tallmon D, Jordan S, Taberlet P. **2003**. The power and promise of population genomics: from genotyping to genome typing. *Nature reviews genetics* **4**: 981–994.

Luu K, Bazin E, Blum MGB. **2017**. pcadapt: an R package to perform genome scans for selection based on principal component analysis. *Molecular Ecology Resources* **17**: 67–77.

Maechler M, Rousseeuw P, Struyf A, Hubert M, Hornik K. **2012**. Cluster: cluster analysis basics and extensions. *R package version* **1**: 56.

Marvier M, Kareiva P, Neubert MG. **2004**. Habitat Destruction, Fragmentation, and Disturbance Promote Invasion by Habitat Generalists in a Multispecies Metapopulation. *Risk Analysis* **24**: 869–878.

May M, Jąkalski M, Novotná A, *et al.* 2020. Three-year pot culture of *Epipactis helleborine* reveals autotrophic survival, without mycorrhizal networks, in a mixotrophic species. *Mycorrhiza* **30**: 51–61.

McCormick MK, Jacquemyn H. **2014**. What constrains the distribution of orchid populations? *New Phytologist* **202**: 392–400.

McCormick MK, Lee Taylor D, Juhaszova K, Burnett Jr RK, Whigham DF, O'neill JP. **2012**. Limitations on orchid recruitment: not a simple picture. *Molecular Ecology* **21**: 1511–1523.

McCormick MK, Whigham DF, Canchani-Viruet A. **2018**. Mycorrhizal fungi affect orchid distribution and population dynamics. *New Phytologist* **219**: 1207–1215.

McCormick MK, Whigham DF, O'Neill J. **2004**. Mycorrhizal diversity in photosynthetic terrestrial orchids. *New Phytologist* **163**: 425–438.

McCormick MK, Whigham DF, O'Neill JP, *et al.* 2009. Abundance and distribution of *Corallorhiza odontorhiza* reflect variations in climate and ectomycorrhizae. *Ecological Monographs* **79**: 619–635.

Médail F, Diadema K. **2009**. Glacial refugia influence plant diversity patterns in the Mediterranean Basin. *Journal of Biogeography* **36**: 1333–1345.

van der Meer S, Jacquemyn H, Carey PD, Jongejans E. **2016**. Recent range expansion of a terrestrial orchid corresponds with climate-driven variation in its population dynamics. *Oecologia* **181**: 435–448.

Merow C, Smith MJ, Silander Jr JA. **2013**. A practical guide to MaxEnt for modeling species' distributions: what it does, and why inputs and settings matter. *Ecography* **36**: 1058–1069.

Molnár A, Sramkó G. **2012**. *Epipactis albensis* (Orchidaceae): a new species in the flora of Romania. *Biologia* **67**: 883–888.

Muir AP, Biek R, Thomas R, Mable BK. **2014**. Local adaptation with high gene flow: temperature parameters drive adaptation to altitude in the common frog (*Rana temporaria*). *Molecular Ecology* **23**: 561–574.

Nathan R, Schurr FM, Spiegel O, Steinitz O, Trakhtenbrot A, Tsoar A. **2008**. Mechanisms of long-distance seed dispersal. *Trends in Ecology & Evolution* **23**: 638–647.

Nei M, Maruyama T, Chakraborty R. **1975**. The bottleneck effect and genetic variability in populations. *Evolution*: 1–10.

Nenadic O, Greenacre M. **2007**. Correspondence analysis in R, with two-and three-dimensional graphics: the ca package. *Journal of statistical software* **20**.

Normand S, Ricklefs RE, Skov F, Bladt J, Tackenberg O, Svenning J-C. **2011**. Postglacial migration supplements climate in determining plant species ranges in Europe. *Proceedings of the Royal Society B: Biological Sciences* **278**: 3644–3653.

Nosil P, Funk DJ, Ortiz-Barrientos D. **2009**. Divergent selection and heterogeneous genomic divergence. *Molecular ecology* **18**: 375–402.

Oehl F, Schneider D, Sieverding E, Burga CA. **2011**. Succession of arbuscular mycorrhizal communities in the foreland of the retreating Morteratsch glacier in the Central Alps. *Pedobiologia* **54**: 321–331.

Ogura-Tsujita Y, Yukawa T. **2008**. *Epipactis helleborine* shows strong mycorrhizal preference towards ectomycorrhizal fungi with contrasting geographic distributions in Japan. *Mycorrhiza* **18**: 331–338.

Oksanen J, Blanchet F, Friendly M, *et al.* 2019. vegan: Community Ecology Package. R package version 2.5-6. 2019.

Ongaro S, Martellos S, Bacaro G, Agostini AD, Cogoni A, Cortis P. **2018**. Distributional pattern of Sardinian orchids under a climate change scenario. *Community Ecology* **19**: 223–232.

Orsini L, Andrew R, Eizaguirre C. **2013**. Evolutionary ecological genomics. *Molecular Ecology* **22**: 527–31.

Otero JT, Flanagan NS. **2006**. Orchid diversity – beyond deception. *Trends in Ecology & Evolution* **21**: 64–65.

Otto-Bliesner BL, Marshall SJ, Overpeck JT, Miller GH, Hu A. **2006**. Simulating Arctic climate warmth and icefield retreat in the last interglaciation. *science* **311**: 1751–1753.

Parisod C. **2022**. Plant speciation in the face of recurrent climate changes in the Alps. *Alpine Botany* **132**: 21–28.

Park DS, Ellison AM, Davis CC. 2018. Mating system does not predict niche breath. *Global Ecology and Biogeography* **27**: 804–813.

Pärtel M, Öpik M, Moora M, *et al.* 2017. Historical biome distribution and recent human disturbance shape the diversity of arbuscular mycorrhizal fungi. *New Phytologist* **216**: 227–238.

Paul JR, Sheth SN, Angert AL. 2011. Quantifying the impact of gene flow on phenotype-environment mismatch: a demonstration with the scarlet monkeyflower *Mimulus cardinalis*. *The American Naturalist* **178**: S62–S79.

Peakall R. 2007. Speciation in the Orchidaceae: confronting the challenges. *Molecular Ecology* **16**: 2834–2837.

Peakall R, Smouse P. 2012. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research—an update. *Bioinformatics* **28**: 2537.

Petanidou T, Godfree RC, Song DS, Kantsa A, Dupont YL, Waser NM. 2012. Self-compatibility and plant invasiveness: Comparing species in native and invasive ranges. *Perspectives in Plant Ecology, Evolution and Systematics* **14**: 3–12.

Peter CI. 2009. Pollination, floral deception and evolutionary processes in *Eulophia* (Orchidaceae) and its allies.

Peterson ML, Kay KM. 2015. Mating system plasticity promotes persistence and adaptation of colonizing populations of hermaphroditic angiosperms. *The American Naturalist* **185**: 28–43.

Peterson AT, Soberón J, Pearson RG, *et al.* 2011. *Ecological niches and geographic distributions (MPB-49)*. Princeton University Press.

Petraitis PS. 1979. Likelihood measures of niche breadth and overlap. *Ecology* **60**: 703–710.

Pfeifer M, Passalacqua NG, Bartram S, *et al.* 2010. Conservation priorities differ at opposing species borders of a European orchid. *Biological Conservation* **143**: 2207–2220.

Pfeifer M, Wiegand K, Heinrich W, Jetschke G. 2006. Long-term demographic fluctuations in an orchid species driven by weather: implications for conservation planning. *Journal of Applied Ecology* **43**: 313–324.

Phillips SJ. 2005. A brief tutorial on Maxent. *AT&T Research* **190**: 231–259.

Phillips SJ, Anderson RP, Dudík M, Schapire RE, Blair ME. 2017. Opening the black box: An open-source release of Maxent. *Ecography* **40**: 887–893.

Phillips RD, Dixon KW, Peakall R. 2012. Low population genetic differentiation in the Orchidaceae: implications for the diversification of the family. *Molecular Ecology* **21**: 5208–5220.

Phillips SJ, Dudík M. **2008**. Modeling of species distributions with Maxent: new extensions and a comprehensive evaluation. *Ecography* **31**: 161–175.

Phillips SJ, Dudík M, Schapire RE. **2018**. Maxent software for modeling species niches and distributions.

Phillips RD, Peakall R, Niet T van der, Johnson SD. **2020**. Niche Perspectives on Plant–Pollinator Interactions. *Trends in Plant Science* **25**: 779–793.

Pigot AL, Tobias JA. **2013**. Species interactions constrain geographic range expansion over evolutionary time. *Ecology Letters* **16**: 330–338.

POWO. **2023**. *Orchidaceae*. http://powo.science.kew.org/taxon/urn:lsid:ipni.org:names:30000046-2. 10 Mar. 2023.

QGIS Development Team. **2019**. QGIS Geographic Information System.

Qiao H, Saupe EE, Soberón J, Peterson AT, Myers CE. **2016**. Impacts of Niche Breadth and Dispersal Ability on Macroevolutionary Patterns. *The American Naturalist* **188**: 149–162.

R Core Team. **2021**. R: A language and environment for statistical computing.

Randin CF, Engler R, Normand S, *et al.* 2009. Climate change and plant distribution: local models predict high-elevation persistence. *Global Change Biology* **15**: 1557–1569.

Randle AM, Slyder JB, Kalisz S. **2009**. Can differences in autonomous selfing ability explain differences in range size among sister-taxa pairs of *Collinsia* (Plantaginaceae)? An extension of Baker's Law. *New Phytologist* **183**: 618–629.

Rasmussen H. **2008**. Terrestrial orchids: from seed to mycotrophic plant. *Terrestrial orchids: from seed to mycotrophic plant.*

Rasmussen HN, Dixon KW, Jersáková J, Těšitelová T. **2015**. Germination and seedling establishment in orchids: a complex of requirements. *Annals of Botany* **116**: 391–402.

Read DJ. **1991**. Mycorrhizas in ecosystems. *Experientia* **47**: 376–391.

Reed PB, Peterson ML, Pfeifer-Meister LE, *et al.* 2021. Climate manipulations differentially affect plant population dynamics within versus beyond northern range limits. *Journal of Ecology* **109**: 664–675.

Reisch C, Rosbakh S. **2021**. Patterns of genetic variation in European plant species depend on altitude. *Diversity and Distributions* **27**: 157–163.

Rengel Z. **2011**. Soil pH, Soil Health and Climate Change. In: Singh, B., Cowie, A., Chan, K. (eds) Soil Health and Climate Change. *Soil Biology* **29**. Springer, Berlin, Heidelberg

Rewicz A, Jaskuła R, Rewicz T, Tończyk G. 2017. Pollinator diversity and reproductive success of *Epipactis helleborine* (L.) Crantz (Orchidaceae) in anthropogenic and natural habitats. *PeerJ* **5**: e3159.

Riahi K, van Vuuren DP, Kriegler E, *et al.* 2017. The Shared Socioeconomic Pathways and their energy, land use, and greenhouse gas emissions implications: An overview. *Global Environmental Change* **42**: 153–168.

Riebesell JF. 1982. Arctic-Alpine Plants on Mountaintops: Agreement with Island Biogeography Theory. *The American Naturalist* **119**: 657–674.

Rodríguez Casal A, Pateiro López B. 2010. Generalizing the convex hull of a sample: the R package alphahull.

Sakamoto Y, Ogura-Tsujita Y, Ito K, *et al.* 2016. The tiny-leaved orchid *Cephalanthera subaphylla* obtains most of its carbon via mycoheterotrophy. *Journal of plant research* **129**: 1013–1020.

Saupe EE, Barve V, Myers CE, *et al.* 2012. Variation in niche and distribution model performance: The need for a priori assessment of key causal factors. *Ecological Modelling* **237–238**: 11–22.

Saupe EE, Qiao H, Hendricks JR, *et al.* 2015. Niche breadth and geographic range size as determinants of species survival on geological time scales. *Global Ecology and Biogeography* **24**: 1159–1169.

Scheffers BR, De Meester L, Bridge TC, *et al.* 2016. The broad footprint of climate change from genes to biomes to people. *Science* **354**.

Schiebold JM-I, Bidartondo MI, Karasch P, Gravendeel B, Gebauer G. 2017. You are what you get from your fungi: nitrogen stable isotope patterns in *Epipactis* species. *Annals of Botany* **119**: 1085–1095.

Schiebold JM, Bidartondo MI, Lenhard F, Makiola A, Gebauer G. 2018. Exploiting mycorrhizas in broad daylight: partial mycoheterotrophy is a common nutritional strategy in meadow orchids. *Journal of Ecology* **106**: 168–178.

Schmitt T, Varga Z. 2012. Extra-Mediterranean refugia: the rule and not the exception? *Frontiers in Zoology* **9**: 1–12.

Schoener TW. 1968. The *Anolis* lizards of Bimini: resource partitioning in a complex fauna. *Ecology* **49**: 704–726.

Schoener TW. 1970. Nonsynchronous spatial overlap of lizards in patchy habitats. *Ecology* **51**: 408–418.

Schönswetter P, Stehlik I, Holderegger R, Tribsch A. 2005. Molecular evidence for glacial refugia of mountain plants in the European Alps. *Molecular Ecology* **14**: 3547–3555.

Schwartz MW. **2012**. Using niche models with climate projections to inform conservation management decisions. *Biological Conservation* **155**: 149–156.

Selosse M-A, Faccio A, Scappaticci G, Bonfante P. **2004**. Chlorophyllous and Achlorophyllous Specimens of *Epipactis microphylla* (Neottieae, Orchidaceae) Are Associated with Ectomycorrhizal Septomycetes, including Truffles. *Microbial Ecology* **47**.

Selosse M-A, Martos F. **2014**. Do chlorophyllous orchids heterotrophically use mycorrhizal fungal carbon? *Trends in Plant Science* **19**: 683–685.

Selosse M-A, Roy M. **2009**. Green plants that feed on fungi: facts and questions about mixotrophy. *Trends in plant science* **14**: 64–70.

Sexton JP, Montiel J, Shay JE, Stephens MR, Slatyer RA. **2017**. Evolution of Ecological Niche Breadth. *Annual Review of Ecology, Evolution, and Systematics* **48**: 183–206.

Shay JE, Pennington LK, Montiel-Molina JAM, Toews D, Hendrickson B, Sexton J. **2021**. Rules of plant species ranges: applications for conservation strategies. *Frontiers in Ecology and Evolution*: 664.

Shefferson RP, Bunch W, Cowden CC, *et al.* 2019. Does evolutionary history determine specificity in broad ecological interactions? *Journal of Ecology* **107**: 1582–1593.

Shefferson RP, Jacquemyn H, Kull T, Hutchings MJ. **2020**. The demography of terrestrial orchids: life history, population dynamics and conservation. *Botanical Journal of the Linnean Society* **192**: 315–332.

Shefferson RP, Kull T, Tali K. **2008**. Mycorrhizal interactions of orchids colonizing Estonian mine tailings hills. *American Journal of Botany* **95**: 156–164.

Shefferson RP, Taylor DL, Weiß M, *et al.* 2007. The evolutionary history of mycorrhizal specificity among lady's slipper orchids. *Evolution* **61**: 1380–1390.

Sheth SN, Morueta-Holme N, Angert AL. **2020**. Determinants of geographic range size in plants. *New Phytologist* **226**: 650–665.

Silva DP, Macêdo ACBA, Ascher JS, De Marco P. **2015**. Range increase of a Neotropical orchid bee under future scenarios of climate change. *Journal of Insect Conservation* **19**: 901–910.

Slatkin M. **1987**. Gene flow and the geographic structure of natural populations. *Science* **236**: 787–792.

Slatyer RA, Hirst M, Sexton JP. **2013**. Niche breadth predicts geographical range size: a general ecological pattern. *Ecology Letters* **16**: 1104–1114.

Smith SE, Read DJ. **2010**. *Mycorrhizal symbiosis*. Academic press.

Soberón J. **2007**. Grinnellian and Eltonian niches and geographic distributions of species. *Ecology Letters* **10**: 1115–1123.

Soberón J, Nakamura M. **2009**. Niches and distributional areas: Concepts, methods, and assumptions. *Proceedings of the National Academy of Sciences* **106**: 19644–19650.

Squirrell J, Hollingsworth PM, Bateman RM, Tebbitt MC, Hollingsworth ML. **2002a**. Taxonomic complexity and breeding system transitions: conservation genetics of the *Epipactis leptochila* complex (Orchidaceae). *Molecular Ecology* **11**: 1957–1964.

Squirrell J., Hollingsworth PM, Bateman RM, Tebbitt MC, Hollingsworth ML. **2002b**. Taxonomic complexity and breeding system transitions: conservation genetics of the *Epipactis leptochila* complex (Orchidaceae). *Molecular Ecology* **11**: 1957–1964.

Sramkó G, Paun O, Brandrud MK, Laczkó L, Molnár A, Bateman RM. **2019**. Iterative allogamy–autogamy transitions drive actual and incipient speciation during the ongoing evolutionary radiation within the orchid genus *Epipactis* (Orchidaceae). *Annals of Botany* **124**: 481–497.

Stebbins GL. **1957**. Self fertilization and population variability in the higher plants. *The American Naturalist* **91**: 337–354.

Steinbauer MJ, Field R, Grytnes J, *et al.* 2016. Topography-driven isolation, speciation and a global increase of endemism with elevation. *Global Ecology and Biogeography* **25**: 1097–1107.

Steinbauer MJ, Grytnes J-A, Jurasinski G, *et al.* 2018. Accelerated increase in plant species richness on mountain summits is linked to warming. *Nature* **556**: 231–234.

Stevanović V, Stevanović B 1995. Basic climatic, geological and pedological factors of the diversity of terrestrial ecosystems in Yugoslavia. *Biodiversity of Yugoslavia with review of internationally important species*. 75-95.

Stewart JR, Lister AM. **2001**. Cryptic northern refugia and the origins of the modern biota. *Trends in Ecology & Evolution* **16**: 608–613.

Stinchcombe JR, Hoekstra HE. **2008**. Combining population genomics and quantitative genetics: finding the genes underlying ecologically important traits. *Heredity* **100**: 158–170.

Štípková Z, Kindlmann P. **2021**. Orchid Extinction over the Last 150 Years in the Czech Republic. *Diversity* **13**: 78.

Štípková Z, Romportl D, Černocká V, Kindlmann P. **2017**. Factors associated with the distributions of orchids in the Jeseníky Mountains, Czech Republic. *European Journal of Environmental Sciences* **7**: 135–145.

Štípková Z, Tsiftsis S, Kindlmann P. **2020**. Pollination Mechanisms are Driving Orchid Distribution in Space. *Scientific Reports* **10**: 850.

Štípková Z, Tsiftsis S, Kindlmann P. **2021**. Distribution of Orchids with Different Rooting Systems in the Czech Republic. *Plants* **10**: 632.

Suetsugu K, Yamato M, Miura C, *et al.* 2017. Comparison of green and albino individuals of the partially mycoheterotrophic orchid *Epipactis helleborine* on molecular identities of mycorrhizal fungi, nutritional modes and gene expression in mycorrhizal roots. *Molecular Ecology* **26**: 1652–1669.

Svenning J, Normand S, Skov F. **2008**. Postglacial dispersal limitation of widespread forest plant species in nemoral Europe. *Ecography* **31**: 316–326.

Swarts ND, Dixon KW. **2009**. Terrestrial orchid conservation in the age of extinction. *Annals of botany* **104**: 543–556.

Swarts ND, Sinclair EA, Francis A, Dixon KW. **2010**. Ecological specialization in mycorrhizal symbiosis leads to rarity in an endangered orchid. *Molecular Ecology* **19**: 3226–3242.

Takebayashi N, Morrell PL. **2001**. Is self-fertilization an evolutionary dead end? Revisiting an old hypothesis with genetic theories and a macroevolutionary approach. *American Journal of Botany* **88**: 1143–1150.

Tayleur C, Caplat P, Massimino D, *et al.* 2015. Swedish birds are tracking temperature but not rainfall: evidence from a decade of abundance changes. *Global Ecology and Biogeography* **24**: 859–872.

Taylor DL, Bruns TD. **1997**. Independent, specialized invasions of ectomycorrhizal mutualism by two nonphotosynthetic orchids. *Proceedings of the National Academy of Sciences* **94**: 4510–4515.

Taylor D, Bruns T, Leake J, Read D. **2003**. Mycorrhizal specificity and function in myco-heterotrophic plants. *Mycorrhizal ecology*: 375–413.

Taylor A, Keppel G, Weigelt P, Zotz G, Kreft H. **2021**. Functional traits are key to understanding orchid diversity on islands. *Ecography* **44**: 703–714.

Tedersoo L, Bahram M. **2019**. Mycorrhizal types differ in ecophysiology and alter plant nutrition and soil processes. *Biological Reviews* **94**: 1857–1880.

Tedersoo L, Bahram M, Zobel M. **2020**. How mycorrhizal associations drive plant population and community biology. *Science* **367**: eaba1223.

Telwala Y, Brook BW, Manish K, Pandit MK. **2013**. Climate-Induced Elevational Range Shifts and Increase in Plant Species Richness in a Himalayan Biodiversity Epicentre. *PLOS ONE* **8**: e57103.

Těšitelová T, Jersáková J, Říhová G, Selosse M. **2012**. Symbiotic germination capability of four *Epipactis* species (Orchidaceae) is broader than expected from adult ecology. *American Journal of Botany* **99**: 1020–1032.

The Euro+Med Plantbase Project.
http://ww2.bgbm.org/EuroPlusMed/PTaxonDetail.asp?NameCache=*Epipactis*&PTRefFk=8000000. 22 Mar. 2022.

Thompson K, Gaston KJ, Band SR. **1999**. Range size, dispersal and niche breadth in the herbaceous flora of central England. *Journal of Ecology* **87**: 150–155.

Thuiller W. **2007**. Climate change and the ecologist. *Nature* **448**: 550–552.

Thuiller W, Albert C, Araújo MB, *et al.* 2008. Predicting global change impacts on plant species' distributions: Future challenges. *Perspectives in Plant Ecology, Evolution and Systematics* **9**: 137–152.

Thuiller W, Lavorel S, Araujo MB. **2005**. Niche properties and geographical extent as predictors of species sensitivity to climate change. *Global Ecology and Biogeography* **14**: 347–357.

Toljander JF, Eberhardt U, Toljander YK, Paul LR, Taylor AF. **2006**. Species composition of an ectomycorrhizal fungal community along a local nutrient gradient in a boreal forest. *New Phytologist* **170**: 873–884.

Townsend Peterson A. **2011**. Ecological niche conservatism: A time-structured review of evidence. *Journal of Biogeography* **38**: 817–827.

Townsend Peterson A, Soberón J. **2012**. Species Distribution Modeling and Ecological Niche Modeling: Getting the Concepts Right. *Natureza & Conservação* **10**: 102–107.

Tranchida-Lombardo V, Cafasso D, Cristaudo A, Cozzolino S. **2011**. Phylogeographic patterns, genetic affinities and morphological differentiation between *Epipactis helleborine* and related lineages in a Mediterranean glacial refugium. *Annals of Botany* **107**: 427–436.

Tremblay RL, Ackerman JD, Zimmerman JK, Calvo RN. **2005**. Variation in sexual reproduction in orchids and its evolutionary consequences: a spasmodic journey to diversification. *Biological Journal of the Linnean Society* **84**: 1–54.

Tribsch A. **2004**. Areas of endemism of vascular plants in the Eastern Alps in relation to Pleistocene glaciation. *Journal of Biogeography* **31**: 747–760.

Tsiftsis S, Štípková Z, Kindlmann P. **2019**. Role of way of life, latitude, elevation and climate on the richness and distribution of orchid species. *Biodiversity and Conservation* **28**: 75–96.

Tsiftsis S, Tsiripidis I, Karagiannakidou V, Alifragis D. **2008**. Niche analysis and conservation of the orchids of east Macedonia (NE Greece). *Acta Oecologica* **33**: 27–35.

Turenne CY, Sanche SE, Hoban DJ, Karlowsky JA, Kabani AM. **1999**. Rapid identification of fungi by using the ITS2 genetic region and an automated fluorescent capillary electrophoresis system. *Journal of clinical microbiology* **37**: 1846–1851.

Tzedakis PC. **2004**. The Balkans as Prime Glacial Refugial Territory of European Temperate Trees In: Griffiths HI, Kryštufek B, Reed JM, eds. *Balkan Biodiversity: Pattern and Process in the European Hotspot*. Dordrecht: Springer Netherlands, 49–68.

Valavi R, Guillera-Arroita G, Lahoz-Monfort JJ, Elith J. **2021**. Predictive performance of presence-only species distribution models: a benchmark study with reproducible code. *Ecol. Monogr*.

Van der Putten WH, Macel M, Visser ME. **2010**. Predicting species distribution and abundance responses to climate change: why it is essential to include biotic interactions across trophic levels. *Philosophical Transactions of the Royal Society B: Biological Sciences* **365**: 2025–2034.

Van Liedekerke M, Jones A, Panagos P. **2006**. ESDBv2 Raster Library—A Set of Rasters Derived from the European Soil Database Distribution v2. 0. *European Commission and the European Soil Bureau Network, CDROM, EUR* **19945**.

Ventre Lespiaucq A, Jacquemyn H, Rasmussen HN, Méndez M. 2021. Temporal turnover in mycorrhizal interactions: a proof of concept with orchids. *New Phytologist* **230**: 1690–1699.

Vilas A, Pérez-Figueroa A, Quesada H, Caballero A. **2015**. Allelic diversity for neutral markers retains a higher adaptive potential for quantitative traits than expected heterozygosity. *Molecular Ecology* **24**: 4419–4432.

Vogt-Schilb H, Munoz F, Richard F, Schatz B. **2015**. Recent declines and range changes of orchids in Western Europe (France, Belgium and Luxembourg). *Biological Conservation* **190**: 133–141.

Vogt-Schilb H, Těšitelová T, Kotilínek M, Sucháček P, Kohout P, Jersáková J. **2020**. Altered rhizoctonia assemblages in grasslands on ex-arable land support germination of mycorrhizal generalist, not specialist orchids. *New Phytologist* **227**: 1200–1212.

Wang D, Lerou J, Nuytinck J, Gomes SI, Jacquemyn H, Merckx VS. **2022**. Root-associated Fungi in Orchidaceae: Diversity, Phylogeny, Ecology, and Outstanding Questions. *bioRxiv*: 2022–12.

Warren DL, Beaumont LJ, Dinnage R, Baumgartner JB. **2019**. New methods for measuring ENM breadth and overlap in environmental space. *Ecography* **42**: 444–446.

Warren D, Dinnage R, Matzke N. **2017**. ENMTools: analysis of niche evolution using niche and distribution models. R package (Version 0.2).

Warren DL, Glor RE, Turelli M. **2008**. Environmental Niche Equivalency Versus Conservatism: Quantitative Approaches to Niche Evolution. *Evolution* **62**: 2868–2883.

Warren DL, Glor RE, Turelli M. **2010**. ENMTools: a toolbox for comparative studies of environmental niche models. *Ecography* **33**: 607–611.

Warren DL, Matzke NJ, Cardillo M, *et al.* 2021. ENMTools 1.0: an R package for comparative ecological biogeography. *Ecography* **44**: 504–511.

Waud M, Brys R, Van Landuyt W, Lievens B, Jacquemyn H. **2017**. Mycorrhizal specificity does not limit the distribution of an endangered orchid species. *Molecular Ecology* **26**: 1687–1701.

Weiß M, Waller F, Zuccaro A, Selosse M-A. **2016**. Sebacinales – one thousand and one interactions with land plants. *New Phytologist* **211**: 20–40.

Wells T. **1991**. Demographic and biological studies on *Ophrys apifera*: some results from a 10 year study. *Population ecology of terrestrial orchids*.

White TJ, Bruns T, Lee S, Taylor J. **1990**. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR protocols: a guide to methods and applications* **18**: 315–322.

Wickham H. **2011**. ggplot2. *Wiley interdisciplinary reviews: computational statistics* **3**: 180–185.

Wickland DP, Battu G, Hudson KA *et al.* 2017. A comparison of genotyping-by-sequencing analysis methods on low-coverage crop datasets shows advantages of a new workflow, GB-eaSy. *BMC Bioinformatics* **18**: 586.

Wiens JJ, Graham CH. **2005**. Niche conservatism: integrating evolution, ecology, and conservation biology. *Annu. Rev. Ecol. Evol. Syst.* **36**: 519–539.

Williams JL, Jacquemyn H, Ochocki BM, Brys R, Miller TE. **2015**. Life history evolution under climate change and its influence on the population dynamics of a long-lived plant. *Journal of Ecology* **103**: 798–808.

Wu Z, Li X, Xie D, *et al.* 2020. Spatial genetic structuring in a widespread wetland plant on a plateau: Effects of elevation-driven geographic isolation and environmental heterogeneity. *Freshwater Biology* **65**: 1596–1607.

Xing X, Gao Y, Zhao Z, *et al.* 2020. Similarity in mycorrhizal communities associating with two widespread terrestrial orchids decays with distance. *Journal of Biogeography* **47**: 421–433.

Yu F, Groen TA, Wang T, Skidmore AK, Huang J, Ma K. **2017**. Climatic niche breadth can explain variation in geographical range size of alpine and subalpine plants. *International Journal of Geographical Information Science* **31**: 190–212.

Zellweger F, De Frenne P, Lenoir J, *et al.* 2020. Forest microclimate dynamics drive plant responses to warming. *Science* **368**: 772–775.

Zhang S-B, Chen W-Y, Huang J-L, Bi Y-F, Yang X-F. **2015a**. Orchid species richness along elevational and environmental gradients in Yunnan, China. *PLoS One* **10**: e0142621.

Zhang Z, Yan Y, Tian Y, Li J, He J-S, Tang Z. **2015b**. Distribution and conservation of orchid species richness in China. *Biological Conservation* **181**: 64–72.

Zhou T, Jin X-H. **2018**. Molecular systematics and the evolution of mycoheterotrophy of tribe Neottieae (Orchidaceae, Epidendroideae). *PhytoKeys*: 39–49.

Supplementary material

S1. GBIF accession DOIs for raw occurrence data

Species	Occurrence points		GBIF DOIs for raw occurrence data
	Before aggregation	After aggregation ($10km^2$)	
E. albensis	107	16	https//doi.org/10.15468/dl.21fhx2, 14 Jun 2019
E. atrorubens	18009	2376	https//doi.org/10.15468/dl.8kls5q, 14 Jun 2019
E. dunensis	65	15	https//doi.org/10.15468/dl.xnceaw, 13 Jan 2020
E. fageticola	241	45	https//doi.org/10.15468/dl.dtlv5o, 14 Jun 2019
E. helleborine	45354	6304	https//doi.org/10.15468/dl.aicdym, 1 Jul 2019
E. kleinii	78	46	https//doi.org/10.15468/dl.jhucjg, 14 Jun 2019
E. leptochila	1730	375	https//doi.org/10.15468/dl.o0lsxl, 14 Jun 2019
E. lusitanica	31	22	https//doi.org/10.15468/dl.zyio7o, 13 Jan 2020
E. microphylla	2509	683	https//doi.org/10.15468/dl.zrwah5, 13 Jun 2019
E. muelleri	4645	852	https//doi.org/10.15468/dl.gkcadj, 14 Jun 2019
E. palustris	20024	2152	https//doi.org/10.15468/dl.4ob1do, 13 Jun 2019
E. phyllanthes	414	85	https//doi.org/10.15468/dl.xmoewx, 14 Jun 2019
E. purpurata	2888	551	https//doi.org/10.15468/dl.4wzvzg, 14 Jun 2019
E. tremolsii	515	211	https//doi.org/10.15468/dl.spzmui, 14 Jun 2019

S2. Variable loadings of PCAs (Principal Components Analyses) performed on two sets of soil variables (biochemical and physical soil). The resulting two axes of each PCA were used as predictor variables in the ecological niche model.

Biochemical variables	PC1	PC2	Soil texture	PC1	PC2
Potassium	0.590	0.181	Clay	0.537	0.026
Nitrogen	0.094	-0.851	Course fragments	0.198	-0.640
CEC (Cation exchange capacity)	0.533	-0.169	Sand	-0.594	-0.179
Phosphorus	0.040	-0.444	Silt	0.534	0.259
pH	0.597	0.135	Bulk density (compaction)	-0.188	0.701

S3. Post-hoc pairwise species comparisons for PERMANOVA

Comparison	Sums of squares	*F*	R^2	*p*-value
E. atrorubens vs E. helleborine	1.293662	3.652447	0.119157	0.001
E. atrorubens vs E. kleinii	0.878721	2.249391	0.089087	0.001
E. atrorubens vs E. leptochila	1.086677	2.955844	0.105733	0.001
E. atrorubens vs E. microphylla	0.790406	1.99999	0.068965	0.005
E. atrorubens vs E. muelleri	0.842647	2.308955	0.087763	0.002
E. atrorubens vs E. palustris	1.109148	2.896423	0.093746	0.001
E. atrorubens vs E. purpurata	0.871089	2.630124	0.127489	0.002
E. atrorubens vs E. tremolsii	0.910546	2.354023	0.080194	0.003
E. helleborine vs E. kleinii	1.254278	3.227607	0.127939	0.001
E. helleborine vs E. leptochila	1.366732	3.746437	0.135024	0.001
E. helleborine vs E. microphylla	1.171613	2.976246	0.102713	0.001
E. helleborine vs E. muelleri	1.098879	3.036589	0.116628	0.001
E. helleborine vs E. palustris	1.488821	3.907769	0.126433	0.001
E. helleborine vs E. purpurata	0.869793	2.67579	0.135994	0.001
E. helleborine vs E. tremolsii	0.954773	2.480378	0.087091	0.001
E. kleinii vs E. leptochila	0.96378	2.357244	0.105435	0.001
E. kleinii vs E. microphylla	0.514975	1.173209	0.050628	0.267
E. kleinii vs E. muelleri	1.025033	2.514607	0.116879	0.001
E. kleinii vs E. palustris	1.007013	2.385672	0.093977	0.001
E. kleinii vs E. purpurata	1.157262	3.040623	0.189558	0.002
E. kleinii vs E. tremolsii	0.795812	1.85662	0.077824	0.011
E. leptochila vs E. microphylla	0.985029	2.396958	0.090804	0.002
E. leptochila vs E. muelleri	1.061316	2.803123	0.117763	0.001
E. leptochila vs E. palustris	1.14619	2.890184	0.103627	0.001
E. leptochila vs E. purpurata	1.070637	3.116058	0.172005	0.001
E. leptochila vs E. tremolsii	0.886569	2.208148	0.084254	0.007
E. microphylla vs E. muelleri	0.856603	2.08914	0.083269	0.003
E. microphylla vs E. palustris	0.954705	2.262309	0.077311	0.002
E. microphylla vs E. purpurata	1.004229	2.573617	0.131484	0.001
E. microphylla vs E. tremolsii	0.705715	1.650708	0.059699	0.031
E. muelleri vs E. palustris	1.056963	2.675199	0.100288	0.001
E. muelleri vs E. purpurata	0.681385	2.020361	0.126112	0.008
E. muelleri vs E. tremolsii	0.883842	2.208694	0.087616	0.002
E. palustris vs E. purpurata	1.049176	2.82495	0.135652	0.001
E. palustris vs E. tremolsii	1.158996	2.80219	0.094026	0.001
E. purpurata vs E. tremolsii	1.031007	2.735794	0.138621	0.001

S4. Niche overlap (Schoener's D) in environmental space between *Epipactis* species

	E. atr	*E. hel*	*E. klei*	*E. lep*	*E. mic*	*E. mue*	*E. pal*	*E. pur*	*E. tre*
E. atr	1	x	x	x	x	x	x	x	x
E. hel	0.647	1	x	x	x	x	x	x	x
E. kle	0.097	0.069	1	x	x	x	x	x	x
E. lep	0.467	0.366	0.126	1	x	x	x	x	x
E. mic	0.421	0.421	0.178	0.410	1	x	x	x	x
E. mue	0.521	0.616	0.033	0.341	0.470	1	x	x	x
E. pal	0.670	0.810	0.089	0.388	0.442	0.629	1	x	x
E. pur	0.382	0.358	0.114	0.460	0.363	0.378	0.392	1	x
E. tre	0.105	0.145	0.209	0.106	0.212	0.120	0.127	0.102	1

S5. Niche overlap (Schoener's D) in geographic space between *Epipactis* species

	E. atr	*E. hel*	*E. klei*	*E. lept*	*E. micr*	*E. muel*	*E. pal*	*E. pur*	*E. tre*
E. atr	1.000	x	x	x	x	x	x	x	x
E. hel	0.751	1.000	x	x	x	x	x	x	x
E. kle	0.340	0.324	1.000	x	x	x	x	x	x
E. lep	0.505	0.421	0.253	1.000	x	x	x	x	x
E. mic	0.549	0.516	0.380	0.578	1.000	x	x	x	x
E. mue	0.614	0.546	0.348	0.607	0.762	1.000	x	x	x
E. pal	0.781	0.842	0.317	0.486	0.529	0.572	1.000	x	x
E. pur	0.618	0.543	0.279	0.554	0.503	0.671	0.576	1.000	x
E. tre	0.276	0.316	0.386	0.276	0.520	0.371	0.287	0.207	1.000

S6. Jaccard similarity of mycorrhizal fungal communities between *Epipactis* species

	E. atr	*E. hel*	*E. klei*	*E. lept*	*E. micr*	*E. muel*	*E. pal*	*E. pur*	*E. tre*
E. atr	1.000	x	x	x	x	x	x	x	x
E. hel	0.122	1.000	x	x	x	x	x	x	x
E. kle	0.116	0.077	1.000	x	x	x	x	x	x
E. lep	0.221	0.149	0.133	1.000	x	x	x	x	x
E. mic	0.202	0.146	0.209	0.183	1.000	x	x	x	x
E. mue	0.226	0.180	0.128	0.188	0.212	1.000	x	x	x
E. pal	0.241	0.159	0.111	0.214	0.183	0.218	1.000	x	x
E. pur	0.168	0.165	0.110	0.171	0.222	0.235	0.229	1.000	x
E. tre	0.160	0.200	0.091	0.210	0.212	0.177	0.156	0.132	1.000

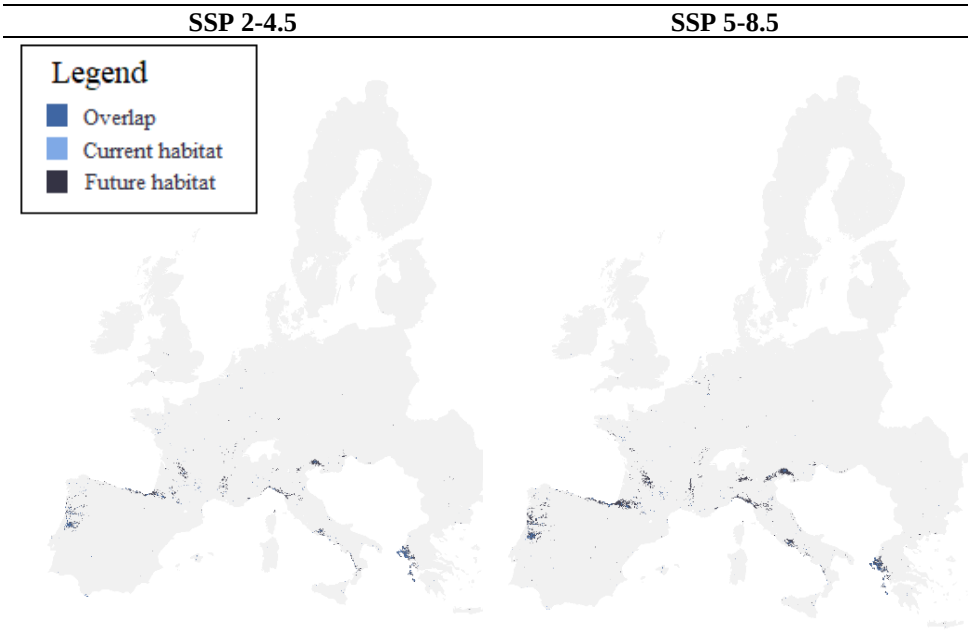
S7. Mean Gibbs p with standard error for *Epipactis* species in near-present conditions and two future climate scenarios (SSP 2-4.5 & SSP 5-8.5) in 2061-2080 and Levins' *B2* niche breadth in geographic and environmental space

Species	Mean Gibbs p			$B2_{geo}$	$B2_{env}$
	Near-present	SSP 2-4.5	SSP 5-8.5		
E. albensis	0.0050±0.0005	0.0075±0.0008	0.0087±0.0009	0.6640	0.8032
E. atrorubens	0.1711±0.0135	0.1515±0.0108	0.1333±0.0098	0.8482	0.6887
E. dunensis	0.0172±0.0018	0.0179±0.0018	0.0231±0.0024	0.6092	0.1631
E. fageticola	0.0031±0.0004	0.0033±0.0004	0.0033±0.0004	0.3926	0.7497
E. helleborine	0.2880±0.0219	0.3525±0.0267	0.3671±0.0282	0.8524	0.7676
E. kleinii	0.0050±0.0007	0.0047±0.0006	0.0041±0.0005	0.6998	0.2792
E. leptochila	0.0145±0.0021	0.0107±0.0016	0.0085±0.0013	0.5831	0.6154
E. lusitanica	0.0026±0.0006	0.0090±0.0019	0.0128±0.0024	0.7095	0.9031
E. microphylla	0.0441±0.0058	0.0465±0.0060	0.0448±0.0056	0.8050	0.5386
E. muelleri	0.0554±0.0069	0.0540±0.0068	0.0489±0.0054	0.7267	0.6163
E. palustris	0.1978±0.0173	0.2171±0.0176	0.2114±0.0167	0.8535	0.6581
E. phyllanthes	0.0060±0.0009	0.0116±0.0016	0.0144±0.0020	0.8391	0.6320
E. purpurata	0.0319±0.0046	0.0206±0.0023	0.0138±0.0015	0.6662	0.5072
E. tremolsii	0.0113±0.0022	0.0158±0.0023	0.0178±0.0023	0.7190	0.3547

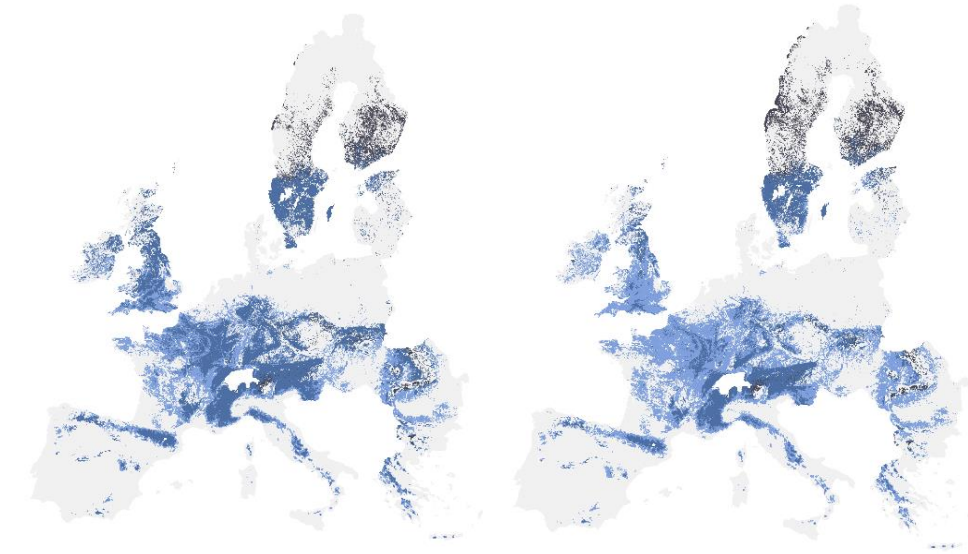
S8. Area of suitable habitat (in 50 km^2 units) above the Maximum Test Sensitivity and Specificity threshold and overlap of current and future habitat areas for *Epipactis* species in near-present conditions and two future climate scenarios (SSP 2-4.5 & SSP 5-8.5) in 2061-2080.

Species	Habitat range			Range overlap with current	
	Current	SSP 2-4.5	SSP 5-8.5	SSP 2-4.5	SSP 5-8.5
E. albensis	2385	6939	10539	2379	2334
E. atrorubens	317184	214085	143342	182088	104310
E. dunensis	43026	45409	65680	42946	42440
E. fageticola	2047	2498	3184	2035	1570
E. helleborine	385645	462798	439304	310072	270798
E. kleinii	5986	5701	3833	5251	3509
E. leptochila	47869	32277	24684	29384	21445
E. lusitanica	7440	51423	87987	7440	7440
E. microphylla	113254	147815	142499	85288	60998
E. muelleri	172179	164138	129545	120446	85446
E. palustris	336987	320853	274912	248167	185529
E. phyllanthes	9583	24185	31859	9583	9583
E. purpurata	101232	32411	11477	16632	4504
E. tremolsii	24465	41097	49072	21886	19846

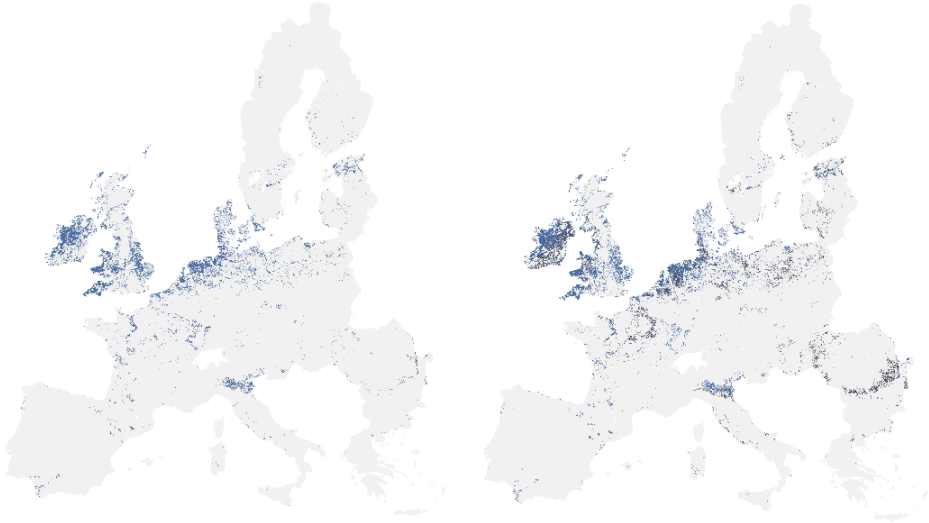
S9. Latitudinal shifts in *Epipactis* habitat from current conditions to conditions predicted for 2061-2080 by two climate models, SSP 2-4.5 and SSP 5-8.5.



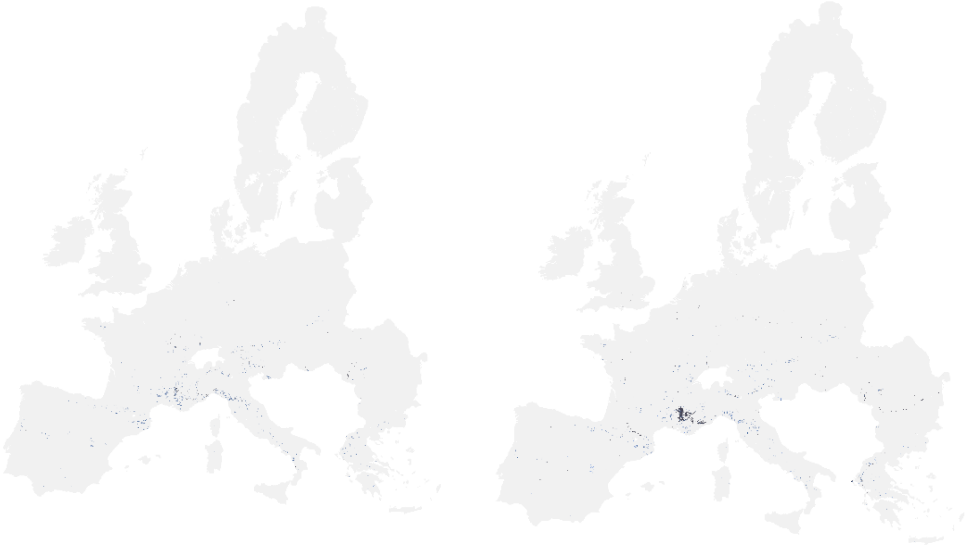
E. albensis



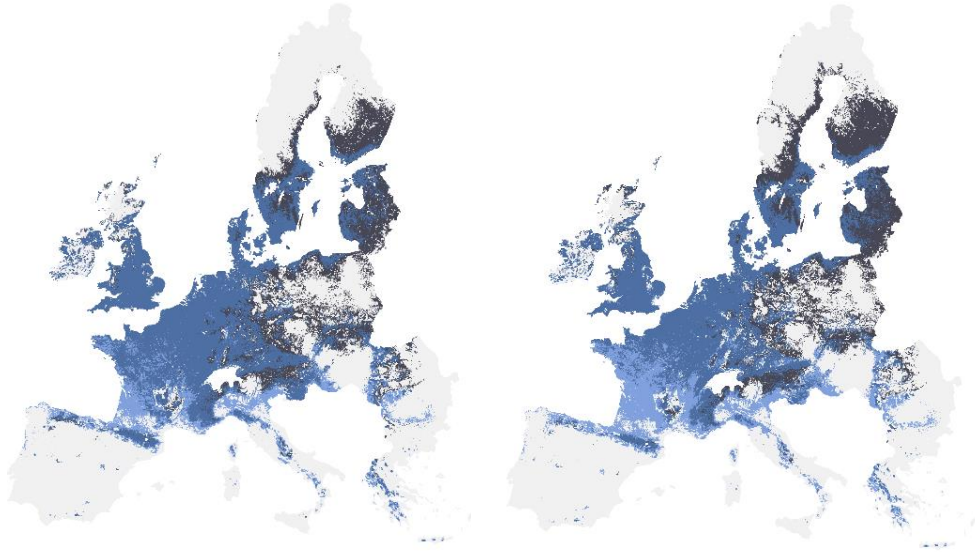
E. atrorubens



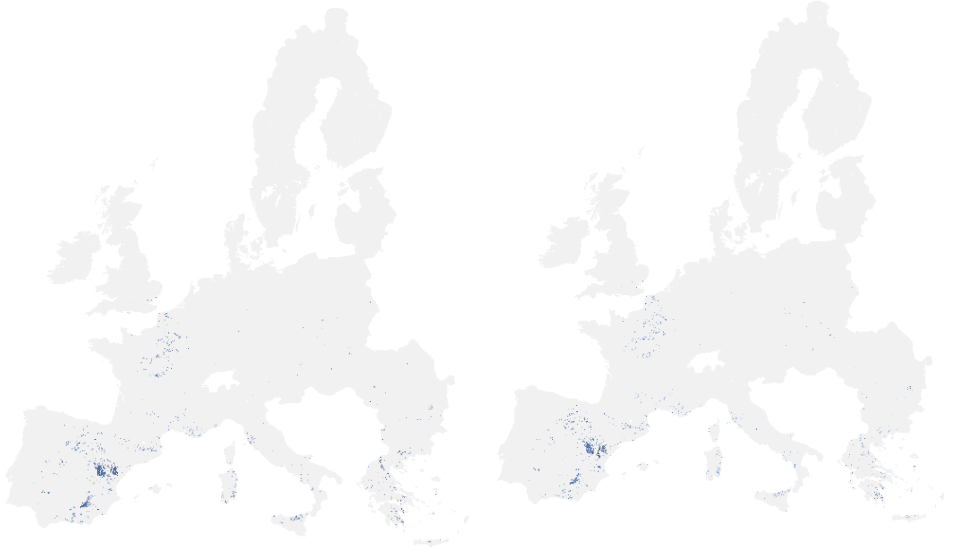
E. dunensis



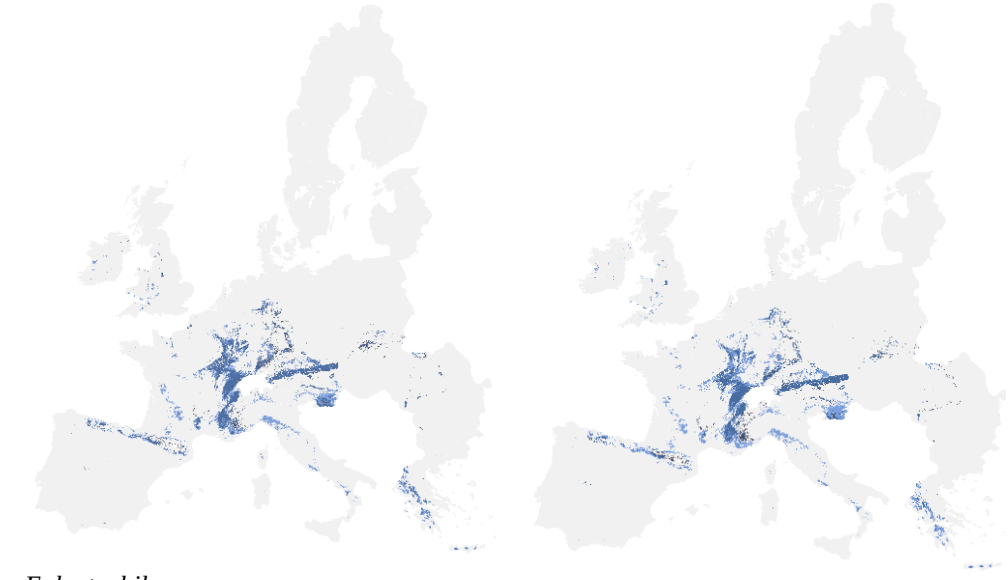
E. fageticola



E. helleborine



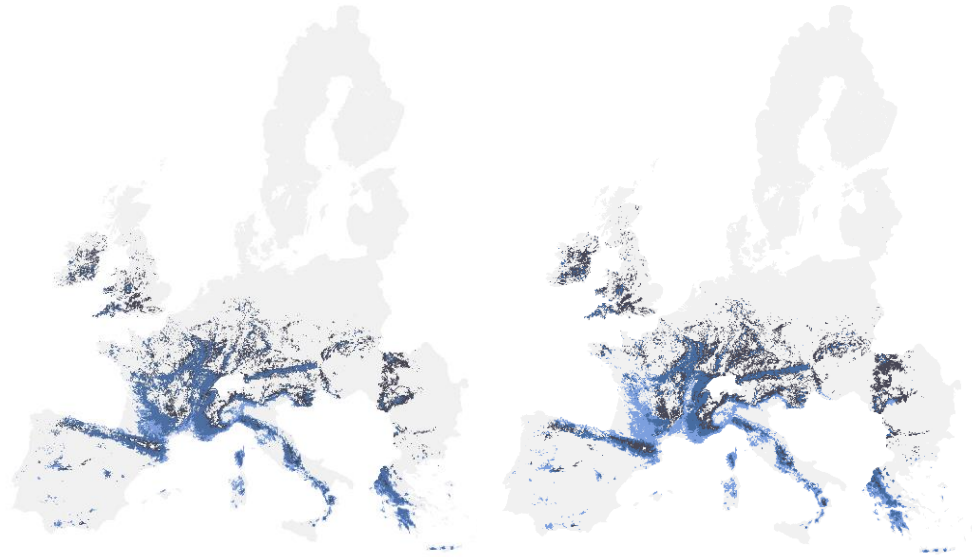
E. kleinii



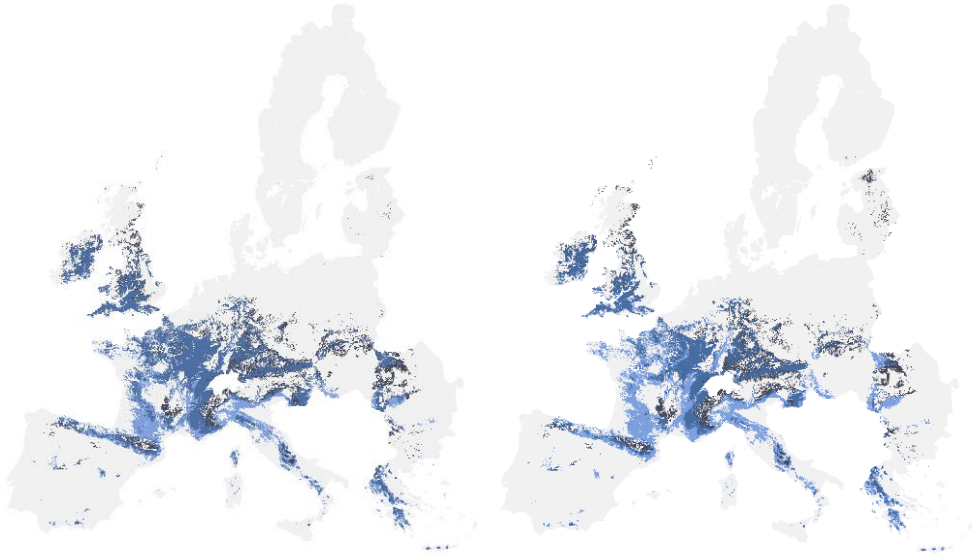
E. leptochila



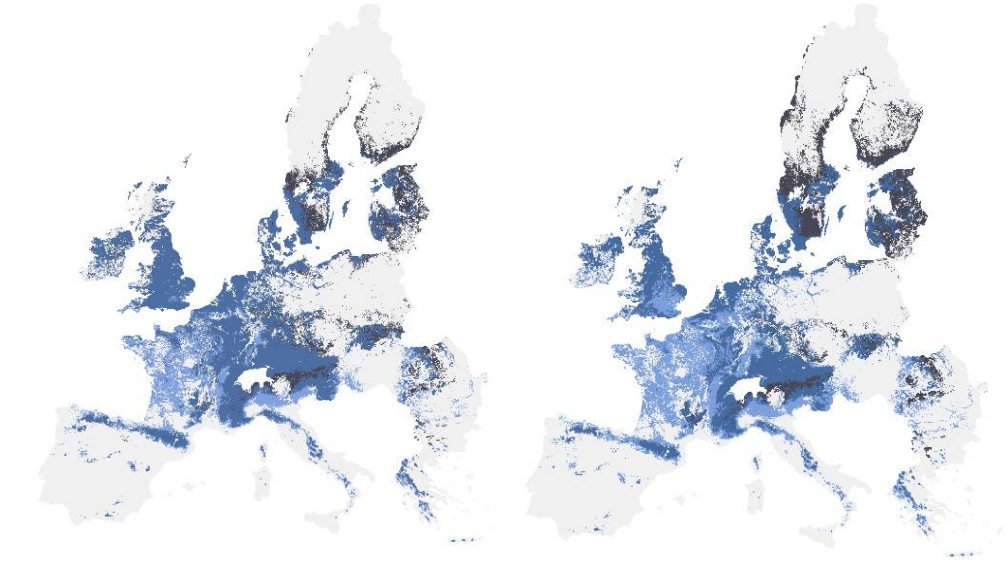
E. lusitanica



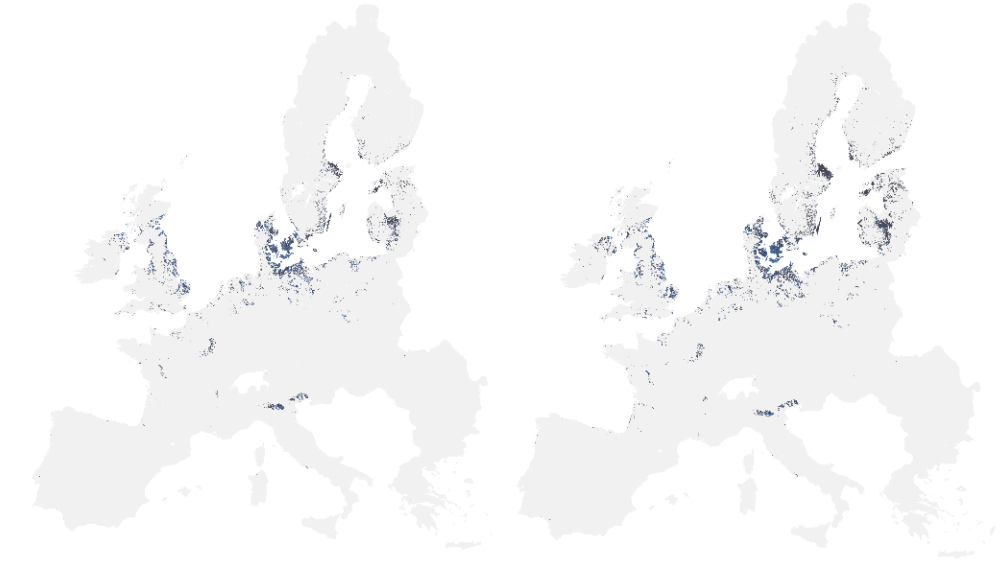
E. microphylla



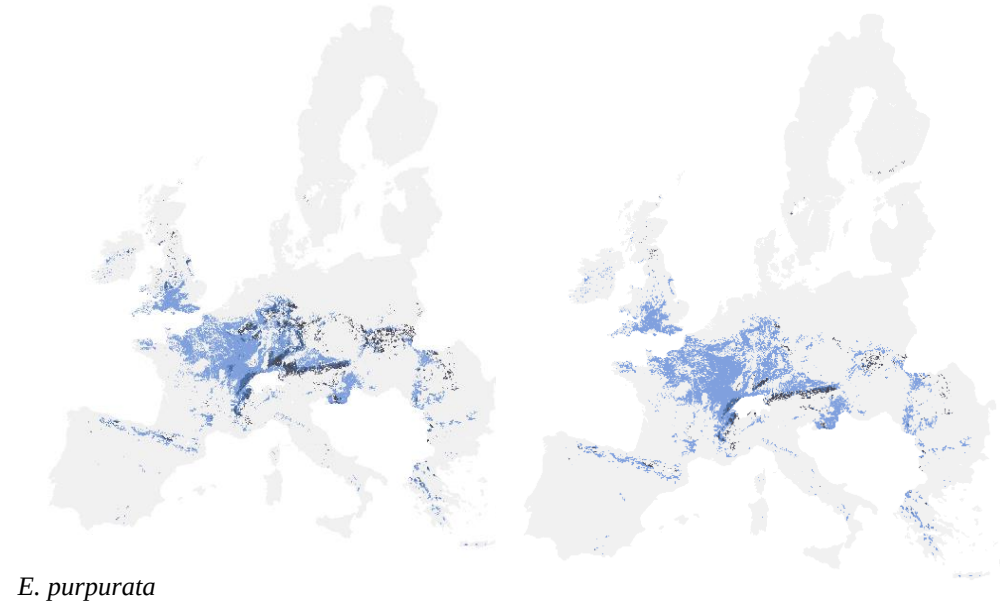
E. muelleri



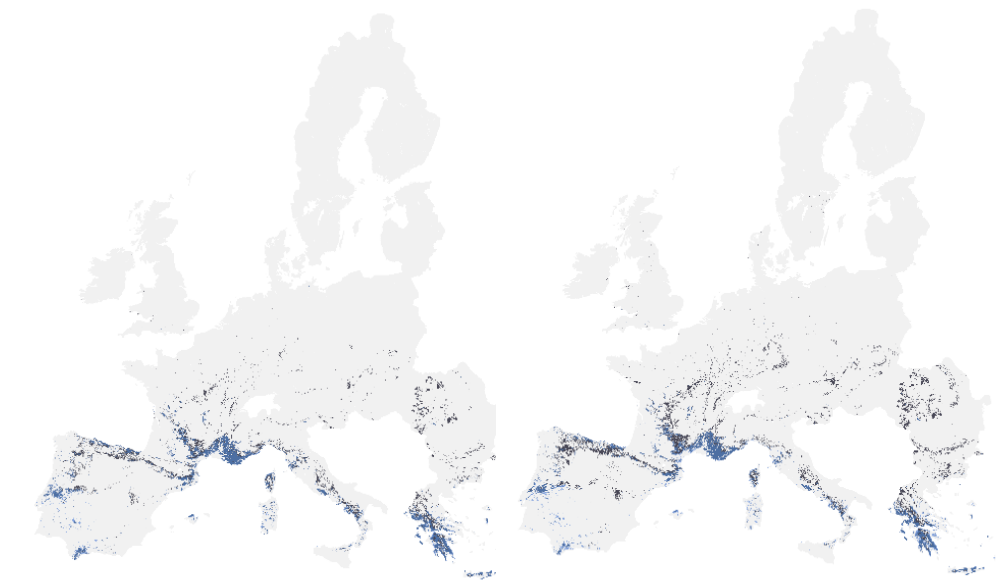
E. palustris



E. phyllanthes



E. purpurata



E. tremolsii

S10. DNA extraction, sequencing and filtering

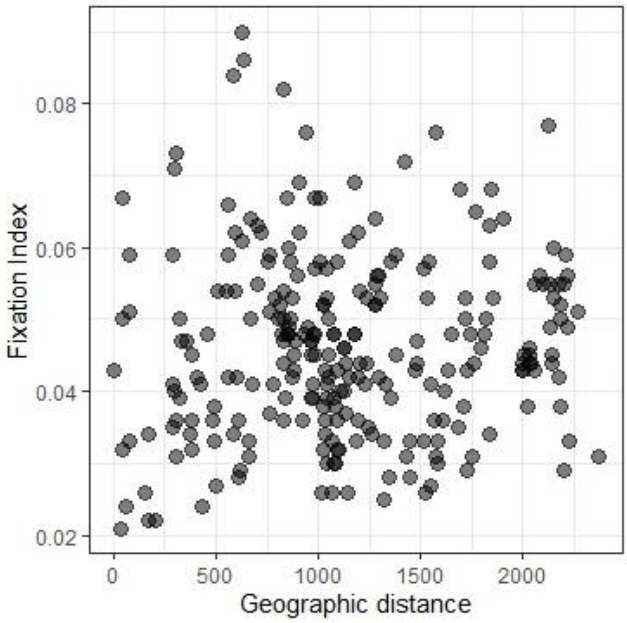
DNA was extracted with a sbeadx maxi plant kit with 1 % thioglycerol added to Lysis buffer PN, and digested with the MsII restriction enzyme. Common and unique barcoded adapters with overhangs complementary to the cut site were ligated to each sample and samples were pooled in equimolar ratios. The pooled library was PCR-amplified and sequenced on an Illumina NovaSeq 6000 SP FC, resulting in ~ 1.5 million reads per sample. Illumina reads were demultiplexed and quality filtered using the Illumina bcl2fastq 2.17.1.14 software. The filtered reads were aligned against the cluster reference using Bowtie2 v. 2.2.3 (Langmead and Salzberg 2012), and samples were genotyped using Freebayes v1.0.2-16 (Garrison and Marth 2012). SNPs (single nucleotide polymorphisms) were retained if they were represented in 5 % of the samples, and had an allele frequency of at least 10 % and a read count of 8, which resulted in 193 257 SNPs.

Genotypes were visualised, sorted and further filtered using Tassel 5 (Bradbury *et al.* 2007). Individuals with more than 30 % data missing and SNP loci with more than 20 % data missing were removed from the dataset. Data were also filtered to retain one SNP per locus. The sequence data from the variant analysis were converted from vcf into other formats for further analysis using PGDSpider2 (Lischer and Excoffier 2012).

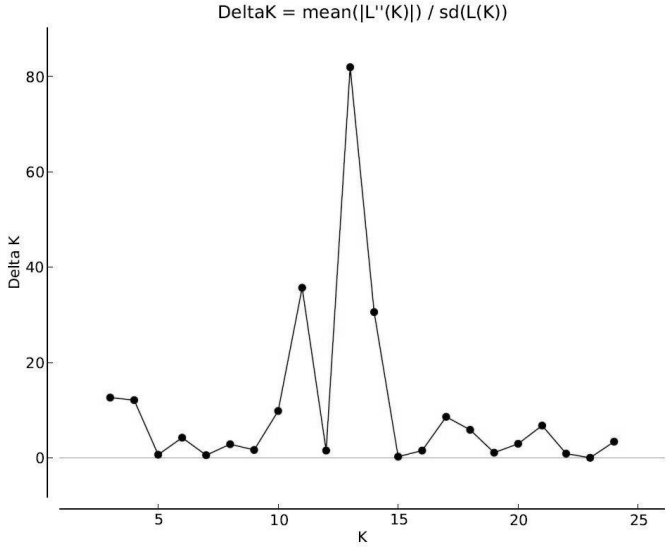
S11. Mean Gibbs p values (habitat suitability) of near-present climate conditions and mean genetic diversity with standard errors of 23 *Epipactis helleborine* populations sampled in Europe (BE=Belgium, CZ=Czech Republic, DK=Denmark, ES=Estonia, FR=France, GK=Greece, HU=Hungary, IT=Italy, PL=Poland, SK=Slovakia, SL=Slovenia). Diversity was measured using allelic richness (*AR*) and observed and expected heterozygosity (H_o, H_e) of a rarefied sample.

Population	No. of samples	Mean Gibbs p value (near-present)	*AR*	H_o	H_e
BE-A	10	0.729±0.007	1.397±0.006	0.588±0.011	0.385±0.006
BE-B	10	0.688±0.004	1.392±0.006	0.575±0.011	0.381±0.006
CZ-A	11	0.356±0.004	1.381±0.006	0.569±0.011	0.371±0.006
DK-A	9	0.655±0.009	1.376±0.007	0.569±0.012	0.363±0.006
ES-A	11	0.670±0.013	1.390±0.006	0.576±0.011	0.380±0.006
ES-B	11	0.565±0.010	1.387±0.006	0.571±0.011	0.377±0.006
ES-C	11	0.402±0.012	1.396±0.006	0.591±0.011	0.386±0.006
FR-B	9	0.688±0.006	1.389±0.007	0.592±0.012	0.375±0.006
FR-C	8	0.392±0.006	1.385±0.007	0.567±0.012	0.371±0.006
FR-D	8	0.692±0.006	1.392±0.006	0.572±0.011	0.378±0.006
GK-A	8	0.208±0.008	1.382±0.007	0.566±0.012	0.366±0.007
GK-B	8	0.237±0.014	1.390±0.007	0.572±0.012	0.375±0.006
GK-C	11	0.429±0.016	1.353±0.008	0.544±0.013	0.340±0.007
GK-D	8	0.385±0.010	1.384±0.007	0.565±0.012	0.369±0.006
HU-A	8	0.524±0.006	1.399±0.006	0.577±0.011	0.383±0.006
HU-B	10	0.549±0.010	1.384±0.007	0.579±0.012	0.372±0.006
IT-A	5	0.208±0.011	1.389±0.007	0.561±0.012	0.366±0.007
IT-B	5	0.208±0.011	1.403±0.008	0.569±0.013	0.374±0.007
IT-C	5	0.385±0.029	1.395±0.008	0.551±0.012	0.368±0.007
PL-A	11	0.388±0.006	1.380±0.006	0.571±0.012	0.37±0.006
PL-B	11	0.385±0.013	1.384±0.006	0.567±0.011	0.375±0.006
SK-B	9	0.390±0.011	1.388±0.006	0.572±0.011	0.373±0.006
SL-A	9	0.372±0.015	1.389±0.007	0.576±0.012	0.375±0.006
Mean	9	0.457±0.010	1.387±0.007	0.571±0.012	0.373±0.006

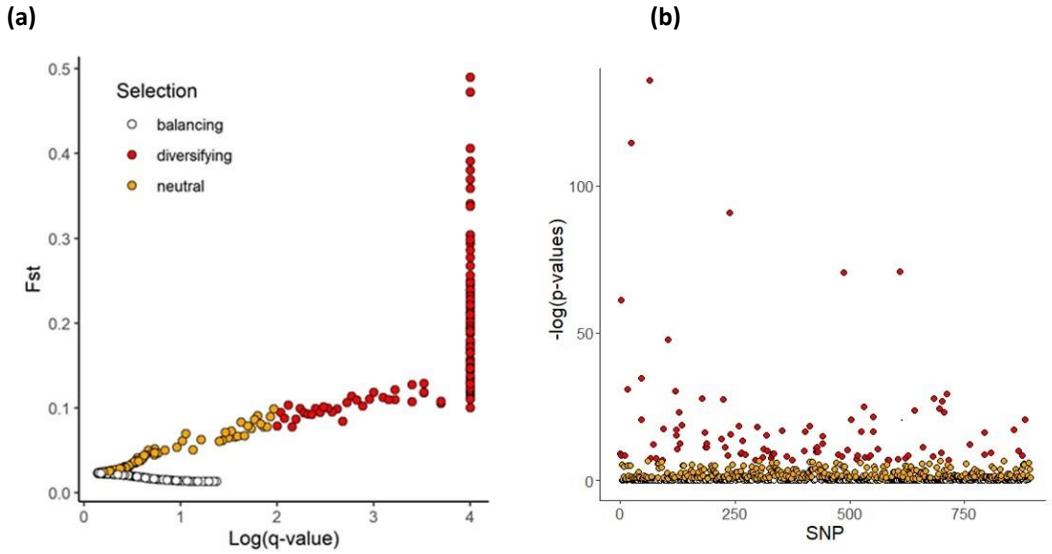
S12. Results of a Mantel test, showing the relationship between pairwise log-transformed geographic distance and F_{st} values among *E. helleborine* populations in Europe.



S13. Delta K graph indicating the most likely number of geneti c clusters in the sample of *E. helleborine*, detected by the Evanno method (Evanno *et al.* 2005). The highest peak in delta K indicates the most likely number of genetic clusters (K) in the sample.



S14. Results of Bayescan (a.) and PCAdapt (b.) for detecting genetic outliers in a SNP dataset of *E. helleborine* samples collected in Europe. Diversifying outlier SNPs are indicated in red, neutral non-adaptive SNPs in orange and balancing SNPs in white.



Publication list

Evans A, Jacquemyn H. **2020**. Impact of mating system on range size and niche breadth in *Epipactis* (Orchidaceae). *Annals of Botany*.

Evans A, Janssens S, Jacquemyn H. **2020**. Impact of Climate Change on the Distribution of Four Closely Related Orchis (Orchidaceae) Species. *Diversity* **12**: 312.

Jacquemyn H, Brys R, Waud M, Evans A, Figura T and Selosse MA. 2021. Mycorrhizal communities and isotope signatures in two partially mycoheterotrophic orchids. *Frontiers in Plant Science* **12**: 618140.

Evans A, Jacquemyn H. **2022**. Range Size and Niche Breadth as Predictors of Climate-Induced Habitat Change in *Epipactis* (Orchidaceae). *Frontiers in Ecology and Evolution* **10**.

Evans A, de Kort H, Brys R, *et al.* 2023. Historical biogeography and local adaptation explain population genetic structure in a widespread terrestrial orchid. *Annals of Botany*: mcad010.


Alexandra Evans