RESTORATION GENETICS: THE IMPACT OF REINTRODUCTION, SOIL SEED BANK AND RECOLONIZATION ON GENETIC VARIATION

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SUMMARY

The present thesis is dedicated to the assessment, analysis and evaluation of genetic consequences of three common restoration approaches, using data collected in three particular restoration projects and one experiment.

In **Chapter one**, the general context of the PhD. Thesis is outlined. Here, issues concerning global habitat and biodiversity loss and its roots are briefly discussed, as well as efforts to counter these detrimental trends using legal frameworks, policies, and financial support. Further, the term biodiversity is introduced, as well as methods to measure its intraspecific (=genetic) component, using tools of conservation genetics. Molecular methods commonly applied in the context of conservation and population genetics are shortly outlined, with a particular focus on methods used in my PhD. thesis.

Last but not least, widely used restoration approaches were introduced and shortly described, especially those studied in my work from the genetic point of view.

In **Chapter two**, the rare species (re)introduction and related matters was outlined, i.e. not only the genetic makeup of newly founded populations and their source, but also the issue of seed sourcing strategies. In particular it is focused on the selection of appropriate seed sourcing procedures along a highly dynamic river system and possible transfer of diaspores in a critically endangered *Myricaria germanica* not only from distant populations located within the same river catchment, but also on possibilities of diaspore exchange between the catchments.

Chapter three is dedicated to the impact of seeding, planting, as well as the combined reintroduction approach of seeding plus planting, on genetic properties of restored populations of a rare and endangered herb *Armeria maritima* ssp. *elongata*. This chapter is a pilot study that summarizes results of a practical reintroduction project, started in 1998.

In **Chapter four** the outcomes of the spontaneous recolonization process in restored calcareous grasslands, created after scrub and woodland removal was studied. Here, the main risk to genetic makeup of restored populations was the possible founder effect and associated erosion of genetic diversity in newly founded populations, as well as inflated genetic differentiation between them, possibly reinforced by random genetic drift. After twenty-five years since the forest clearing, the results of recolonization using three common grassland species with contrasting mating systems was investigated. These

settings allow to gain further information regarding suitability of (re)colonization in the context of restoration genetics.

Further, in **Chapter five** restoration possibilities related to the soil seed bank were assessed. For this purpose, *Origanum vulgare* populations from the two soil layers with the aboveground populations were compared. Specifically, it was analysed if there were any significant differences concerning heterozygosity levels in the soil vs. vegetation, as reported in previous research. Furthermore, levels of differentiation between the soil and the current populations, as well as differentiation among the populations from the soil and the aboveground populations at different study sites were studied, respectively.

Finally, in **Chapter six** the results of the previous four chapters were summarized and reviewed in the context of natural processes which take place in the studied habitats. Using the four studies in population genetics, conclusions that might be useful in a context of future restoration efforts were formulated.

CHAPTER ONE: GENERAL INTRODUCTION




Biodiversity under siege

Our planet´s biodiversity is under siege. Anthropogenic pressure – be it in terms of industrialization, land-use intensification, invasion of alien species or climate change (Clavel et al., 2011; Cardinale et al., 2012; Dirzo et al., 2014) – imposes the main threats to both natural and semi-natural ecosystems (Sih et al., 2000; Vellend, 2003; Klausmeyer and Shaw, 2009; Sánchez-Bayo and Wyckhuys, 2019; Chase et al., 2020). In this context, theory of the Anthropocene geological epoch came up, suggesting essential changes in the relationship between humans and the Earth (Crutzen P.J. and Stoermer E.F., 2000; Lewis and Maslin, 2015; Exposito-Alonso et al., 2022). The rate of species extinction is currently as much as one hundred times that of the "normal rate" throughout geological time and is frequently termed the sixth mass extinction (Barnosky et al., 2011; Pimm et al., 2014; Cowie et al., 2022).

Ecological restoration – a political priority

Recognizing two decades of failure to attain goals and targets in ecosystem restoration and to mitigate the species extinction trajectory, parties to the Convention on Biological Diversity, i.e. the key global conservation policy mechanism, developed a strategy for jointly safeguarding nature, the 'Post-2020 Global Biodiversity Framework' (CBD, 2018; Mace et al., 2018), to bend the curve of biodiversity loss. However, the zero draft was heavily criticized due to the poorly elaborated genetic diversity targets and indicators, referring to only domestic animal breeds and crops (CBD, 2020). The new strategic document should explicitly commit to preserving genetic diversity within all species, both wild and domesticated, and to implement strategies to preserve adaptive potential of populations of species (Hoban et al., 2020; Laikre et al., 2020). In addition, necessary indicators should be defined to assess the progress toward this goal (Hunter et al., 2018). The last draft version of the Post-2020 global biodiversity framework has already implemented goals to maintain genetic diversity of populations and their adaptive potential (Goal A, CBD, 2022). Hence there is a glimpse of hope that improvements in genetic diversity targets and indicators are possible, as the 15th Conference of the Parties to the convention takes place in December 2022.

The European Union, in its turn, made clear commitments to halt biodiversity loss (EU, 2011), using legal frameworks, such as the Birds and Habitats Directives (EC, 1992), policies, such as the EU Biodiversity Strategy for 2030 (EC, 2020), and financial mechanisms, such as the LIFE programme. These legal frameworks, policies and financial support fostered efforts without precedent globally, and yielded the Natura 2000 network (European Commission, Directorate-General for Environment, Sundseth, K., 2008), the world's largest network of protected areas (Hermoso et al., 2022). However, these major achievements turned insufficient to halt biodiversity loss at the continental scale (EEA, 2020). This is not the only environmental EU´s battlefield, since the impacts of global change on society, biodiversity and ecosystem services continue to accelerate (IPBES, 2019).

In Central Europe, the most serious threats to biodiversity are land use changes. Furthermore, continuing large-scale drainage

and nutrient accumulation in the soil, as well as overfertilization of water. Additionally, the ongoing climatic change may pose a risk to many plant species, for instance, long-term shifts in composition of mountain plant communities were detected, clearly attributed to the climate change (Skálová et al., 2022).

Three levels of biodiversity

The term biodiversity, short for biological diversity, first appears in the mid-1980s (Tangley, 1985; Wilson, 2011). Biologists frequently define the term a totality of inherited variation in all the organisms of a selected area (Wilson, 2010). Biodiversity manifests itself at different levels: it comprises the diversity of ecosystems, species, and genes (Larsson, 2001). Especially the intraspecific diversity is of a fundamental importance to the long-term population survival prospects, as it defines its fitness and evolutionary potential in changing climates and environments (Des Roches et al., 2018). However, ironically the intraspecific genetic level of biodiversity was overlooked for a long time not only because its importance was not sufficiently recognized, but also due to the difficulties to quantify it (compare Laikre et al., 2020).

Therefore, pragmatic genetic indicators for maintaining genetic diversity and adaptive capacity of species are urgently required for appropriate monitoring of changes in genetic diversity (Hunter et al., 2018). Indicators might be, first, the number of populations with effective population size above versus below 500 (Franklin, 1980; Beissinger and McCullough, 2002; Jamieson and Allendorf, 2012), second, the rates of loss of distinct populations within species, and, last but not least, the number of species and populations in which genetic diversity has been monitored using DNA-based methods (Hoban et al., 2020; Laikre et al., 2020). Although molecular ecology and conservation genetics are rapidly evolving fields, much wider knowledge is still urgently needed to plan and to implement rescue measures before it is too late.

Genetic analyses allow to identify the genetically most valuable populations across the distribution range for future evolutionary development (Shafer et al., 2015; Mijangos et al., 2015b; van Rossum et al., 2020).

Conservation genetics

Former conservation efforts have dealt with populations from a taxonomic, evolutionary, or mathematical point of view. Modern efforts include genetic studies, providing conservation scientists and practitioners with much more information about the diversity within and among populations. About fifty years ago, Crow and Kimura (1970) and Ohta and Kimura (1973) predicted the relationship between population size and genetic marker diversity, and thus laid foundations of conservation genetics. Their work fostered massive research effort focusing on the impact of population dynamics and especially small population size on population performance, viability, and evolutionary potential. So far, conservation genetics provided several best-supported paradigms to assist rescue measures related to threatened species (Teixeira and Huber, 2021; Willi et al., 2022). Moreover, the advanced status of the science field is evidenced by high profile publications (Allendorf et al., 2013; Frankham et al., 2015).

Conservation genetics, similar to the restoration ecology, aims to close the gap

between theory and practice (van Andel and Aronson, 2012; Holderegger et al., 2019). Without genetics, we may scatter valuable resources on a population that is not endangered – or a population in which recovery prospect reduced to nil. Further, reintroduction efforts require information regarding suitable seed source populations (Guerrant Jr and Kaye, 2007; Godefroid et al., 2011; Godefroid et al., 2016; Kaulfuß and Reisch, 2017). Moreover, valuable insights into how various restorations approaches worked from the genetic point of view can be gained, thus informing and/or improving future restoration efforts.

Specific genetic techniques are used to study the genomes of a species regarding specific conservation issues as well as general population structure. This analysis can be done in two ways, with current DNA of individuals or historic DNA (Wayne and Morin, 2004). Techniques to analyse the differences between individuals and populations include, inter alia, RFLP, AFLP, RAPD, SSCP, SSR, SNPs and DNA sequencing. These techniques focus on different areas of plant and animal genomes. The specific information that is required indicates which techniques are used and which parts of the genome are analysed (Wan et al., 2004). Molecular markers allow us to answer a wide range of population genetic issues related to gene flow (Hudson et al., 1992; Edelaar and Bolnick, 2012), parentage (García et al., 2002), and population structuring (Cockerham and Weir, 1993; Meirmans and Hedrick, 2011).

In this thesis, the genetic markers AFLP and SSR were applied. First, amplified fragment length polymorphisms (AFLPs) are PCR-based markers for the quick screening of genetic diversity. It is based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA (Vos et al., 1995). AFLP methods promptly generate hundreds of replicable markers from DNA of any organism; thus, they provide high-resolution genotyping of fingerprinting quality. The cost and time efficiency, replicability and resolution of AFLPs are better or equal to those of RAPD, RFLP, and SSR. The main disadvantage lies in the fact that AFLPs essentially generate dominant markers (Vos et al., 1995; Mueller and Wolfenbarger, 1999; Meudt and Clarke, 2007).

Secondly, microsatellites (SSR) are tracts of tandemly repeated DNA motifs ranging in length from one to six nucleotides, and are typically repeated 5–50 times (Richard et al., 2008; Tautz, 1989). As a co-dominant marker, SSR is an efficient tool to detect heterozygosity. Currently, microsatellites together with SNPs are the most commonly used markers in population genetic studies (Narum et al., 2008; DeFaveri et al., 2013; Puckett, 2017; Flanagan and Jones, 2019). Despite the higher precision of SNPs, microsatellites, due to their fast mutation rate and a high degree of polymorphism, effectively detect population processes and patterns and are superior for relatedness estimation, micro-scale structuring detection and in species with reduced genetic diversity (Narum et al.2008; DeFaveri et al., 2013; Hauser et al., 2021). The main drawback of the method is that microsatellites suffer null alleles and homoplasy (Rousset, 1996; Estoup et al., 2002; Selkoe and Toonen, 2006).

Restoration methods and their genetic implications

In terrestrial ecosystems, restoration mostly means (re)establishment of plant communities, which mostly constitute the base of subsequent trophic levels. Generally, there are two options to restore populations or habitats: first, to allow for natural regeneration via spontaneous colonization or using soil seed banks. The necessary, self-evident prerequisites for this approach are preserved diaspore sources in surroundings or in the soil (Bakker et al., 1996b; Poschlod et al., 1998a; Isbell et al., 2019). The second possibility suggests deliberate plant introductions. In fragmented landscapes, natural succession often leads to incomplete/poor recovery of vegetation, due to the absence of natural habitats that could serve as diaspore sources (Bullock et al., 2007; Suding, 2011; Isbell et al., 2019; Prach et al., 2015a; Isbell et al., 2019). Especially in fragmented landscapes or intensively managed agriculture areas, plant introduction became a major restoration tool (Hölzel et al., 2012).

There is a common agreement that restoration should prioritize native species (McDonald et al., 2016). However, there is a substantial debate on which sources or provenance will ensure the best restoration outcome (Guerrant Jr and Kaye, 2007; Broadhurst et al., 2008; Breed et al., 2013; Kettenring et al., 2014; Prober et al., 2015; Bucharova et al., 2017).

Spontaneous habitat recovery, by its very nature, entirely satisfies demands related to the regionality and local adaptation of seeds. This restoration approach makes good use of natural, locally available seed sources – either from the soil or from the seed rain (Willems and Bik, 1998). In comparison to other restoration methods, natural colonization due to the seed dispersal in space (seed rain) and time (seed bank) intrinsically involves the advantage of matching the gene pool of nearby populations (McKay et al., 2005).

Natural colonization on fellow lying old fields (review Rejmanek and van Katwyk, 2005), abandoned quarry areas (Ilves et al., 2015) or following scrub or woodland removal (Kiefer and Poschlod, 1996; Kiefer, 1998b; Blanckenhagen and Poschlod, 2005) imposes an important grassland restoration tool. This method is a natural and low cost way of grassland restoration (Prach and Hobbs, 2008), as it relies on spontaneous seed dispersal processes from locally available propagule sources (Redhead et al., 2014; Bakker et al., 1996c; Kirmer et al., 2008; Redhead et al., 2014). However, a crucial role in colonization of restored grasslands by target species is the availability of source populations in the close surrounding landscape (Prach et al., 2015; Aavik and Helm, 2018; Kiss et al., 2021). Moreover, the process of natural regeneration requires sufficient time to be successful (Kiefer and Poschlod, 1996; Blanckenhagen and Poschlod, 2005; Prach et al., 2015; Wagner et al., 2019; Prach et al., 2015a). In light of conservation genetics, a possible founder effect in immigrant populations during the colonization process (Slatkin, 1977a; Wade and McCauley, 1988; Whitlock and McCauley, 1990) might undermine the confidence in this method (compare Vandepitte et al., 2012). Owing to the limited size of founder groups, possible local loss of genetic diversity and random genetic drift may occur (Franklin, 1980; Franklin and Frankham, 1998; Vandepitte et al., 2012).

Soil seed bank (ssb) has been shown to be a useful tool of restoration in types of

habitats with high unpredictability of biotic and abiotic conditions such as deserts (Zaghloul et al., 2013; Bossuyt and Honnay, 2008), whereas for other habitat types such as grasslands, its potential to recover target plant communities is limited (Willems and Bik, 1998; Davies and Waite, 1998; Bisteau and Mahy, 2005; Bossuyt and Honnay, 2008; Wakshum et al., 2018). However, individual species and their populations might be restored using natural seed sources from the soil seed banks. The impact of ssb on genetic makeup of recovered or standing populations is widely discussed (e.g., Cabin et al., 1998; Mahy et al., 1999; Mandak et al., 2006a; Honnay et al., 2008; Ottewell et al., 2011; Schulz et al., 2018; Summers et al., 2018; Yang et al., 2021), implicating mitigation of the consequences of habitat fragmentation and protection of species from genetic drift and population genetic differentiation (Honnay et al., 2008).

Similarly to the two previously described methods, restoration via topsoil removal works based on natural recovery from soil seed banks and/or from the seed rain. It turned out to be a promising method in habitats with high conservation value, but affected by eutrophication of the upper soil horizon and densely overgrown by dominant species (e.g., *Calamagrostis epigejos* in psammophilous vegetation, or, *Phragmites communis* and expansive *Sphagna* in wet meadows and fens, respectively). In these particular cases, seed banks and diaspore rain proved to be essential sources of recovery (Bakker et al., 2012; Řehounková et al., 2021; Singh et al., 2021).

The second type of ecological restoration applies germplasm material deliberately collected in wild plant populations or produced for restoration purposes, and spread on target sites. This approach enables faster habitat or population restoration than the spontaneous recovery. At the same time, applying this method, an artificial gene flow is induced and, therefore, its implementation should be carefully considered (Mijnsbrugge et al., 2010; Bischoff et al., 2010; Bucharova et al., 2019; Höfner et al., 2021; Carvalho et al., 2021). The local provenance was traditionally regarded to be the best choice (Hamilton, 2001), which is based on the assessment that the most plant species comprise genetically differentiated populations, at least partly due to adaptations to local conditions. As a consequence, locally adapted plants mostly perform better than plants from alien provenances, a phenomenon called local adaptation (Leimu & Fischer, 2008; Oduor et al., 2016; Bucharova, Durka, et al., 2017). However, with ongoing climate change, local provenancing has been increasingly criticized, since the plants are adapted to the past and current local climate conditions. As the climate changes, local adaptation is expected to lag behind (Anderson and Wadgymar, 2020). Some experts thus suggest to complement the local provenance by plants from populations that are adapted to predicted climate and thus, to improve the performance of a restored population exposed to the climate change (Vitt et al., 2010; Corlett and Westcott, 2013; Prober et al., 2015). However, this premise does not consider myriads of biotic interactions, which reflect the plant provenance (Bucharova, 2017; Bucharova et al., 2022).

To restore habitats and populations, seeds, juvenile plants or other vectors can be used. A technique widely used for centuries, was translocation of plant material, e.g. green

hay, raked litter, threshed seeds or barn chaff (Kaulfuß and Reisch, 2021; Wagner et al., 2021). This approach has been applied to introduce target species and thus to enhance species richness in degraded grasslands, or to initiate new grasslands (Kiehl et al., 2010; Coiffait-Gombault et al., 2011; Albert et al., 2019). Kaulfuß and Reisch (2021) detected, that this restoration approach is well suited to preserve the composition of species-rich grasslands and the natural genetic pattern of typical grassland plant species.

Planting seedlings, mature plants or belowground parts, or sowing seeds collected in wild populations, respectively, are techniques often applied to additionally enhance species richness and propagule availability in sites restored using other methods (Guerrant Jr and Kaye, 2007). Especially the use of juveniles and adult plants usually yields faster maturity and population establishment than seed sowing (Godefroid et al., 2011; Dalrymple et al., 2012) but requires more time and financial costs. Moreover, it also implies multiple steps in a production process and may unintentionally cause genetic erosion (Basey et al., 2015). Seeds, in their turn, can be applied especially if target species produce large amounts of seeds (Guerrant Jr and Kaye, 2007). From the genetic point of view, application of seeds versus plants in ecological restoration schemes was only scarcely investigated so far (St. Clair et al., 2020).

Currently, large-scale restoration programmes require huge amounts of regionally adapted seed. Regional seed mixtures aim to answer this situation (Mitchley et al., 2012; Durka et al., 2019; Höfner et al., 2021; Kaulfuß et al., 2022), either in high-diversity or low-diversity option (Kirmer et al., 2012). They are especially vital in cases when the local species pool does not enable spontaneous grassland regeneration via natural diaspore input (Willems and Bik, 1998; DiLeo et al., 2017). This method helps to restore grassland communities in a comparatively short time horizon (Kövendi-Jakó et al., 2019). However, Lesica and Allendorf (1999) framed one of the central restoration dilemmas: either the use of a single source material for local restorations of less disturbed sites – or genetically variable mixtures originating from multiple populations but threatening genetic makeup of surrounding natural populations, and thus acceptable to only restore severely disturbed locations. Bucharova et al. (2019) reframed the previous concept of Lesica and Allendorf, promoting the *regional admixture provenancing* as an approach compromising both demand of high genetic diversity and adaptive potential *and* local adaptations in plants originating from the same region as the target site.

After collecting, seeds are usually propagated in agricultural orchards to enhance the supply (Ladouceur et al., 2018). This common approach imposed a new cluster of issues, dealing e.g. with unintentional adaptations during the propagation process (Basey et al., 2015; Espeland et al., 2017; Nagel et al., 2019b). However, these issues would go far beyond the scope of the present thesis.

RESTORING POPULATIONS OF THE CRITICALLY ENDANGERED SHRUB *MYRICARIA GERMANICA* ALONG THE ALPINE RIVER ISAR BY REINTRODUCTION – IS THERE AN IMPACT ON GENETIC VARIATION?

Reintroduced plants of *Myricaria germanica*

at the querry ponds in the alluvial floodplains of the Isar River

Abstract

The vegetation along alpine rivers belongs to the most endangered habitats in the Alps. Many species occurring on gravel banks drastically declined due to hydrologic changes related to anthropogenic regulation. In the recent decade strong restoration efforts have been made to prevent the alpine river key stone plant species *Myricaria germanica* from extinction. The impact of restoration on genetic variation has, however, not been investigated so far, although potential founder and bottleneck effects may have an impact on restoration success. In our study we analysed, therefore, genetic diversity and differentiation within and among natural and restored populations of *M. germanica* along the alpine rivers Isar and Lech in SE Germany using molecular markers. Genetic diversity and differentiation of natural *M. germanica* populations varied strongly and we observed no isolation by distance, as to be expected for a species from a highly dynamic river habitat. Genetic diversity of restored populations was slightly lower but comparable to genetic diversity of natural populations along the Isar River. Moreover, genetic differentiation between the source and the restored populations was not significant. Genetic variation of the restored populations reflected, hence, the natural genetic pattern of *M. germanica*. Further populations should, therefore, be restored to counteract the ongoing decline of this iconic alpine river plant species.

Key words

Alpine river, conservation genetics, genetic diversity, gravel bank, reintroduction, restoration

Introduction

Ecological restoration is a powerful tool to counteract the shrinkage of natural habitats and biodiversity, including hidden and irreversible loss of genetic diversity. One of the most important restoration instruments is species reintroduction (IUCN/SSC, 2013) with the aim to establish viable and self-sustainable populations, which will produce progeny in their own and persist into future decades. However, reintroduction is a complex venture, and its success depends on many factors. One of these factors is the genetic variation of both the seed source and restored populations (Guerrant Jr, 1996; Guerrant Jr and Kaye, 2007; Godefroid et al., 2011; Drayton and Primack, 2012). Before restoration starts, an appropriate source population for restoration has to be chosen. This is an important step, because insufficient genetic diversity of the source population may lead to low rates of establishment and a limited performance in restored populations (Dostálek et al., 2010). Especially small-sized, isolated populations may suffer from low levels of genetic variation (Busch and Reisch, 2016; Leimu and Fischer, 2008; Keller and Waller, 2002).

Moreover, using seeds from only a few individuals for restoration may cause a bottleneck effect in the restored population (; Robichaux et al., 1997; Godefroid et al., 2011) which may hamper the population´s ability to undergo adaptive changes, as a response to changing environments (Brown and Briggs, 1991; Frankham et al., 2010). Therefore, Brown and Briggs (1991) suggest to sample at least 50-100 individuals, randomly chosen per population. On this way, at least one copy would be recovered of the more common alleles, i.e. with frequency in excess of 0.05-0.10 in the sample with an ensured probability of 90-95%.

Sourcing across multiple populations aims to counteract the genetic impoverishment and captures an inter-population genetic diversity (Brown and Briggs, 1991; Guerrant Jr and Kaye, 2007; Gabel et al., 2017). Plants or seeds from diverse source populations yield significant amounts of variation in reintroduction success (Godefroid et al., 2011). This approach is often used in large-scale projects aiming to sustainably enhance adaptation capability and survival prospects of restored populations in changing climate and environments (Havens et al., 2015; St. Clair et al., 2020). In particular cases, however, this approach might be counterproductive. This applies especially if a distinct population structure is intended as a conservation target. In reintroduction or reinforcement projects, which aim to preserve a unique genetic structure of natural populations, it is appropriate to collect seeds in a single population to reach the restoration goal (Betz et al., 2013; Kaulfuß and Reisch, 2017). Further, if a species exhibits strong population differentiation, the multi-source approach may cause outbreeding depression and, subsequently, viability decreases in the progeny (Montalvo and Ellstrand, 2001).

In our study, we examined the impact of restoration by reintroduction on the genetic variation of the critically endangered plant species *Myricaria germanica* Desv. This species naturally occurs on sand and gravel banks along Alpine streams. Due to anthropogenic river regulation the natural river dynamics changed drastically in the recent decades. As a consequence, habitat loss and a rapid decline of population density in *M.*

germanica occurred (Kudrnovsky, 2013; Harzer et al., 2018).

In *M. germanica*, several studies focused on its phenology and habitat (Lener et al., 2013; Egger et al., 2017), as well as population dynamics (Sitzia et al., 2016), the role of inundation frequency (Gostner et al., 2017), and the species´ colonization potential (Fink et al., 2017). Genetic variation and structure of natural populations were studied by Wiedmer and Scheidegger (2014), Werth and Scheidegger (2014) and Werth et al. (2014). Furthermore, several studies informed about reintroduction efforts, which took place in Austria and Italy (Kudrnovsky, 2013; Michelion and Sitzia, 2015), as well as in Germany (Harzer et al., 2018). Woellner et al. (2021) studied results of natural colonization on genetic makeup of a spontaneously established population.

The impact of reintroduction on the genetic variation of restored *M. germanica populations*, has, however, not been analysed so far. This means that data on the genetic diversity and structure of restored populations are not available at the moment – a gap we aim to close in our study. We analysed natural and restored populations of *M. germanica* along the alpine river Isar and compared their variation with populations along the river Lech to evaluate the genetic variation of the species in a broader range.

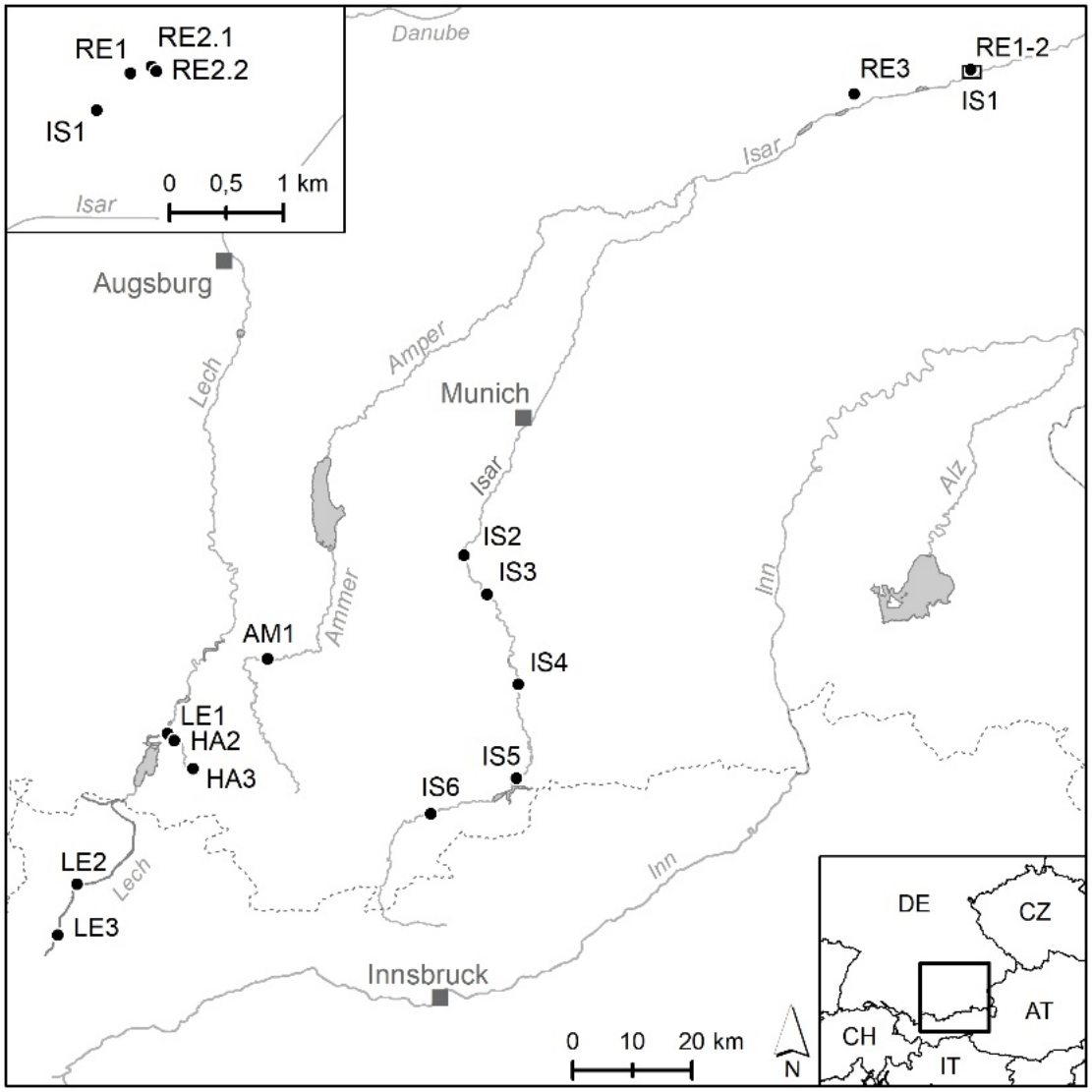
More specifically we addressed the following questions: (i) How large is genetic diversity within and genetic differentiation among natural populations of *M. germanica* along the rivers Isar and Lech? (ii) Are there differences between natural and restored populations concerning genetic diversity? How strong is genetic differentiation between natural and restored populations? (iii) Which conclusions can be drawn for the reintroduction of *M. germanica* along the lower course of river Isar and the seed sourcing strategy applied for restoration?

Materials & Methods

Species description

The German Tamarisk, *Myricaria germanica* L. (Desv.) is a perennial deciduous shrub, 60-200 (250) cm high and has a mixed mating system with frequent selfing (Kubitzki and Bayer, 2003; Werth and Scheidegger, 2014). Flowers develop in terminal or lateral racemes, which appear usually on main branches. The racemes may be vernal (produced early in the season from the woody stems) or aestival, i.e. produced later on the growth of the current year (Tutin, 1968). The species flowers from June to August and is pollinated by generalist insects (Tutin, 1968). The German Tamarisk is a light-dependent germinator. The structure of seeds enables a facilitated spread by wind. Moreover, seeds are floatable as well, thus, dispersal through water is possible (Bill, 2000). *M. germanica* is a diploid, 2n=24 (Váchová, M., Májovský, J., 1978). The species is adapted to a frequent change of dry and moist periods as well as periodic flooding. It usually occurs on sand and gravel bars and other open biotopes, especially along alpine streams. Here, the shrub is exposed to the influence of extreme physical conditions. *M. germanica* also occurs in secondary habitats, such as newly established gravel pits, former quarries or railroad embankments (Marinov et al., 2017). Its distribution ranges from

Figure 1. Geographic location of the study populations of the German Tamarisk (*Myricaria germanica*).



Central Europe including Fennoscandia extending from the Pyrenees, Central Italy, South Ukraine and Lower Wolga to the mountains of Central Asia. The life span varies approximately between 10 and 20 years (Ellenberg et al., 2010). In the last decades, several reintroduction projects aimed to prevent the species from final extinction (Latzin and Schratt-Ehrendorfer, 2005; Egger et al., 2010; Kudrnovsky, 2013; Lener et al., 2013; Riehl and Zehm, 2018).

Study design and sampled populations

We investigated sixteen populations in total, of which twelve were natural, three restored and one offspring population descending from one of the restored populations (Fig.1). The natural populations comprised i) six populations growing along the river Isar, ii) five populations along the river Lech and iii) one remaining natural population situated at the river Ammer. One of the natural Isar populations (IS1) was used as a seed source for

restoration. We investigated three restored populations originating from IS1. One of them (RE2.1) produced progeny, which spontaneously emerged in its vicinity. We enclosed this offspring population (RE2.2) into the study. If available, sixteen plants per population were sampled. Terminal branches lacking buds or flowers were collected, wrapped in teabags and immediately dried over silica gel. Dried plant material was stored in boxes containing an active silica gel until the DNA extraction.

Restoration took place on the lower course of the Isar River. Here, the last surviving natural population was used as a seed source. It was composed of mostly senile shrubs, which flowered and fruited unregularly. Despite the small and shrinking size of the population, it was used as a single source to preserve the purity of the seed origin. By doing this, a strict implementation of native genotypes was kept on the local scale (Bischoff et al., 2006; Mijnsbrugge et al., 2010). Mature diaspores were collected during several consecutive years and seeded immediately after collection. Plants were cultivated in a nursery in the same region for two years. After that, the young shrubs were planted along the gravel pits situated within the flood-plain area of the Isar River. The populations RE1 and the RE2.1 were located near the natural seed source population IS1, whereas the RE3 was planted twenty km away from it (Fig.1).

Molecular analyses

We analysed a total of 228 plants (14-16 individuals per population; in populations consisting of a lower number of plants we confined our analysis to the number of available individuals). Genomic DNA for AFLPs was isolated from dried plant material following the CTAB protocol (Rogers and Bendich, 1994), adapted as described in previous studies (Reisch et al., 2005). Concentration of DNA stock solutions were diluted with water to 7.8 ng/µl and used for AFLP analyses. The AFLP analyses were conducted according to the protocol from Beckmann Coulter, including slight modifications as described in former studies (Reisch, 2007; Bylebyl et al., 2008).

Pre-selective amplifications were run with just one selective nucleotide (MseI-C and EcoRI-A).

Selective amplification was carried out using three primer combinations MseI-CAA/EcoRI-AAC (D2), MseI-CTA/EcoRI-AAG (D3), MseI-CAT/EcoRI-ACA (D4). The amplification products were diluted as follows: 2-fold (D2) and 5-fold (D4) using TE0.1 buffer for DNA. The fluorescence-labelled samples were separated by capillary gel electrophoresis on an automated sequencer (GenomLab GeXP, Beckman Coulter). Raw data were examined applying the GeXP software (Beckman Coulter) and analysed using the software Bionumerics 4.6 (Applied Maths). Across all samples, each band was scored as either present or absent. Estimation of genotyping error rate was used to detect reproducibility of molecular analyses (Bonin et al., 2004) with a result of 4.57%.

AFLP statistics

Based on the AFLP data, a binary matrix was created using the software Bionumerics 4.6 (Applied Maths). Present bands of a particular length were classified as 1, in case of absence as 0. Based upon the 0/1 matrix,

the percentage of polymorphic bands across the whole dataset was calculated as a ratio n_i/N, where n_i is the number of bands which are polymorphic N represents the total number of fragments (bands). To reduce statistical bias, all monomorphic bands across all individuals were then excluded from following analyses (Keiper and McConchie, 2000; Li et al., 2005).

Genetic diversity within populations was calculated using program AFLPsurv (Vekemans (2002). Allelic frequencies were computed using Bayesian method with non-uniform prior distribution (Zhivotovsky, 1999). The approach of Lynch and Milligan (1994) was employed which uses Nei´s gene diversity (H) as a measure of genetic diversity, computed according to formula $H=1-\sum(p_i)^2$ where p_i represents the allele frequency (Nei, 1973). We tested the impact of population size on genetic diversity applying a Spearman´s rank correlation coefficient.

Differences in genetic diversity between the natural populations along the Isar River and restored populations were tested using the T-test implemented in R (RStudio-1.1.414 2009). We carried out the Shapiro-Wilk normality test beforehand.

Analyses of molecular variance (AMOVA) based on pairwise Euclidian distances between samples were conducted in a program GenAlex 6.41 (Peakall and Smouse, 2006). Significance levels were based on 999 permutations. We tested differentiation between the two river catchments, among populations within the catchments and within populations. Pairwise Φ_{PT} values for all populations were calculated. We conducted the Mantel test to assess the isolation-by-distance (IBD). To do so, pairwise genetic distances (ΦPT) among populations were correlated with geographical distances between the populations (Mantel, 1967).

Distances among populations were computed as Nei´s standard (Ds) with non-uniform prior distribution of allele frequencies using AFLP-surv (Vekemans, 2002). Based on Ds distances, a consensus Neighbor-Net graph was constructed applying the program SplitsTree4 (Huson and Bryant, 2006).

Patterns of genetic similarities between individuals were analyzed in the software GenAlEx 6 (Peakall and Smouse 2006) using principal coordinate analyses (PCoA) based on a squared Euclidean distance matrix.

The genetic structure of populations was inferred using Bayesian cluster analysis implemented in STRUCTURE 2.3.4. (Pritchard et al., 2000, Pritchard et al., 2009). The population structure of the data set was assessed and individuals were assigned to groups. Assuming that the data set consists of an unknown number of K groups, 100.000 Markov Chain Monte Carlo simulations with a burn-in period of 10.000 iterations were run to identify the number of groups. Analyses for the predefined value of K were computed 20 times for each value of K from 1 to 16 (Falush et al., 2007). We applied the program Structure Harvester (Earl and vonHoldt, 2012b) to summarize results. Group assignment was an *ad hoc* quantity procedure computing ΔK (Evanno et al., 2005). The best estimate of K for the given data set was specified according to the model, which gave the consistent results for multiple runs as well as the highest probability of the data. To assess gene flow among the studied populations as well as immigration events from

outside, we employed the program AFLPOP 1.2 (Duchesne and Bernatches, 2002). An assignment test implemented in this program calculated, which population was the most probable source for a particular individual. We used settings as follows: the allocation procedure of unknown origin; marker frequencies of zero were replaced according to formula [1/(sample size +1)]; allocation procedure of individuals was conducted at three levels, employing three various minimal log-likelihood differences, MLDs (Raffl et al., 2006; Pollux et al. 2009). AFLPOP allocates an individual to a source population X if the likelihood of this individual within X is at least 10^{MLD} times as high as that of the next most likely source population (Duchesne and Bernatches, 2002). The MLD were set at 0.5 (i.e. an individual was only allocated if its likelihood was 3 times as large as that of the next largest for an allocation to take place). When testing allocations under less rigorous conditions, MLDs were set at 0.3 (i.e. 2 times more likely) and 0 (an individual was allocated to the most probable source population). CA, i.e. correctly assigned specimen, means that an individual was allocated to its own population of origin. MA, mismatched assigned specimen was an individual most probably originating from other study population. NA, i.e. not assigned, means that an individual was most likely originating from a population not included in our study. The number of simulated genotypes for calculating P values was set to 500.

Results

Genetic diversity

AFLP genotyping of 229 plants resulted in 209 fragments (CAA-AAC: 67 fragments, CTA-AAG: 71 fragments, CAT-ACA: 71 fragments), of which 44.02 % were polymorphic. Genetic diversity within the populations of the Lech catchment, measured as the Nei´s Gene Diversity H ranged from 0.11 to 0.20 (H_{MEAN} = 0.13±0.04). The highest value of genetic diversity showed the population LE1 located at the estuary of the Halblech River into the Lech River (Tab.1). Genetic diversity H within the natural populations the Isar catchment varied from 0.10 to 0.19 (H_{MEAN} 0.13 ± 0.03). The highest value reached the population IS1 situated on the lower course of the river Isar. Genetic diversity of the natural population along the Ammer River was 0.07. Within the restored populations, genetic diversity ranged from 0.09 to 0.14 (H_{MEAN} = 0.11±0.02). Genetic diversity within the restored and the natural populations along the Isar River was similar, the difference was not significant (t-test, $p>0.05$). We found no significant positive correlation between the population size and genetic diversity. The natural offspring population RE2.2 situated next to the restored population RE2.1 harboured similar levels of genetic diversity as the natural seed source population IS1 ($H_{RE2.2}$=0.17, H_{IS1}=0.19).

Table 1. Genetic variation within populations of the German Tamarisk. Number, name, abbreviation, acronym of natural/restored populations (N/R) and region (SW, Swabia; TIR, Tirol; LB, Lower Bavaria; UB, Upper Bavaria). Given are also geographic position (latitude and longitude) of the study populations, an indication of the population size (PS), the number of analysed individuals (n) and the genetic variation within populations measured as unbiased Nei's Gene Diversity (H).

Nr	Name	Abb.	N/R	Reg.	Lat. (N)	Long. (E)	PS	n	H
Natural Lech									
1	Helfenwang	LE1	N	SW	47.658836	10.782436	1000	15	0.20
2	Forchach	LE2	N	TIR	47.429682	10.586864	100	16	0.12
3	Martinau	LE3	N	TIR	47.352924	10.545933	30	14	0.11
4	Eschenberg	HA2	N	SW	47.647453	10.796745	300	15	0.09
5	Im Laich	HA3	N	SW	47.605983	10.840262	50	15	0.12
Mean Lech									**0.13±0.04**
Natural Isar									
6	Rosenau	IS1	N	LB	48.658284	12.572688	5	5	0.19
7	Puppling	IS2	N	UB	47.930992	11.438931	200	15	0.10
8	Geretsried	IS3	N	UB	47.873019	11.490216	300	16	0.16
9	Gaissach	IS4	N	UB	47.737731	11.561908	50	16	0.11
10	Sylvenstein	IS5	N	UB	47.596638	11.558305	100	14	0.11
11	Ochsensitz	IS6	N	UB	47.54244	11.368965	>2000	16	0.13
12	Kohlbach	AM1	N	UB	47.772878	11.002495	15	13	0.07
Mean Isar									**0.13±0.03**
Mean natural									**0.13±0.04**
Restored Isar									
13	Rosenau	RE1	R	LB	48.661202	12.576762	-	16	0.09
14	Rosenau	RE2.1	R	LB	48.661710	12.579271	-	16	0.09
15	Postau	RE3	R	LB	48.626697	12.316344	-	13	0.14
Mean restored									**0.11±0.02**
Natural offspring population of the RE2.1									
16	Rosenau	RE2.2		LB	48.661710	12.579271	-	15	0.17
T-test (Isar and restored)									**p>0.05**

Table 2. Molecular variance among populations of *Myricaria germanica,* calculated using analyses of molecular variance (AMOVA). Df indicates degree of freedom, SS the sum of squares, MS the mean squares, % the proportion of genetic variation, Φ_{PT} the level of genetic differentiation. Levels of significance are based on 999 iteration steps and are indicated by three ($p < 0.001$), or one ($p<0.05$) asterisk.

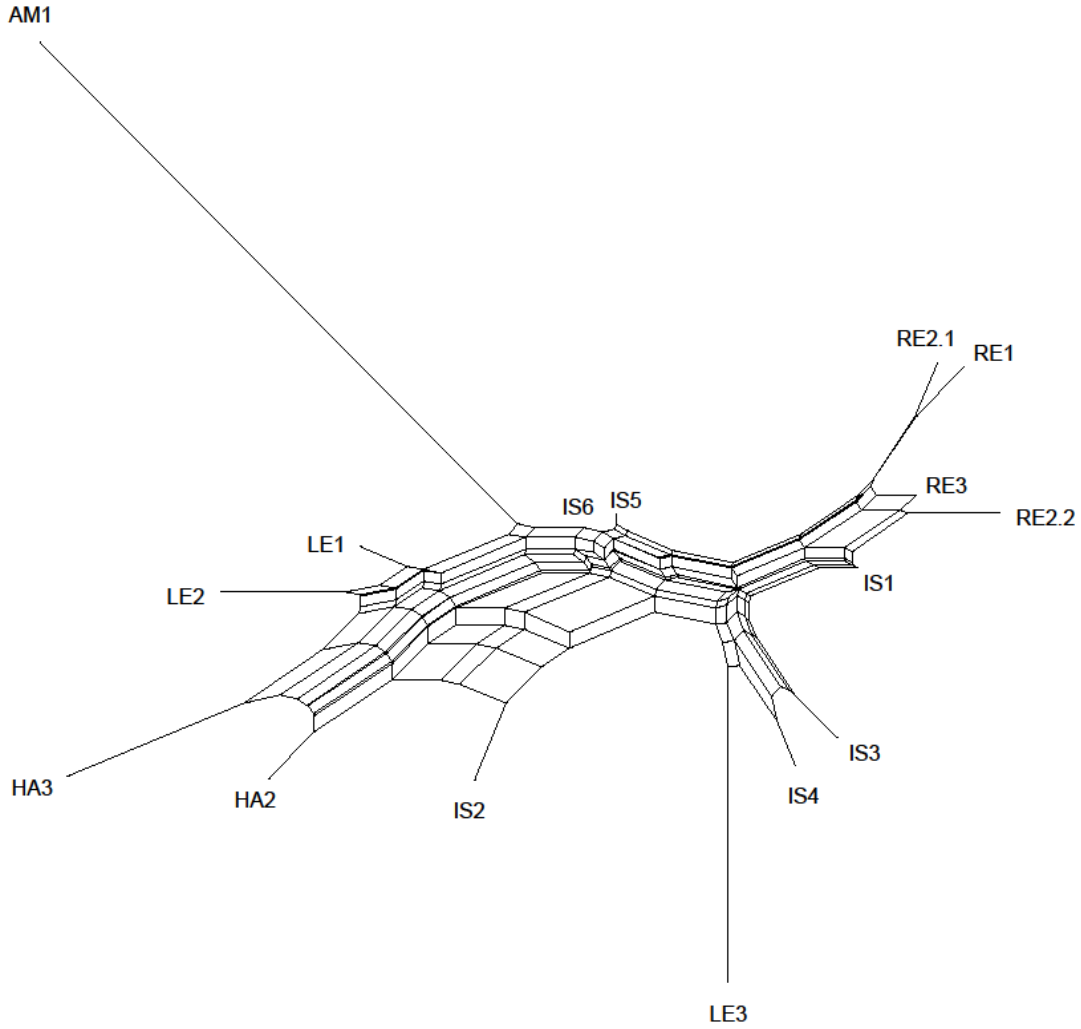
Source of variation	df	SS	MS	%	Φ_{PT}
All natural populations					
Among populations	11	269.5	24.5	31	0.31***
Within populations	158	518.1	3.3	69	
Isar (without Ammer)					
Among populations	5	79.5	15.9	21	0.21***
Within populations	76	267.8	3.5	79	
Lech					
Among populations	4	94.9	23.7	29	0.29***
Within populations	70	232.3	3.3	71	
Lech and Isar					
Between the Lech and the Isar River	1	36.8	36.8	4	
Among Populations within the groups	10	232.8	23.3	24	0.32***
Within Populations	158	518.1	3.2	72	
Restored and the natural population IS1					
Among Groups	1	6.3	6.3	8	
Among Populations	2	9.8	4.9	4	0.12*
Within Populations	46	137.1	2.9	88	

Genetic differentiation, structure and gene flow among populations

Genetic differentiation among all the natural populations measured as Φ_{PT} was 0.31. Genetic differentiation among the Isar populations (without its tributary, the Ammer River) was Φ_{PT}=0.21, among the Lech and Halblech populations Φ_{PT}=0.29. The three-level AMOVA revealed a differentiation of 4% between the Isar and the Lech catchment (Tab.2). The difference between the natural seed-source population IS1 and the restored populations was modest (8%) and not significant, genetic differentiation among the restored populations was 4% (p=0.03). We found no significant differentiation between the natural offspring population RE2.2 and the natural seed source population IS1 (Tab. S1).

Among the natural populations, correlation between pairwise genetic distances Φ_{PT} and geographic distances was not significant. We computed both direct-line and along-river distances. The Isar and the Lech catchments were analysed together and separately. Within the Lech catchment, a slight but non-significant correlation between geographic and genetic distances was indicated (Mantel test R^2=0.0975, p=0.18).

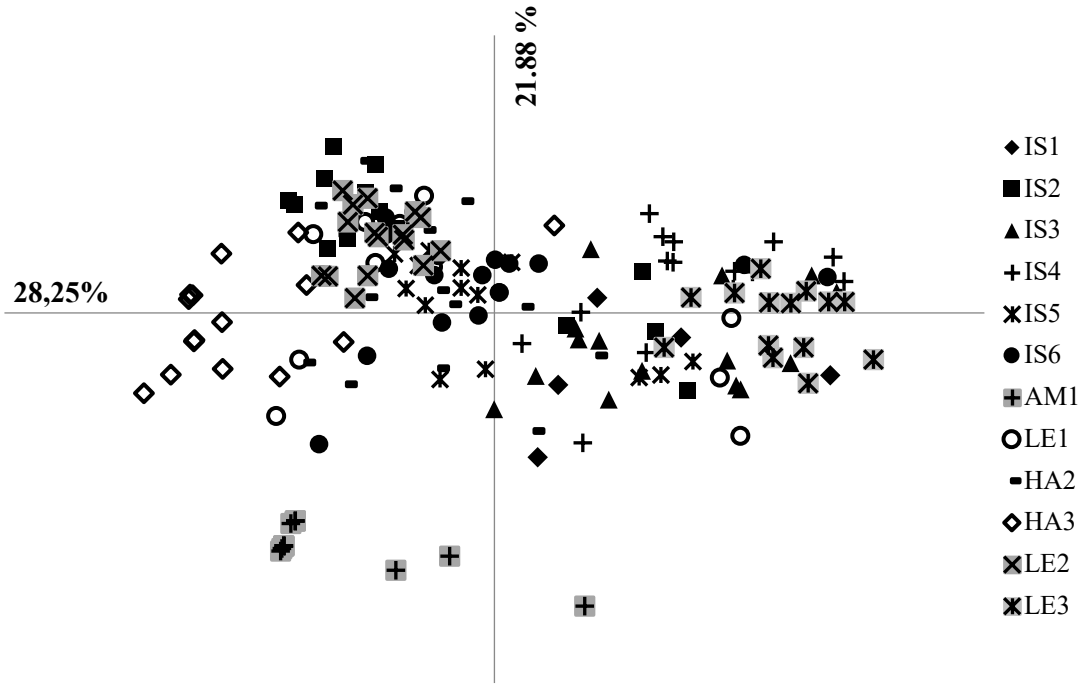
Figure 2. Neighbor-Network of all studied *M. germanica* populations based on the AFLP data. The natural seed-source population IS1 together with the restored populations RE1, RE2.1, RE3 and the natural offspring population RE2.2 are associated in one cluster, visible on the upper right side of the network.



The Neighbor-Net analysis revealed that the natural seed source population IS1, the restored populations RE1, RE2.1, RE3, and the natural offspring population RE2.2 formed one consistent cluster, visible on the right side of the graph (Fig.2). The rest of the populations did not reflect geographic configuration such as proximity or allegiance to a particular river catchment. Solely the Ammer population AM1 formed a separate branch distinct from all other populations.

Principal coordinates analysis was computed across all individuals from the natural populations of *M. germanica* (Fig.3).

Figure 3. Principal coordinates analysis computed across all individuals from the natural populations of *M. germanica*, based on AFLPs data. The first axis explained 28.25 % of variance, the second axis explained 21.88% of variance. The most natural populations are not clearly separated from each other, thus reflecting dispersal events a gene flow among the populations within and among the Isar and the Lech catchments



The first axis explained 28.25 % of variance, the second axis explained 21.88% of variance. The results of the PCoA analysis were similar to the NeighborNet as well as Bayesian analysis, i.e. we observed no population grouping according to the river catchment. The majority of the natural populations were not clearly separated from each other, with an exception of the population AM1.

The Bayesian cluster analysis Structure assessed the best partitioning of K=2 (Evanno et al., 2005). Within the two inferred clusters, populations from the both river catchments were mixed. The natural seed source as well as the restored populations were assigned to the same cluster (Fig.S1a, S1b).

(mean 8.8%) of individuals (from the populations IS3, IS4, IS5, IS6 and LE2) were

The assignment tests detected four groups of individuals. The first group originated from the same population, and was “correctly assigned” (Tab. S2). In some cases, a high percentage of individuals (from 60 to 100%) originated from their own population (IS2, IS3, IS4, AM, HL2, HL3 and LE3), but in other cases (IS1, IS5, IS6, LE1 and LE2) the percentage of individuals originating from the same site was low (from 7 to 20%). The second group of individuals did not originate from their own populations, i.e. these individuals were “mismatched allocated”. From this group, 13 to 40% (mean 23.5%) of individuals (IS1, IS2, IS5, LE1) were allocated to upstream populations, whereas 6 to 19%

allocated to downstream situated populations. Further, the third group of individuals

(7-20%, mean 12%) were allocated to populations outside of their own catchment (from the populations IS1, IS4, IS6, LE1, LE3 and HL2). Finally, the fourth group could not be allocated to any of the studied populations (0-81%, mean 33%) (Tab.4). All calculations were done using MLDs of 0.5 (Tab.2S).

Discussion

Genetic diversity and differentiation of the natural populations

Detected mean genetic diversity within the natural populations was in concordance with values previously reported for plant species with similar life history traits (Reisch and Bernhardt-Römermann, 2014). Genetic diversity in the Asian congener species Myricaria laxiflora varied more widely, but reached similar mean values (Liu et al., 2006). Previous research suggests that population size might be a good indicator of high genetic diversity within populations (Leimu et al., 2006). The positive correlation between the population census size and genetic diversity was postulated by theory and frequently observed (Frankham, 1996). However, in our study we did not find this relationship. A lack of correlation between the population size and genetic diversity was often observed in riparian species living in dynamic habitats, including *M. germanica* (Tero et al., 2003; Prentis et al., 2005; Liu et al., 2006; Werth and Scheidegger, 2014), as well as for non-riparian species (Dostálek et al., 2010; Kaulfuß and Reisch, 2017). Possible rea-sons may be a good dispersal potential of *M. germanica* as well as a considerably high seed output (Fink et al., 2017). In an extreme case, a large population may originate from a single individual. In line with this, the smallest natural population in our study IS1 contained the highest levels of genetic diversity. This population served as a seed donor of the restored populations.

Genetic differentiation among natural populations of *M. germanica* was comparable to levels of differentiation detected for rare taxa as well as for taxa with mixed mating system (Nybom and Bartish, 2000; Reisch and Bernhardt-Römermann, 2014). For the Asian congener *Myricaria laxiflora*, even higher levels of genetic differentiation were revealed among populations (Liu et al., 2006). Genetic differentiation between adjacent populations along the river Isar, measured as ΦPT, varied considerably (ΦPT ranged from 0.00 to 0.33). Along the river Isar, many natural populations disappeared due to habitat destruction. These gaps implicate missing “stepping stones” formerly enabling gene flow along the river corridor, which are now lacking. This pattern may also apply to widely natural habitats, for instance, along the river Tagliamento. Here, also high levels of isolation among natural populations of M. germanica were observed, caused by unsuitable dry conditions prevailing on the long stretches of the river course (Werth et al., 2014). Between the Isar and the Lech catchments, we observed low levels of differentiation, identifying dispersal events that likely occurred across the two inferred river systems (Fer and Hroudova, 2009).

Bayesian cluster analysis, as well as assignment tests, NeighborNet and the PCoA corroborated the results of the AMOVA analysis. Bayesian cluster analysis of population structure revealed two gene pools across all studied populations. Notably, the two gene pools were not confined to one of the studied

river catchments, i.e. contained populations from the both of them. Similarly, assignment tests suggested that several dispersal events between the Isar and the Lech catchments occurred. Comparable results were repeatedly reported in previous research on riparian and aquatic species (Fer and Hroudova, 2009; Ngeve et al., 2017) including *M. germanica*. Thus, our results did not give a hint for the `one catchment-one gene-pool` theory as proposed, tested and rejected by Werth and Scheidegger (2014). Dispersal events were most probably facilitated by zoochory or human activities.

Assignment tests revealed a high proportion of individuals in populations originating from the same site, especially within the populations on the middle course of the river Isar, along the river Halblech and Ammer, as well as one population on the upper course of the river Lech (LE3). Here, the biggest portion of the seed output sunk within the same population. This is in concordance with research on wind dispersal of *M. germanica,* where dispersal kernel reached 10-25 m distance from the fruiting shrub (Fink et al., 2017). On the other hand, five populations in our study showed much higher proportion of individuals from outside the own population. These populations occupied more dynamic or downstream situated segments of the studied rivers. Here, an input of diaspores from adjacent populations or accumulation of genotypes from upstream river sections seem to be the most probable reason.

Principal coordinate analysis showed that almost all of the natural populations with one exception (AM1) were not clearly separated from each other, thus reflecting dispersal events and gene flow among them. The NeighborNet analysis also supported these findings. Further, we found no significant correlation between genetic and geographic distances among the six populations along the river Isar. The lack of this correlation was frequently observed for riparian and aquatic species (Prentis et al., 2005; Pollux et al., 2009; Honnay et al., 2010), albeit it was identified for *M. germanica* in Switzerland (Werth and Scheidegger, 2014). In our study, the lack of correlation might be a consequence of both long-distance dispersal events, and, in some populations, the result of the absence of gene flow due to the missing “stepping stones”.

Genetic diversity and differentiation of restored populations

Generally, within the restored populations, we detected a slightly lower level of the genetic diversity compared with the group of the natural populations along the river Isar. The difference was, however, not significant. A decreased level of genetic diversity in restored populations was reported in many studies (Liu et al., 2008; Zucchi et al., 2018). On the contrary, restored populations frequently maintain similar or even higher levels of genetic diversity in comparison with their source populations (Betz et al., 2013; Millar et al., 2019a; Millar et al., 2019b). General conclusions are, therefore, difficult to draw, since the effects of restoration on genetic diversity seem to depend on species and project specific traits. The most probable reason for the lower levels of genetic diversity within the restored populations of *M. germanica* seem to be fluctuations in seed production of particular individuals within the seed source population. When seeds were collected for restoration, only a part of the shrubs flowered and fruited regularly, due to the senile stage of their life cycle. Thus, it

was not always possible to cultivate seedlings from the entire source population every year.

In the close vicinity of RE2.1, a spontaneous offspring population RE2.2 emerged in the years 2015-2016. Notably, the levels of genetic diversity comprised in the offspring population were nearly twice as high as detected in R2.1. Most probably, the genetically variable population IS1 acted as a gene contributor and enhanced genetic diversity of the offspring population R2.2 via pollen transport.

The seed source population IS1 and the restored populations were genetically not differentiated significantly, which means that seed collection and plant production for restoration did not cause genetic drift. Negligible levels of genetic differentiation between natural and restored populations were reported also in previous research (Pierson et al., 2007; Ritchie and Krauss, 2012; Betz et al., 2013; Millar et al., 2019a; Millar et al., 2019a).

The NeigborNet tool confirmed genetic similarity of the natural seed-source population IS1 and the restored populations RE1, RE2.1, RE3 including the offspring population RE2.2. However, we observed differences in genetic differentiation between the seed source population IS1 and the respective restored populations. Genetic differentiation was higher between IS1 and RE1, as well as between IS1 and RE2.1, respectively, but zero between IS1 and RE3. This may be explained not only by unequal seed production among the seed donor individuals, but possibly also by viability differences of collected seeds (Liu et al., 2008; Basey et al., 2015). A similar situation occurred during the restoration of the endangered plant species Pulsatilla vernalis. Here, slight differences in genetic differentiation between natural and restored individuals were found in three of nine populations, which is ascribed to the fact that with-in the three populations not all individuals were fertile and could be sampled (Betz et al., 2013). The population RE2.2, which emerged as an offspring of the population RE2.1 exhibited surprisingly high genetic similarity with the natural source population IS1. This supports our assumption that the pollen transport occurred between the nearby populations IS1 and RE2.1. We therefore emphasize the importance of presence of a donor (source) population near the restored stands, especially in long-lived species (Pierson et al., 2007). The spatial proximity of such a genetically di-verse source population can counteract a possible founder or bottleneck event in a progeny of restored populations.

Our study revealed no clear pattern of genetic differentiation among natural populations of the species, not even among the two studied river catchments, which underlines the fact that alpine rivers are a highly dynamic system. It can be strongly assumed that in widely natural river systems, gene flow among *M. germanica* populations could be high. We detected dispersal events that took place across populations irrespective of distance or river catchment. This implicates that multiple seed sourcing across natural populations would not jeopardize the natural genetic pattern of *M. germanica* in the studied area and could be applied along with seed sourcing in local, genetically variable populations. Both approaches would enhance genetic diversity of reintroduced populations, und thus contribute to the long-

term robustness of this iconic alpine river species in changing environments.

Implications for practice

- It is of advantage, where possible, to plant new stands close to the source population(s) to enhance genetic diversity within restored populations. Genetically diverse source populations may act as gene contributors, e.g. via pollen transport.

- Local, genetically variable populations can be appropriate seed sources for future restoration efforts. When possible, as many individuals as feasible should be sourced for seeds to capture genetic diversity comprised in a natural population. This approach would increase the long-term restoration success.

- Multiple seed sourcing from different populations can also be considered to increase genetic diversity of restored populations. In a highly dynamic river system, in which long-distance dispersal events occur, this would not harm the natural genetic pattern of *M. germanica*.

RESTORATION OF *ARMERIA MARITIMA* SSP. *ELONGATA* POPULATIONS BY SOWING AND PLANTING IN A PRACTICAL 20-YEAR RESTORATION PROJECT: IS THERE AN IMPACT ON GENETIC VARIATION?

The natural habitat of *A. maritima* ssp. *elongata*, the nature reserve “Sanddünen bei Offenstetten”, Bavaria, Southwest Germany

Flowering *Armeria maritima* ssp. *elongate*

Abstract

Reintroduction is a frequently used method to restore populations of endangered species. However, it has hardly been tested whether there is a pronounced genetic impact resulting from a propagule type used for restoration. Here, we carried out a pilot study based on results of a 20-years practical experiment. Restoration efforts aimed to start new populations of an endangered herb *Armeria maritima* ssp. *elongata.* The project took place in a nature reserve protecting comprising a declining nutrient-poor sand-dune habitat in Bavaria, SE Germany. To address the knowledge gap, we report the results of a restoration study in which several populations were restored about 20 years ago, using seeds and/or plugs.

To evaluate if there was a strong effect of used propagules on genetic properties of restored populations, we compared three groups of seeded, planted and seeded & planted populations of *A. maritima* ssp. *elongata.*

Using molecular markers (AFLPs), we observed slightly higher levels of genetic variation in restored compared to natural populations, but the difference was not significant. Genetic variation was comparable in populations restored by different techniques and genetic differentiation among natural and restored populations was negligible. Based upon our results, we conclude that both sowing and planting are restoration techniques, which can be used to create variable populations genetically comparable to the natural source populations.

Our study provides a first piece of evidence that both seeds and plants can be applied successfully in population restoration projects. Moreover, species with different life history traits such as mating system, frequency (rarity) or life span should be used to further test the impact of propagules under different experimental settings.

Key words:

Conservation, genetic diversity, genetic differentiation, population enhancement, population size, reintroduction, restoration

Introduction

In the last decades, natural and semi-natural habitats suffered from severe anthropogenic pressure, subsequent fragmentation and decrease in area (Thompson et al., 2019). This applies in particular to nutrient-poor sandy biotopes harbouring numerous highly endangered species, particularly in Central Europe. Especially due to afforestation, land-use intensification and nitrogen deposition, formerly abundant plant species became rare (Lauterbach et al., 2017; Jones et al., 2017; Felipe-Lucia et al., 2020).

As the destruction of wild populations accelerates, so does the need for scientifically informed restoration work (Benayas et al., 2009; Vitt et al., 2010; Havens et al., 2015). One of the major objectives of restoration efforts is to found populations capable to persist and propagate on their own in the wild, as well as to maintain genetic structure of source populations. Conservation science supports the use of genetically diverse germplasm for restorations purposes. High levels of genetic diversity are a vital component of population fitness, resistance against pests and diseases as well as ecosystem services Cardinale et al., 2012; Reynolds et al., 2012). Genetic diversity is also a prerequisite to persist and reproduce under a variety of natural conditions in the wild (Reusch et al., 2005).

In the recent years, new guidelines emerged on how to successfully carry out restoration work using tools of restoration genetics (IUCN/SSC, 2013; Basey et al., 2015; Durka et al., 2019; Havens et al., 2015; Espeland et al., 2017; Nagel et al., 2019a; IUCN/SSC, 2013 Godefroid et al., 2016, compare Mijangos et al., 2015a). However, practice often lags behind theory regarding conservation of genetic diversity in restored populations. Genetic erosion may happen at any stage of the restoration process (Godefroid et al., 2011; Basey et al., 2015). Many studies deal with genetic stochasticity processes such as genetic drift and bottlenecks due to insufficient number of sourced individuals, or founder effect caused by small effective size of restored populations and subsequent genetic drift, inbreeding and loss of evolutionary potential (Leimu and Fischer, 2008; Frankham et al., 2014).

Restoration success may be hampered by demographic processes such as attrition of propagules between initial planting/sowing and reaching maturity. This may be intensified by low genetic diversity, demographic stochasticity and a small population size (Allee effect) (Bowles et al., 2015). These pitfalls may be compensated in different way depending on the restoration method (sowing/planting). Thus, planting can balance the negative effects due to higher survival rates than seeds (Bowles et al., 2015; Dorogina et al., 2014) and due to achieving reproduction maturity earlier, especially in long-lived species. (Albrecht and Maschinski, 2012; Bowles et al., 2015;). Sowing, in turn, produces naturally recruited plants with a long-term viability prospect. Moreover, sowing is easier and less costly procedure than production of nursery-grown plants, which, in turn, may select for less vital individuals (Lesica and Allendorf, 1999).

However, the actual impact of restoration by sowing or planting on the genetic diversity and differentiation of founded populations was investigated only scarcely so far (St. Clair et al., 2020; Bowles et al., 2015).Presumably, in can be assumed that

populations initiated using seeds contain higher levels of genetic diversity, due to the higher number of applied propagules in comparison with pre-grown plants. Populations started with pre-grown plants, in turn, may contain lower levels of genetic diversity due to constraints in the course of cultivation process (Ensslin and Godefroid, 2019).

Regarding genetic differentiation, Kucera et al. (2021) detected shifts in representation of source populations in a multiple-source restoration experiment. In various restoration sites, seeds originating from multiple sources exhibited unequal establishment rates. Hence, resulting source population composition varied vastly, implying that local conditions facilitated some populations over others. It can therefore be assumed, that populations started using seeds will probably show higher levels of differentiation due to various conditions prevailing on a particular site. This presumption, however, was not demonstrated yet.

For this reason, we analysed results of a practical reintroduction project, which took place in Bavaria, SE Germany, in a nature reserve protecting rare nutrient-poor sand dune habitats. In this region, one of the last natural populations of *Armeria maritima* ssp. *elonagata* are located. To safeguard its survival in future, a restoration project has been launched in 1998. In its course, several new populations were started using seed, young plants as well as using both types of propagules. The source and the restored populations occupied the same type of habitat. Population size has been regularly estimated. Since then, only one of the eight studied populations become extinct, most probably due to the extreme summer draught during the years 2018-2019.

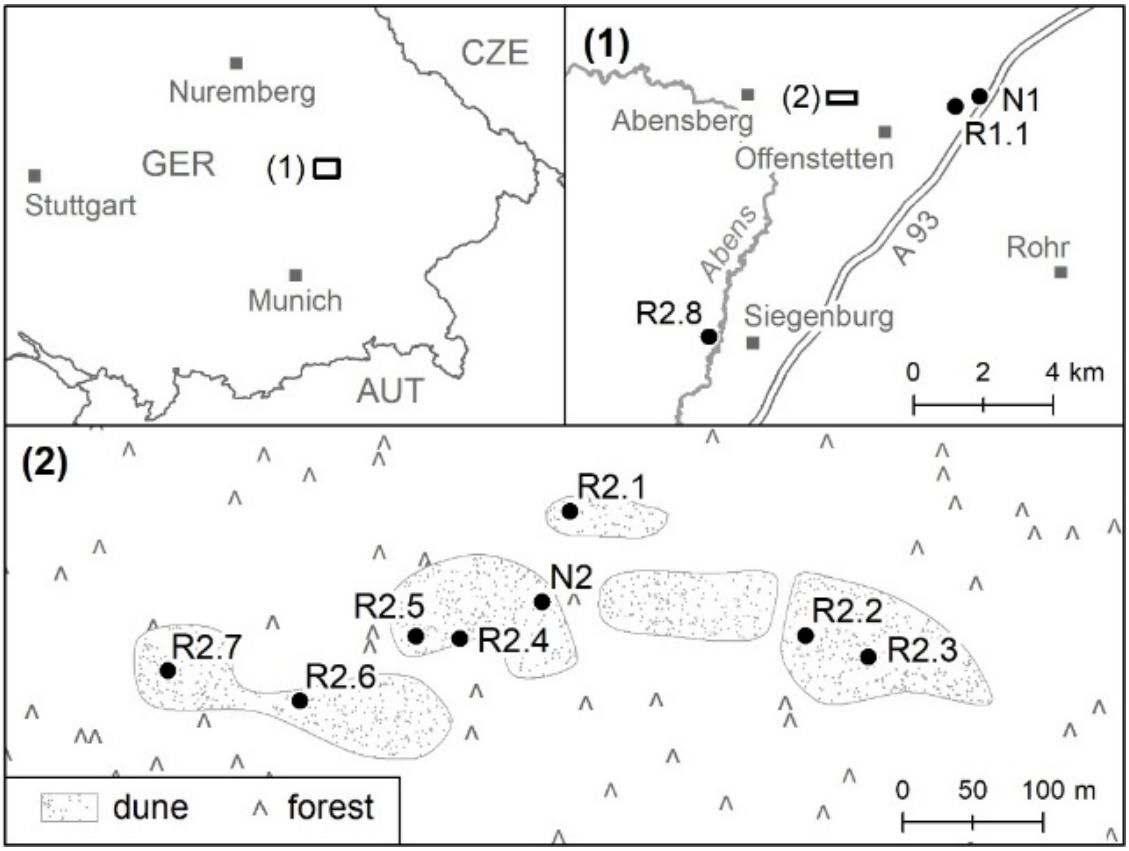
Hence, in our study, we addressed these gaps and asked the following questions: 1. Do the natural and restored populations differ regarding levels of genetic diversity? 2. How high is genetic diversity and differentiation of the *ex situ* population in comparison with its source population? 3. How high is the differentiation among the natural and restored populations? 3. Is there any apparent, detectable impact of the reintroduction method on the genetic makeup of restored populations?

Materials and methods

Study species and design

Armeria maritima ssp. *elongata* (Hoffm.) Bonnier is a perennial, tufted herb confined to nutrient poor soils. The subspecies is predominantly distributed in dry sandy lowlands of the Central Europe, including Germany, Poland and the Czech Republic (Meusel et al., 1978; Kovanda, 1990). In South East Germany, it has a patchy distribution with a shrinking range and was included in the Red List of endangered vascular plants (Scheuerer and Ahlmer, 2003; Bayerisches Staatsministerium für Umwelt,Gesundheit und Verbraucherschutz, 2005). The breeding system of the genus *Armeria* involves

Fig.1. Geographic location of the analysed *Armeria maritima* ssp. *elongata* populations in the study region.



insect-pollinated plants with outcrossing mating system (Lefebvre, 1970; Lefebvre and Chandler-Mortimer, 1984a; Woodell and Dale, 1993). Seeds are usually dispersed by wind but dispersal distances reach only a few meters from maternal plants (Philipp et al., 1992). *A. maritima* (Mill.) Willd. possess a transient soil seed bank (Kleyer et al., 2008). The species can reach an age of over 20 years (Lefebvre and Chandler-Mortimer, 1984b). *A. maritima* ssp. *elongata* is a diploid with 2n=18 (Oberdorfer, 2001).

For our study we analysed natural and restored populations of *A. maritima* ssp. *elongata* near Siegenburg in South Eastern Germany (Fig. 1). The restoration project started in 1998. The study area involved sandy semi-dry and dry grasslands situated in natural sand dunes originating from the last glacial period, as well as on roadside embankments (Fig.1).

We assume that the analysed genetic diversity was still structured by the genotypes that were introduced (rather by that of the offspring). The reason is that the study species can flower a few years after seeding but older individuals produce more seeds and hence have a higher contribution to the population genetic properties.

Plant material for molecular analyses was sampled in natural (N) and restored (R) populations, as well as in one *ex situ* population. The *ex situ* population (R2ex) originating from the natural N2 was cultivated in the Botanical Garden of the Regensburg University since 2004.

The natural population N1 and its related restored population R1.1 were situated on roadside embankments (Fig.1). The natural population N2 and its related restored populations R2.1-R2.8 were located in a sand dune protected area. The shortest linear distance between the two natural populations N1 and N2 was four km from each other. The *ex situ* population R2ex originating from the natural N2 was cultivated in the Botanical Garden of the Regensburg University since 2004. Together with the N1 and N2 populations, the *ex situ* population R2ex was used as a supplementary seed source for the restoration (Tab.1). Restored populations were founded using three methods, (i) sowing, (ii) planting, and (iii) a combined method of sowing and planting.

Molecular analyses

For molecular analyses in each population, fresh leave material from sixteen individuals was collected in situ and dried in tea bags over silica gel. In lab, we analysed a total of 175 plants, fifteen to sixteen individuals per population.

To detect genetic variation, a genome-wide genotyping using AFLPs was employed (Vos et al., 1995). Genomic DNA for AFLPs was isolated from silica gel dried plant material following the CTAB protocol (Rogers and Bendich, 1994), adapted as described in previous studies (Reisch et al., 2005).

AFLP analyses were conducted according to the protocol from Beckmann Coulter, including slight modifications as formerly described (Reisch, 2007). Concentration of DNA stock solutions was measured using a spectrophotometer and the solutions were then diluted with water to a concentration of 7.8 ng/µl.

Double-strand adapters (Biomers) were prepared by adding equal volumes (0.25µl per reaction) of single-strand adapters (M-Ad1 and M-Ad2, each 40µM, and E-Ad1and E-Ad2, each 4µM respectively), heating up to 95°C for 5 min and cooling down to 22°C in a thermal cycler. A volume of 6.4µl DNA solution (7.8µg/ml) was digested by adding a 3.6 µl mixture containing restriction enzymes 2.5U MseI and 2.5U EcoRI. In the same step, adaptors were ligated using 0.5U T4 DNA ligase, 0.1x of ligase buffer (Thermo Scientific), 0.05M NaCl, 0.5µg BSA, 1µM MseI and 0.1µM EcoRI adaptor pairs. The following incubation was performed in a thermal cycler for 2h at 37°C, with the last step of enzyme denaturation at 70°C for 15 min. Products of this reaction were diluted 10fold using 1x TE buffer for DNA (820mM Tris-HCl, pH 8.0; 0.1mM EDTA, pH 8.0).

Subsequent polymerase chain reactions were performed in a reaction volume of 5µl. Pre-selective amplifications were run using 1µl of the diluted restriction-ligation product added to a mixture of 0.25µl primers (Biomers) with just one selective nucleotide (0.25µM MseI-C and 0.25µM EcoRI-A), 1.25µl H2O and a MastermixS for AFLP (PeqLab) containing buffer, dNTP and Taq-Polymerase. PCR parameters were set as follows: heating up to 94°C for 2min followed by 30 cycles of successive denaturing at 94°C for 20 s, annealing at 56°C for 30 s and elongation at 72°C for 2 min. The extension process was terminated for 2 min at 72°C and 30 min by 60°C, with a final cool down to 4°C. Products of the pre-selective DNA amplification were diluted 20fold using TE0.1 buffer for DNA. Selective

amplifications were performed using three primer combinations MseI-CAG/EcoRI-ACC (D2), MseI-CAG/EcoRI-AGG (D3), MseI-CAG/EcoRI-ACA (D4). PCR reactions were run in a total reaction volume of 5µl containing 1.25µl water, 2.5µl MasterMix S (PeqLab comprising buffer, dNTPs and Taq polymerase), 0.25µl of selective 0.25µM MseI and 0.05 µM EcoRI primers (Biomers) and 0.75µl diluted product of pre-selective amplification. We used EcoRI primers, labeled with fluorescent dye (D2, D3 and D4) to enable detection of PCR fragments.

Selective amplifications were carried out according to the following touchdown profile: 2 min at 94°C, 10 cycles of denaturation for 20 s at 94°C, annealing 30 s initiated at 66°C and then reduced for 1°C in each following cycle, 2 min elongation by 72°C. Subsequently, 25 cycles of denaturation for 20 s at 94°C, annealing for 30s at 56°C and 2 min elongation by 72°C, completed by a final extension for 30 min at 60°C and a cool down to 4°C.

Products of the selective PCRs were diluted as follows: 2fold (D2) and 5fold (D4) using TE0.1 buffer for DNA. Subsequently, the PCR products of a given sample (volume of 5 µl, each D2, D3 and D4) were pooled and added to a stop solution consisting of 2µl 3M sodium acetate, 2µl 100 mM Na2EDTA and 1µl glycogen. After precipitation, DNA was pelleted by adding of ethanol (96%, -20°C) in a centrifuge (20 min at 14000 g by 4°C). The supernatant was poured off and the pellets were washed using Ethanol (70%, -20°C). The second centrifugation was conducted at the same conditions (20 min at 14000 g by 4°C). Finally, the pellets were vacuum-dried in vacuum drier and dissolved in a mixture of 24.8 µl Sample Loading Solution (SLS) and 0.2 µl CEQ DNA Size Standard 400 (Beckman Coulter). The fluorescence-labelled samples were separated by capillary gel electrophoresis on an automated sequencer (GenomLab GeXP, Beckman Coulter). Raw data were examined applying the GeXP software (Beckman Coulter), exported as synthetic gel files (.crv files) and analysed applying the software Bionumerics 4.6 (Applied Maths). A genotyping error rate of 2.39% was calculated to control the AFLP procedure quality.

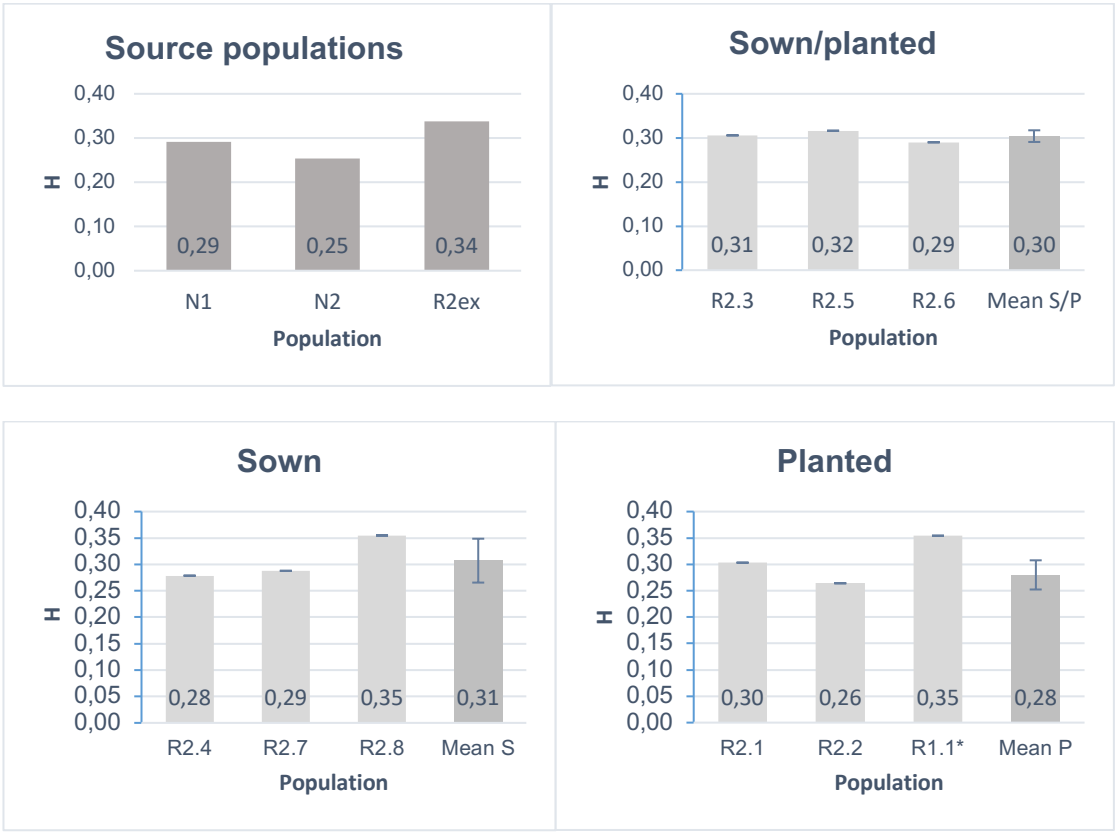
Statistical analyses

Based on the AFLP data, a binary matrix (0/1) was created using the software Bionumerics 4.6 (Applied Maths). If absent, fragments of a particular length were classified as zero and in case of presence as one. Using the 0/1 matrix, the percentage of polymorphic loci (PPL) was calculated across the whole dataset as a ratio n_i/N, where n_i is number of fragments which contain polymorphism, N represents the full number of fragments (loci). Genetic diversity within populations was computed as Shannon´s Information Index calculated following the formula $SI = -\Sigma(p_i * \ln(p_i))$, where p_i is the frequency of the ith fragment in the respective population, based on all scored AFLP fragments. Here, we employed the program Popgene (Yeh et al., 2000). Further, we computed Nei´s gene diversity according to a formula $H=1-\sum(p_i)^2$, where p_i represents the allele frequency (Nei, 1973;

Tab.1. Type of population (natural/restored, N/R), location, sample size (n), population size (p.s.), source population, restoration method, geographic position, and average diversity over loci measured as Nei´s Gene Diversity and Shannon´s information index (SI). *The population R1.1 was not included into the mean calculation of „Planted“, because it originated from another source population N1.

Pop.	Location	n	p.s.	Source	M.	Lat. (N)	Long.	H	SI	DW
Natural populations										
N1	Bachl	16	1000	---	---	48.8143	11.9412	0.29	0.29	0.72
N2	Offenstetten	16	100	---	---	48.8154	11.8875	0.22	0.25	0.69
Mean natural ±SD								**0.26**	**0.27**	**0.71**
SD								0.05	0.03	0.02
Sown populations										
R2.4	Offenstetten	16	20	R2ex	S	48.8152	11.8868	0.27	0.28	0.67
R2.7	Offenstetten	16	18	N2,	S	48.8151	11.8839	0.27	0.29	0.69
R2.8	Dassfeld	16	420	R2ex	S	48.7554	11.8323	0.32	0.35	0.66
Mean sown ±SD								**0.29**	**0.31**	**0.67**
SD								0.03	0.04	0.02
Planted populations										
R2.1	Offenstetten	16	20	N2	P	48.8160	11.8883	0.29	0.30	0.66
R2.2	Offenstetten	16	20	N2,	P	48.8120	11.9322	0.26	0.26	0.67
R1.1	Scheuern	16	1000	N1	P	48.8110	11.9293	0.34	0.35	0.68
Mean planted ±SD								**0.28***	**0.28***	**0.67**
SD								0.02	0.05	0.01
Sown and planted populations										
R2.3	Offenstetten	16	74	N2	S/P	48.8150	11.8909	0.30	0.31	0.68
R2.5	Offenstetten	16	40	N2,	S/P	48.8151	11.8867	0.30	0.32	0.71
R2.6	Offenstetten	15	48	N2,	S/P	48.8149	11.8850	0.29	0.29	0.71
Mean sown&planted ±SD								**0.30**	**0.30**	**0.70**
SD								0.01	0.01	0.02
Mean restored								**0.29**	**0.31**	**0.68**
SD								0.03	0.03	0.02
Ex situ population										
R2ex	Regensburg	16		N2		48.9931	12.0924	0.31	0.34	0.67

Fig.2. Average gene diversity over loci for natural (N1, N2), ex situ (R2ex), sown, sown/planted and planted populations of *Armeria maritima* ssp. *elongata,* measured as Shannon´s information index (SI). The mean values for each method (sown, planted, sown/planted) are shown in the last bar, as well as the standard deviation (* the R1.1 was not included into the mean calculation of the group „Planted“, because it originated from the other source population N1)



Lynch and Milligan, 1994). We used the program AFLPsurv (Vekemans, 2002). Allelic frequencies were computed using Bayesian method with non-uniform prior distribution (Zhivotovsky, 1999). Correlation between population size and genetic diversity was tested using Spearman´s rank correlation coefficient (R Core Team, 2014). Additionally, Frequency-down-weighted-marker-values (DW) were computed according to Schönswetter and Tribsch (2005). Genetic differentiation among populations and groups of populations was detected using analysis of molecular variance AMOVA (Excoffier et al., 1992) implemented in the program GenAlex 6.41 (Peakall and Smouse, 2006). Computation based on pairwise Euclidian distances between samples. Significance values related to variance components rest upon 999 permutations of individuals supposing no genetic structure. We tested partitioning of genetic variation within populations, among populations and between regions, applying the two-level and the three-level hierarchical AMOVAs.

Bayesian cluster analysis was conducted using the program STRUCTURE 2.3.4. (Pritchard et al., 2000, Pritchard et al., 2009). The population structure of the whole data set was inferred based on the clustering of

Tab.2. The results of the analyses of molecular variance (AMOVA) for natural and restored populations of *A. maritima* ssp. *elongata*, within and among populations based on 158 AFLPs fragments. Levels of significance were based on 999 iteration steps and indicated by asterisks (p<0.001, i.e. ***; p<0.01, i.e. **; n.s., i.e. nonsignificant).

Source of molecular variation	df	SS	MS	%	Φ_{PT}
N1 and N2					
Between populations	1	41.6	41.6	14	0.14***
Within populations	30	344.6	11.5	86	
N1 and R1.1					
Between populations	1	17.5	17.5	2	0.02 (n.s.)
Within populations	30	415.1	13.8	98	
N2 and sown populations (R2.4, R2.7, R2.8)					
Between N02 and sown populations	1	14.1	14.1	0	0.03**
Among populations	2	45.8	22.9	5	
Within populations	60	714.4	11.9	95	
N2 and sown&planted populations (R2.3, R2.5, R2.6)					
Between N02 and sown/planted popula-	1	12.2	12.2	0	0.01 (n.s.)
Among populations	2	29.3	14.6	1	
Within populations	59	709.2	12.0	99	
N2 and planted populations (R2.1, R2.2)					
Between N02 and planted populations	1	12.9	12.9	0	0.01 (n.s.)
Among populations	1	12.8	12.8	1	
Within populations	45	514.9	11.4	99	
N2 and R2ex (Botanical garden Regensburg)					
Between populations	1	22.8	22.8	5	0.05**
Within populations	30	369.6	12.3	95	

Fig.3. Results of the Bayesian cluster analysis for *A. maritima* ssp. *elongata.* Proportion of individuals from each sampled population allocated to one of the two groups (K) concluded from the Structure analysis (Pritchard et al., 2000). Individuals from the populations N1 and R1.1 were predominantly assigned to the cluster 1.

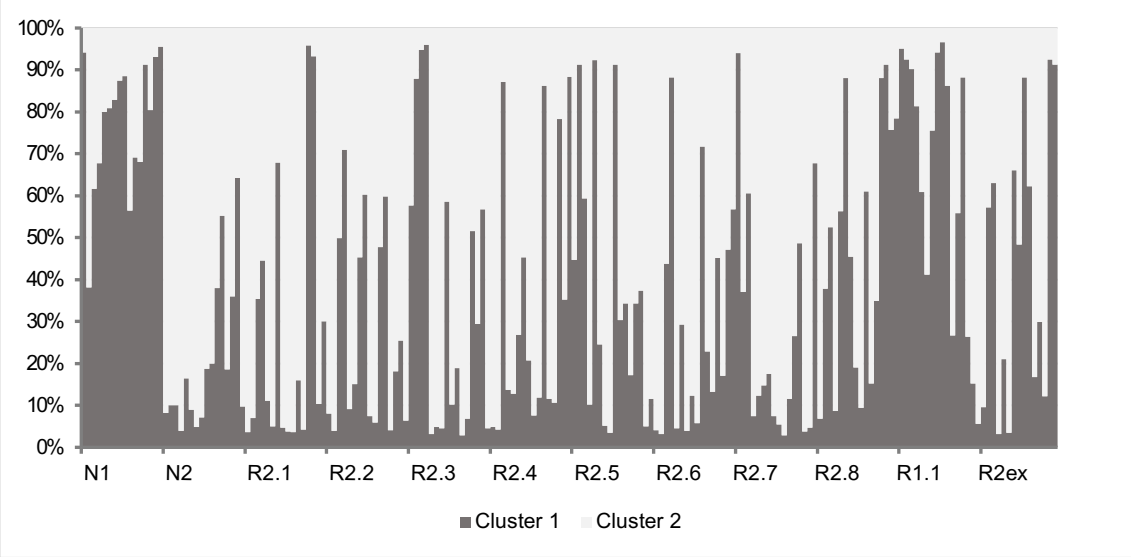
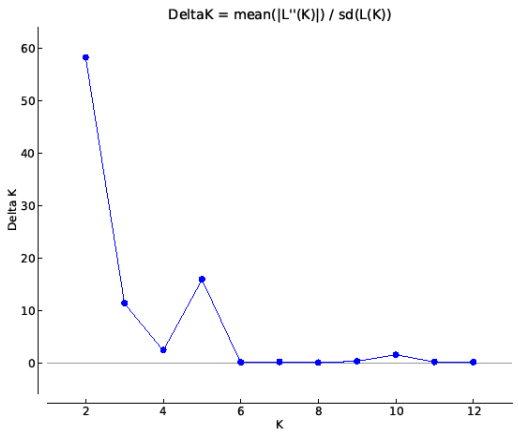
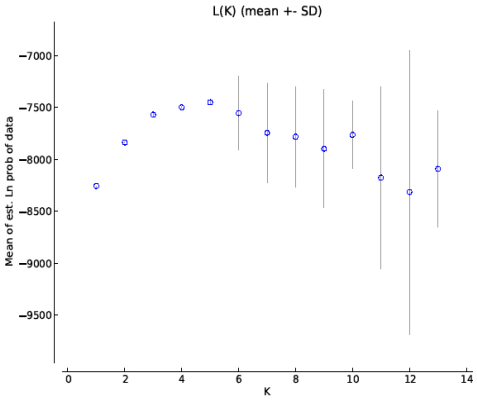


Fig. 4. Results of Bayesian clustering of *A. maritima* ssp. *elongata*. (a) ΔK displayed for each of the tested values of K 1 to 13, based on the second order rate of change of probability with respect to K. The highest peak at K=2 should correspond to the true value of K (b) The graph shows the mean ln Pr(X|K) and ±SD over 20 runs for each value of K.

a)



b)



individuals. An admixture model with correlated allele frequencies was assumed. A number of Markov Chain Monte Carlo simulations was set to 100.000 and the burn-in

period to 10.000 iterations. Number of clusters K was set to 13, which corresponds to the number of populations plus one. Analyses were run independently 20 times for each K to assess the amount of variation of the likelihood for each value of K. The best estimate of K for the given data set was specified according to the model, which gave consistent results for all 20 runs (Kopelman et al., 2015). The program Harvester (Earl and vonHoldt, 2012) was applied to summarize results. An estimate of the posterior probability of the data Pr (X|K) for a particular K was calculated (Pritchard et al., 2000). To identify the real number of clusters K, ad hoc statistic ΔK was used which was calculated as a second order rate of change of probability of the Pr (X|K) with respect to K (Evanno et al., 2005) (Fig. 4).

Distances among populations as Nei´s standard (Ds) with non-uniform prior distribution of allele frequencies were computed according to Lynch and Milligan (1994) employing the pro-gram AFLP-surv (Vekemans, 2002). Based on the Ds distances, a consensus Neighbor-Net graph was constructed applying program SplitsTree4 (Huson and Bryant, 2006). Patterns of genetic similarities between individuals were analysed in the software GenAlEx 6 (Peakall and Smouse 2006) using principal coordinate analyses (PCoA) based on a squared Euclidean distance matrix.

Results

Genetic diversity

AFLP analysis revealed 158 clear bands in *A. maritima* ssp. *elongata* across all populations, of which 84.2% were polymorphic. Levels of genetic diversity measured as the Nei´s gene diversity H and Shannon´s Information Index SI within the two natural populations were HN1=0.29, HN2=0.22 and SIN1=0.29, SIN2=0.25, respectively. Within the reintroduced populations, the levels of genetic diversity ranged between $H_{R2.2}$=0.26 and $H_{R1.1}$=0.34 ($SI_{R2.2}$=0.26 and $SI_{R1.1}$=0.35) (Tab.1). The *ex situ* population exhibited a genetic diversity of H_{R2ex}= 0.31 (SI_{R2x}=0.34).

Overall, the lowest values of genetic diversity were found within the natural population N2 (H_{N2}=0.22, SI_{N2}=0.25), the highest within the planted population R1.1 ($H_{R1.1}$=0.34, $SI_{R1.1}$=0.35).

Most diverse were the populations R2.8 (sown), R1.1 (planted) and R2ex (*ex situ*). Here, genetic diversity H ranged from 0.31 to 0.34 (SI from 0.34 to 0.35).

The average level of genetic diversity detected within the natural populations was H_N=0.26±0.05 (SI_N=0.27±0.03) and within the restored populations H_R=0.29 (SI_R=0.31). The average level of genetic diversity within the groups of sown, planted and sown/planted populations were similar (Tab.1, Fig.2). In the group of planted populations, the R1.1 was not included in the mean calculation, because it originated from the other source population N1 (Fig.2).

We have also calculated the Frequency-down-weighted-marker-values (DW) which indicates accumulation of rare fragments in populations. DW within the two natural populations was DW_{N1}=0.72, DW_{N2}=0.69. Within the reintroduced populations, the levels of DW ranged between $DW_{R2.1,\ R2.8}$=0.66 and $DW_{R2.5,\ R2.6}$=0.71 (Tab. 1). The *ex situ*

Fig.5. Consensus NeighborNet of all *Armeria maritima* ssp. *elongata* populations based on the AFLPs data. The natural population N2 together with its offspring restored populations are situated on the left side of the graph, slightly separated from the natural N1 and its offspring restored population R1.1.

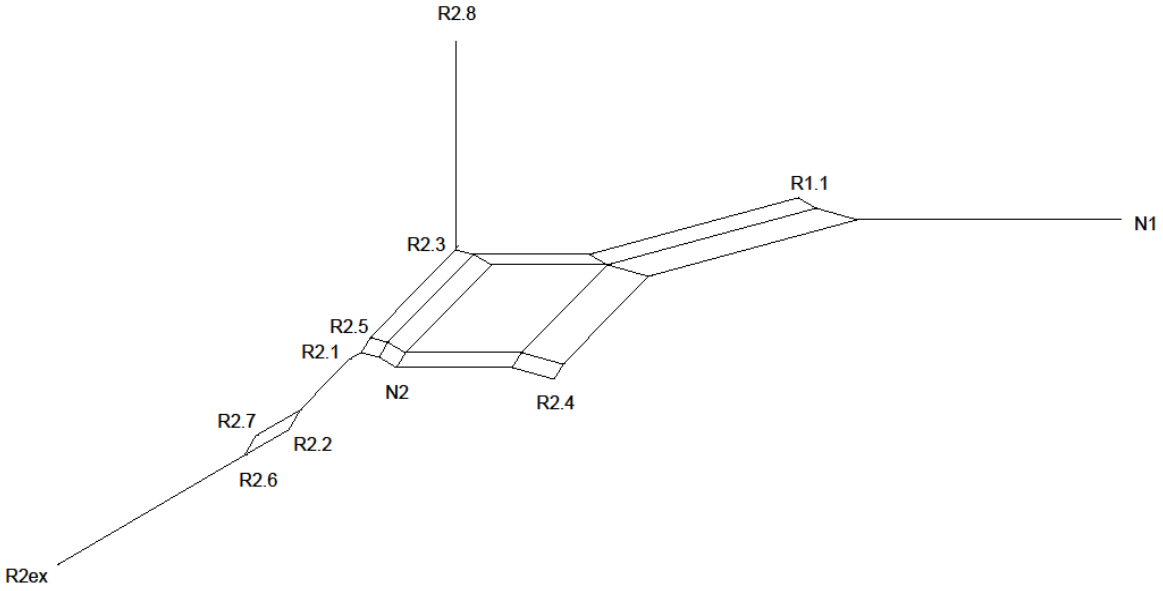
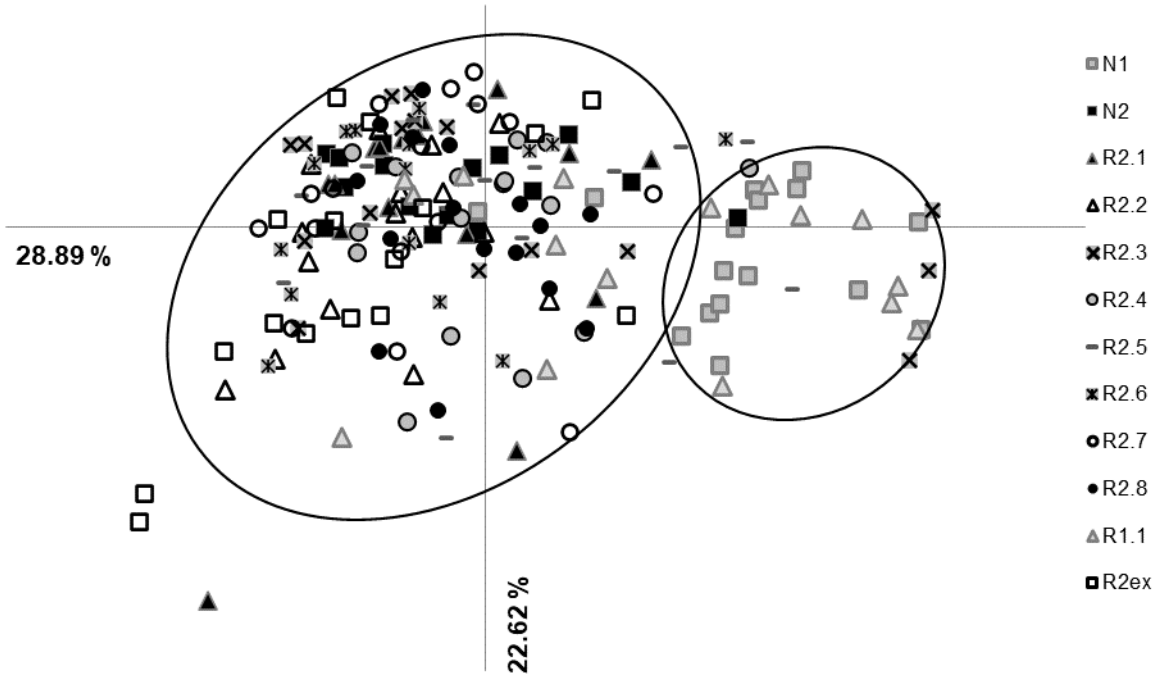


Fig. 6. Results of the principal coordinates analysis (PCoA) based on AFLP data of *A. maritima* ssp. *elongata.*



population exhibited a genetic diversity of DW_{R2ex}= 0.67.

Genetic differentiation

The highest levels of differentiation among populations were observed between the both natural populations N1 and N2 (Φ_{PT} = 0.14, p<0.001) (Tab.2). Genetic differentiation between the natural population N1 and its offspring restored population R1.1 was low and not significant (2%, p=0.09). Genetic differentiation between the natural source population N2 and the *ex situ* population R2ex was somewhat higher (5%, p<0.01). The three-level hierarchical AMOVA was carried out according to the reintroduction method (Tab.2). The values of genetic differentiation between the natural population N2 and the groups of restored populations (sown, planted, as well as sown and planted) were negligible. Within the group of sown populations, we observed the highest differentiation among populations (5%). In contrast, within the groups of planted as well as sown and planted populations only minimal levels of differentiation among the populations were detected (1%).

The Bayesian cluster analysis STRUCTURE (Pritchard et al., 2000) indicated a peak of Evanno *ad hoc* statistic ΔK at K=2 (Fig.4a) (Evanno et al., 2005). This implicates that the dataset most probably consists of two genetic clusters. One cluster included predominantly individuals of the source population N1 and its related restored population R1.1 The second cluster comprised all remaining populations, i.e. N2 and the restored populations originating from the N2, including the *ex situ* R2ex population (Fig.3).

The network constructed using the program SplitsTree4 (Huson and Bryant, 2006) displayed the two natural populations and their related restored populations in two groups (Fig.5). The natural population N2 together with its descendant restored populations constitutes a cluster on the left side of the graph. The natural N1 and its related restored population R1.1 is visible on the right side of the graph, slightly separated from the rest of the populations.

The multivariate analysis PCoA revealed only a slight clustering of populations, according to the source populations N1 and N2 and its corresponding restored populations (Fig. 6).

Discussion

Genetic diversity

The mean genetic diversity within the natural populations of *A. maritima* ssp. *elongata* was higher (0.26±0.05) than mean observed values for species with outcrossing reproductive strategies (0.19±0.08) as well as for rare species (0.12±0.07) (Reisch and Bernhardt-Römermann, 2014). Similarly, higher levels of gene diversity than refered for other taxa were observed in previous studies in *Armeria maritima* (Vekemans et al., 1992; Weidema et al., 1996). Two traits may have particularly contributed to the enhanced diversity levels of this species: the outcrossing habit of breeding and a long span of life. Cross fertilizing in plants promotes gene flow due to the allele exchange within and among populations. Additionally, in long-lived species mixed generations persist together. This means that the initial generation still acts as a gene contributor, thus enhancing genetic diversity in offspring

populations (Pierson et al., 2007; Powolny et al., 2016).

In contrast, congener species *A. maderensis* substantiated apparently low levels of the within-population genetic diversity (Piñeiro et al., 2009), most probably due to historic events (isolated evolution on islands precluding gene flow, long-term small populations, possible founder effects following colonization of ancestors from mainland and volcanic eruptions) but also overgrazing by herbivores.

One of the major concerns related to the long-term success of restoration efforts remains the loss of genetic diversity (Aavik et al., 2012; Burgarella et al., 2007; Liu et al., 2008; Robichaux et al., 1997; Nagel et al., 2019a; Basey et al., 2015; Espeland et al., 2017; Havens et al., 2015), which implies lower adaptation capability and reduced evolutionary potential. Higher genetic variation means better long-term survival prospects in changing environments. In our study, restored populations comprised slightly higher levels of genetic diversity than natural populations. Similar results were reported in many other studies (Betz et al., 2013; Dittberner et al., 2019; Pierson et al., 2007; Ritchie and Krauss, 2012; St. Clair et al., 2020).

Several reasons may have contributed to the observed patterns of slightly higher levels of genetic diversity within restored populations. First, the natural seed source population N2 and the populations restored using propagules originating from the N2 were situated in the same protected area, close to each other (Fig.1). This means that gene flow may have occurred frequently (Darvill et al., 2004) and contributed to higher genetic variation comprised in offspring populations, in comparison to source populations (Morris et al., 2002; Pierson et al., 2007). Second, the N2 population went possibly through a bottleneck process during its existence. Bottleneck events result in a loss of alleles and, subsequently, decrease of genetic diversity. This may be the reason, why genetic diversity is lower in N2 compared to the restored populations originating from N2. Third, lower genetic variation found in N2 might be a consequence of competition processes occurring on the site, which was densely occupied by this population for a long time (compare Snaydon, 1978).

In the case of the restored R1.1, the seed source was not only the natural N1 but also the supplementary propagule source, the *ex situ* population R2ex. Moreover, the large population size might have contributed to the high genetic diversity in R1.1. Fourth, high levels of genetic diversity comprised in the restored populations can be directly associated with the way of the seed collection (Konnert and Ruetz, 2003; Basey et al., 2015; Havens et al., 2015). This was carried out representatively over the whole population across several years (M. Scheuerer, personal communication), which may have increased genetic diversity in the restored populations.

Regarding the restoration methods – sown, planted as well as sown and planted – we did not detect any apparent, strong differences in levels of genetic diversity within all three groups. However, the sown group showed slightly higher levels of genetic diversity than populations founded using plants (Fig.2).

Similar to slightly higher diversity levels were in accordance with our assumption

(compare St. Clair et al., 2020). Even though a high percentage of seedlings normally fail to survive due to unfavourable weather or site conditions as well as due to self-thinning processes in the wild, still much more seeds are often required and used for direct sowing than for plant cultivation in nursery settings. This may facilitate higher diversity levels in sown populations. The cultivation process, in its turn, skips the most vulnerable part of the plant life-cycle in the wild, the seedling stage and thus, the survival percentage in much higher (Drayton and Primack, 2000). One caveat is that cultivation may foster maladaptations and unintended evolution processes, especially when practiced over several generations (Basey et al., 2015; Ensslin and Godefroid, 2019). For instance, dormant seeds might be omitted in a plant nursery, but in the wild they would later become a part of a population.

The highest levels of GD were reached across the restoration methods, i.e., within the populations R2.8 (sown) and R1.1 (planted). The levels of GD were probably affected by the highest number of founding individuals rather than by reintroduction methods. This result implicates that the population size might be more relevant, in terms of genetic diversity, than the reintroduction method *per se.* However, the restricted number of replicates posed a constraint to statistical analyses. Therefore, further research is needed using higher numbers of populations to reveal potential differences.

outcrossing species. Moreover, two processes might have also played a role. First, R2ex was founded using seeds produced by N2. However, surrounding *in situ* restored populations presumably acted as gene donors via pollen transfer, und thus contributed

Concerning the rare alleles, we did not observe any apparent loss of rarity in the restored populations in comparison with their sources. The highest levels of rarity were found in both natural sources as well as in the sown & planted restored populations.

In our study we also analysed the genetic diversity of the *ex situ* population R2ex, which was cultivated since 2004, using seed material originating from the N2 population. As detected for the restored populations, the *ex situ* population R2ex maintained higher levels of genetic diversity than the N2 population. This was a surprising result because the population size of N2 is at least twice as large as that of R2ex. Former studies suggested a clear threat of genetic erosion in *ex situ* cultivations (Enßlin et al., 2011; Rucinska and Puchalski, 2011; Lauterbach et al., 2012; Brütting et al., 2013). Genetic erosion can be caused not only by unrepresentative source population sampling, but also due to genetic drift in small *ex situ* populations, as well as increased selfing rates and inbreeding processes (Ensslin and Godefroid, 2019). In a survey involving 32 studies on *ex situ* cultivations, only one species (perennial, self-incompatible) showed higher genetic diversity than in the wild (Brütting et al., 2013). Here, two traits played a key role: life form and a breeding system. A short life span combined with selfing imposed intrinsic risk of reduced genetic diversity in *ex situ* cultivations. However, *A. maritima* ssp. *elongata* is a perennial, long-lived,

to higher genetic diversity in seeds used to initiate R2ex. (Darvill et al., 2004). Second, N2 possibly went through a slight bottleneck in 2008-2009 and 2012 (Martin Scheuerer, unpublished data), which reduced its genetic diversity. In such a case, generally, *ex situ*

populations may help to maintain genetic diversity lost in the wild (compare Abeli et al., 2020). However, during the *ex situ* cultivation of wild plants trait may be changed as a consequence of relaxed selection pressures in botanic gardens. Thus, changes in response to drought stress were documented in a multi-species experiment (Ensslin and Godefroid, 2020). Presumably the most striking example of changes in traits were differences in seed dormancy between garden-cultivated and wild populations (Schröder and Prasse, 2013; Ensslin et al., 2018). Reduction of variability in dormancy and germination properties may occur very rapidly (Schroder et al., 2013). This may potentially pose a risk to restoration programmes using seeds originating from *ex situ*. Therefore, research is needed in this respect prior to the use of *ex situ*-produced seeds in restoration schemes (Ensslin et al., 2018).

Genetic differentiation

In our study *Armeria maritima* ssp. *elongata* maintained its most genetic variation within populations, a pattern typically observed in long-lived species with outcrossing mating system (Vekemans et al., 1992; Weidema et al., 1996). The highest level of genetic differentiation was detected between the two natural populations N1 and N2. The population N1 colonized a roadside embankment influenced by mowing and winter maintenance

Regarding the restoration method, genetic differentiation between the natural populations and the respective groups of sown, planted as well as sown and planted populations was very low or even absent. St. Clair et al. (2020) detected that nursery-grown plants of *Castilleja laevisecta* used for restoration purposes tended to deliver a more

by salts, whereas N2 occupied natural sand dune habitats. Different conditions may influence phenology of populations, e.g. flowering times (Reisch and Poschlod, 2009), which can cause high levels of genetic differentiation. We assume, however, that the most probable reason of the observed differentiation levels was the spatial distance between the two natural populations. In contrast, genetic differentiation among the natural and the respective restored populations was almost zero (Tab.2). Negligible genetic differentiation among natural and restored or reinforced populations were also reported in previous restoration studies (Betz et al., 2013; Millar et al., 2019b; Pierson et al., 2007; Ritchie and Krauss, 2012). In our study, two main reasons most probably contributed to the negligible levels of differentiation. First, the germplasm used for restoration was collected representatively, but also, secondly, the spatial distance of the natural and the respective restored populations was low and could enable gene flow, most likely via pollen. The seeds of *A.maritima* ssp *elongata* are typically wind-dispersed, but dispersal distances reach usually only a few meters and do not allow to reach an adjacent population (Philipp et al., 1992). Dispersal for longer distances would be possible via epizoochory, e.g., by grazing sheep. This practice was, however, not present in the study area.

consistent representation of (multiple) source populations, especially in the first generation. When using mixed seed sources, growing juveniles under nursery conditions enables better control of source population representation. Seeds, in their turn, produced higher variability in representation of the source populations. This is in accordance with our findings, because we

observed the largest genetic differentiation among the sown populations.

According to the local conditions during the restoration procedure, as well as dormancy and germination properties of seed, a certain fraction of seeds germinates, survives or declines, thus supporting this differentiation pattern (Kucera et al., 2021; compare Seglias 2018). Possibly, the sown population R2.8 in our study additionally contributed to the observed levels of differentiation due to its separate location, because gene flow between R2.8 and the rest of the populations could not take place (site Dassfeld, Fig.1, see also Fig.5).

Genetic differentiation between the natural and the *ex situ* population was low but significant (5%, $p<0.01$) (Tab.2), higher than expected. Brütting et al., 2013 found even higher levels of genetic differentiation between *ex situ* and natural population, albeit in four of five species the original source population was not known precisely. Generally, *ex situ* populations are at risk due to several causes. First, their genetic makeup (and that of seeds which they produce) may change as a consequence of unintended hybridization with congeners, or due to genetic introgression from other accessions of the same species cultivated in the grounds of the same botanical garden. Second, an unintended selection may occur if *ex situ* environments that do not resemble original conditions in the wild (Ensslin 2011). The moderate shift in allele frequencies in R2ex which we observed might arise due to thirteen years of the separate cultivation. During this period, the *ex situ* population lacked any gene exchange with the natural source population N2. We conclude that an improvement should be considered through input of fresh genetic material from the wild.

In our study, all analysed individuals were assigned to two groups using Bayesian cluster analysis Structure (Pritchard et al., 2000). Individuals originating from the natural N1 and its offspring population R1.1 were more frequently assigned to cluster 1, whereas N2 and its descendant restored populations, respectively, were predominantly allocated to the cluster 2 (Fig. 3). This pattern was corroborated by both the NeighborNet analysis and the multivariate PCoA (Fig.5, fig. 6). Both illustrates that the natural genetic pattern in the study region has not been affected negatively by restoration. Seeds have been collected separately in the N1 and N2 populations and have been used consequently to restore populations near the respective natural (source) populations. Obviously, no accidental mixing of seeds from the different natural sources took place.

From our study we conclude, that from the genetic point of view, the restoration of vital, genetically variable and locally differentiated populations of *Armeria maritima* ssp. *elongata* generally worked well. This pilot study based on a practical restoration project have shown that both sowing and planting have proven to be suitable approaches to restore genetically representative populations without losing genetic diversity. This is an important prerequisite to retain their evolutionary potential for a long-term survival, especially in a context of ongoing climate change. However, in future studies, larger restoration projects are needed, using multispecies experiments. Thus, we can gain more detailed insights in how different reintroduction methods may influence genetic patterns in restored populations.

A SOURCE OF HIDDEN DIVERSITY FOR RESTORATION: SOIL SEED BANK AND ABOVEGROUND POPULATIONS OF *ORIGANUM VULGARE* CONTAIN SIMILAR LEVELS OF GENETIC VARIATION

The seedlings of *Origanum vulgare*, emerged from the soil seed bank in our experiment and a mature plant

Abstract

In many landscapes, restoration success depends on the presence of diaspores either in the close surroundings, or directly beneath the sites to be restored. The soil seed bank is, therefore, an important part of ecosystem resilience and constitutes a vital pillar for the regeneration of genetic diversity in many plant populations. However, regeneration from the soil seed bank and the results of restoration can only be considered as successful, when the genetic diversity of restored populations is not eroded and genetic differentiation not inflated.

In our study we compared genetic variation within and among soil seed bank and aboveground populations of *Origanum vulgare*, to test whether genetically variable populations can be restored from the soil seed bank. We explored levels of genetic diversity within above ground populations and the corresponding soil seed banks. Furthermore, we assessed the extent to which the soil seed bank differs genetically from the aboveground population.

Levels of genetic diversity were to a great extent similar in aboveground populations and the corresponding soil seed banks. Only levels of inbreeding were slightly higher in the in the lower layer of the soil seed bank compared to the aboveground populations which is most likely due to selection processes acting against homozygotes accumulating in the seed bank. Furthermore, significant genetic differentiation between the aboveground population and the corresponding seed banks was completely lacking. Across all sites, genetic differentiation between the soil seedbank was similar to that between the aboveground populations, most probably due to the absence of severe climate conditions, strong bottlenecks or disturbance events.
From our study, we conclude that the soil seed bank represents a vital source for the restoration of genetically variable populations of *O. vulgare*.

Key words: soil seed bank, restoration, genetic diversity, Origanum vulgare

Introduction

Soil seed banks are one of the most remarkable characteristics of plants that do not solely rely on seed dispersal in space, but also in time by storing long-term dormant diaspores in the soil (Bakker et al., 1996b; Lamont and Enright, 2000; Blanckenhagen and Poschlod, 2005) With their soil seed banks, plants work against consequences of environmental and demographic stochasticity, which occur across a broad range of climate zones, habitats and life history types (Baskin and Baskin, 2011). Especially for rare species or taxa in environments with harsh and highly unpredictable conditions, seed banks play the role of bet-hedging, one of the pivotal possibilities to reduce long-term risks (Zaghloul et al., 2013). Species occupying dynamic habitats with strong disturbance regimes, such as sites influenced by flood events or forest gaps are known to possess high-density seed banks exceeding 10.000 seeds / m^2 (Kiss et al., 2016; Poschlod and Rosbakh, 2018). In calcareous grasslands, where conditions are relatively stable and potential disturbances are mainly related to grazing or mowing, a subset of species build persistent seed banks but usually of lower densities not exceeding 5.000 seeds / m^2 (Bossuyt and Honnay, 2008a). In particular cases, seed banks of calcareous grasslands can reach up to 8.000 seeds / m^2 (Karlík and Poschlod, 2014; Poschlod and Jackel, 1993).

Currently, the role of seed banks gains in weight in the context of the ongoing climate change that deepens fragmentation processes and fosters area loss of temperate European calcareous grasslands. Previous research revealed that small and fragmented populations are prone to extinction due to environmental and demographic stochasticity and possible genetic erosion, more than large ones (Andrén, 1994; Young et al., 1996; Honnay et al., 2005). In addition, due to genetic consequences of fragmentation, common species seem to suffer more from genetic erosion than rare species (Honnay and Jacquemyn, 2007). Especially outcrossing species, depend on gene flow between populations to maintain genetic variation. Due to the ongoing fragmentation process and increasing distances between remaining populations, chances to exchange pollen are getting rarer. Simultaneously with the fragmentation, decreasing population size causes a further loss of genetic diversity (Honnay and Jacquemyn, 2007).

However, long-lived seeds stored in soil seed banks may help to slow down these processes significantly. Consequently, species with long-lived seeds show a lower fragmentation-caused extinction probability than species with short-lived seeds (Piessens et al., 2005). Stöcklin and Fischer (1999) found that in isolated grassland fragments, species possessing seeds with high longevity (more than 5 years) were less prone to extinction in comparison with taxa with low seed longevity. Tonsor et al. (1993) pointed out that soil seed banks might serve as a “genetic memory” conserving changes in the genetic constitution of populations and may retain alleles through periods in which they were selected against. This is due to unpredictable environmental dynamics and extreme conditions (Zaghloul et al., 2013), disturbances (Bosbach et al., 1982) or

changes in breeding system with temporary or spatial variation in selfing rates (Schulz et al., 2018). Thus, seed banks buffer results of the directional selection triggered by fluctuations of environmental conditions (Templeton and Levin, 1979).

In a restoration context, not only genetic diversity contained in surrounding populations (Iberl et al., 2022) but also the genetic diversity stored in the soil seed bank may potentially contribute to the restoration of genetically variable aboveground populations. For such an approach the level of genetic diversity in the soil seed bank should, however, be at least as high as that of the present aboveground population (Honnay et al., 2008).

Several studies tested the ecological and evolutionary effects, when long-living diaspores are stored in soil. Both in annuals (Lundemo et al., 2009; Falahati-Anbaran et al., 2011; Hanin et al., 2013) and in perennials (Falahati-Anbaran et al., 2011), soil seed banks may extend effective population size and outweigh a random loss of genetic variation due to genetic drift (McCue and Holtsford, 1998). However, previous findings concerning genetic diversity were not consistent. Generally, three situations have been reported: first, higher levels of genetic diversity in the soil seed bank than in the aboveground population (McCue and Holtsford, 1998; Morris et al., 2002), secondly, no significant differences between the soil seed bank and aboveground population (Mahy et al., 1999; Hanin et al., 2013; Plue et al., 2017) and, thirdly, a frequent detection of lower genetic diversity in seed banks than in the aboveground population (Tonsor et al., 1993; Cabin et al., 1998; Zaghloul et al., 2013; Schulz et al., 2018). Deeper insights were provided by a meta-analysis of Honnay et al. (2008), who revealed no evidence, that high levels of genetic diversity were stored in the soil seed bank. This was also confirmed by Mandak et al. (2012) who suggested that the role of the seed bank is rather to maintain than to accumulate genetic diversity. In the study presented here, we analysed the level of genetic variation within and between the soil seed bank and the corresponding aboveground populations of the typical dry grassland species *Origanum vulgare*.

In contrast to many previous studies, we also examined the vertical distribution of genetic variation, since we tested a potential differentiation between upper and lower soil layers. Previously, it has been shown that in the absence of disturbance regimes, deeply buried seeds must be older than those near the surface (Grandin and Rydin, 1998) and deeper soil layers may accumulate seeds with higher longevity (Bekker et al., 1998). Especially the fraction of smaller seeds may fall deeper into the fissures in the soil. Koch et al. (2003) demonstrated that increasing dynamics of the habitat decreased not only similarity between the vegetation and the soil, but also reduced genetic homogeneity of the seed bank populations from different soil layers. Consequently, we tested in our investigation whether upper and lower layers of the soil seed bank differ from each other and the aboveground population in genetic variation. We analysed this in the context of restoration, since the genetic variation stored in the soil seed bank may help to

buffer the detrimental effects of fragmentation (Poschlod et al., 1998b). More specifically, we asked following questions:

1. How large is the genetic diversity maintained in the seed bank in comparison with the aboveground populations?
2. How strong is genetic differentiation between the aboveground populations and their corresponding seed banks and among the soil seedbank and aboveground populations from different study sites?
3. Can genetically variable populations of *O. vulgare* be restored from the soil seed bank, particularly in case of massive disturbance or local extinction?

Materials & Methods

Study species

For our study we selected *Origanum vulgare (Lamiaceae)*, a perennial, long-lived, rhizomatous aromatic herb, confined to dry grasslands, scrub or woodland fringe vegetation and thermophilous oak forests, but occurring also in highly disturbed alluvial meadows (Oberdorfer, 2001; van Looy et al., 2009). The species has a long-term persistent soil seed bank and often produces seed banks of high densities (Poschlod et al., 1991; Kleyer et al., 2008; Kiss et al., 2016).*O. vulgare* successfully colonizes restored calcareous grasslands (Helsen et al., 2013). This is a gynodioecious species which is predominantly outcrossing but selfing is also possible (Klotz et al., 2002). Pollinators are insects, mainly honeybee, bumblebees and hoverflies (Janovsky, 2020). *O. vulgare* builds dry fruits – clusters of four one-seeded nutlets. Seeds are small, oblong and egg-shaped, 0,9-1,3 mm long and smooth (Slavik, 2000). The seeds are usually dispersed by gravity wind and animals (Klotz et al., 2002; Poschlod et al., 1998b). Germination takes place in vegetation gaps during spring.

Study area and sampling design

For our study we selected five calcareous grasslands on the Franconian Jura in Southeastern Germany (Tab. S1). The distance between study sites ranged from 0.2 to 17 km. The study sites are located in an altitude of 360 to 445 metres above sea level and the climate is temperate-subcontinental. The selected grasslands are predominantly surrounded by woodland, scrub, hay production grasslands or arable fields. At each study site we established five sampling plots of 8x10 m where *O. vulgare* occurred. Each plot was divided in 80 subplots (10 rows and 8 columns) of one square meter. Using a chessboard design (Fig. S1), we sampled plant material and soil samples in four subplots per row. Per subplot, two soil samples and leaf material from one individual were collected. Consequently, during summer 2018, leaf material of 40 individuals per location (if available) was sampled, dried and stored over silica gel until further processing in lab. Soil samples were extracted in March 2019 following the recommendations of Bakker et al., 1996a; Bakker et al., 1996b. Sampling during late winter or early spring makes sure that stratification took place in winter, producing more precise information about the soil seed bank. In each subplot, we took two randomly chosen soil samples using a soil corer with an inner diameter of four cm, yielding a sampling area of 2 x 12.56 cm^2 = 25.12 cm^2 and approx. ten cm deep

(corresponds to approx. 0.25 litres) per plot, according to the formula $\pi*r^2$. In total, we sampled 0.25*40=10 litres in each location. The sample volume of more than 6 litres per site corresponds to the recommended sampling volume (Hutchings, 1986). Subsequently, we divided each core into two sections according to the depth from zero to five cm and from five to ten cm, if available, respectively. For each subplot, we separately pooled the core samples from the upper and the lower layers in the cores.

Soil seed bank analysis

Before processing, the soil samples were stored in plastic bags in a cooling chamber at 4°C. The soil was then washed through a sieve cascade consisting of five mm to remove stones and roots and 0.2 mm to eliminate fine-textured soil. This step was essential to reduce the sample volume and to improve conditions for seed germination (Heerdt et al., 1996). We did not detect any apparent differences in stoniness of the soil samples from different study sites. The soil samples were then spread in thin layers of approximately three mm depth into trays filled with sterilized horticultural substrate. To allow natural temperature fluctuations during day and night, as well as to avoid any disturbance caused by birds or mice, samples in the trays were cultivated in a nonheated open cage greenhouse. Samples were watered gently to ensure that seeds would not be washed away. Cultivation took place from April 2019 to June 2020, until no new *O. vulgare* seedlings emerged. These were collected in a juvenile stage, large enough to deliver leaf tissue for molecular analyses. In total, thirty individuals per location were cultivated until flowering from the soil seedbank in order to determine the percentage of female and hermaphrodite individuals of the gynodioecious species (in total 150 individuals). Cultivation lasted from April 2019 to August 2020, until all plants have flowered. The aboveground populations contained on average 14.2% of female plants (range from 7.7 to 21.4%), with median of 15.2% female plants. The seed bank populations comprised on average 15.6%, respectively (varied from 3.7 to 23.9%) and had a median of 19% female plants. The difference between the two groups was not significant (Shapiro-Wilk normality test p-value=0.47, paired t-test p-value = 0.72).

Microsatellite analyses

For molecular analyses, we used leaf material from the aboveground populations as well as from the germinated individuals emerging from both layers of the soil seed bank. Genetic variation of the aboveground population and in the upper and the lower soil layers of the soil seed bank was determined using microsatellites. We extracted nuclear DNA from the silica gel dried leaf material according to the CTAB protocol after Rogers and Bendich (1994) in an adaptation by Reisch (2007). The extracted DNA was diluted with water to a concentration of 7.8ng/µl and then used for microsatellite analysis.

In total we studied 382 samples using nine microsatellite loci, which have been developed for *O. vulgare* by Novak et al. (2008) (OR 10, 12-14, 27, 40, 44, 64, 77). PCR was carried out in two multiplexes of four and five microsatellites respectively, in 10µl reactions containing 3.2µl template DNA (7.8 ng/µl), 0.8 µl H_2O, 0.5 µl forward primer (10µM) multiplex (Beckmann/vwr), 0.5 µl reverse primer

(10µM) multiplex (Beckmann/vwr), 5 µl 2xMaster Mix S (Beckmann/vwr). Thermal cycling profile of Novak et al. (2008) was employed, beginning with denaturation at 95°C for 15 min., followed by 35 cycles of 95°C for 60 s, 59°C for 60 s, 72°C for 2 min, and a final elongation step of 72°C for 9 min. After amplifications of the DNA, 1 µl of PCR product was added to 24.8 µl of the Sample loading solution and 0.2 µl CEQ Size Standard 400 (both Beckmann Coulter). Amplified DNA fragments were sized using capillary gel electrophoresis on an automated capillary electrophoresis machine (GeXP, Beckmann Coulter) and scored with Bionumerics (Applied Maths NV), Version 7.6.

Statistical analyses

Microsatellite data were then checked for scoring errors caused by stutter bands, null alleles and large allele dropout using MICROCHECKER (van OOSTERHOUT et al., 2004). Allele frequencies were computed at the nine loci for each sample site and cohort, i.e. the above ground population and the upper and lower soil layers. We found a homozygote excess in 26% of the populations for the locus OR12 and 13% for the locus OR14, which possibly indicates the presence of null alleles. Therefore, we performed statistical analyses both with and without these loci. As the results calculated using nine, eight and seven loci were similar, we decided to include all nine loci in the analyses.

In a first step, we used all samples from the vegetation and the seed bank to perform analyses of genetic diversity. However, since we distinguished two depth soil layers in the seed bank, thus gaining three cohorts (the aboveground vegetation, the upper and the lower soil layers, respectively) (Tab.1, Tab. S2), we additionally performed an analysis with an equal sample size of ten samples per each cohort. The limiting factor for this analysis was the reduced number of available plants from the lower soil layer. Therefore, the samples from the vegetation and the upper soil layer were randomly chosen from all available samples. For AMOVA, PCoA, NeighborNet and Structure Bayesian cluster analysis, we used the total available number of samples from the aboveground and the seed bank populations including both soil layers.

We employed Microsatellite Analyzer (MSA; Dieringer and Schlötterer, 2003) to convert microsatellite data into appropriate formats for the analyses. Further, we used MSA to compute a chord distance matrix after Cavalli-Sforza and Edwards (1967) which was applied to construct a NeighborNet diagram with the SplitsTree4 software (Huson and Bryant, 2006). The Genepop on the Web software (Rousset, 2008), option 5 was employed to calculate inbreeding index (Fis). Three indices of genetic diversity were calculated using GenAlEx 6.503 (Peakall and Smouse, 2006): mean number of alleles per locus (N_a), expected heterozygosity (H_e) and Shannon´s Information Index (SI, results given in Supplementary). We compared genetic diversity, computed for the seed bank and the vegetation, using paired Student´s t-test. All tests and figures related to the genetic diversity indices were done in R4.0.5 package stats (R Core Team, 2014) using packages car, psych, Lattice and DescTools.

The overall genetic differentiation was analyzed based on Fst employing the analysis of molecular variance AMOVA implemented in GenAlEx 6.503 (Peakall and Smouse,

2006). We conducted a three-level hierarchical analysis to infer differences between the three groups, i.e. the aboveground, the upper and the lower seed bank populations of *O. vulgare*, pooled across all locations. We further inferred differentiation between the vegetation and its corresponding seed bank populations for each location using the two-way AMOVA. Additionally, we separately assessed differentiation between the aboveground and the seed bank populations, across all five study locations. We tested for significance of the detected genetic differentiation based on 999 permutations.

We further performed the Principal coordinate analysis (PCoA) to infer patterns of genetic similarities between individuals, based on co-dominant genotypic distances (GenAlEx 6, Peakall and Smouse, 2006). We proceeded with two approaches: first, we inferred genetic similarities between the three cohorts within each separate study location. Additionally, we analysed each of the three cohorts separately, using samples from all locations belonging to the identical cohort. We thus created three separate diagrams for the vegetation, the upper soil and the lower soil layer (the diagram of the second approach see the Supplementary).

Using the Bayesian cluster analysis implemented in STRUCTURE 2.3.4 (Pritchard et al., 2000), we inferred a general genetic pattern of the whole dataset. We applied an admixture ancestor model without prior population information, assuming correlated allele frequencies. Individuals were clustered with the Bayesian Monte Carlo Markov Chain (MCMC) method using burn-in lengths of 100.000 and 100.000 MCMC sampling repeats. The program estimates a probability of genotypes being distributed into K number of clusters. The maximum number of clusters (K) were specified according to the number of populations plus one. We assumed two cohorts (vegetation and soil) in each of the five study locations to be a population, i.e. we tested a model using 10 + 1 = 11 K. Thirty independent runs for each value of K were carried out (Falush et al., 2003). For all individuals, the membership coefficient for every cluster was calculated. Group assignment was an ad hoc quantity procedure calculating ΔK ad-hoc statistics based on the rate of change in the log probability of data between successive K values (Evanno et al., 2005). We determined the best estimate of K according to the model, which gave the highest value of ΔK and identical results for the multiple runs. We summarized the results using the program Structure Harvester 0.6.34 (Earl and vonHoldt, 2012a) and Cluster Markov Packager Across K (Kopelman et al., 2015). The population membership in in genetic clusters was visualized using ArcGIS 10.8 (Fig.3). We additionally run the STRUCTURE analysis for each study location separately including the three cohorts, i.e. the aboveground and the soil populations with partitioned soil layers. In this case, the highest possible value of K was set at four. We otherwise used the same analysis settings as described above. For each location, we included two possible solutions for K, reflecting, first, the K-value corresponding to the highest probability of the data based on the Evanno approach

(Evanno et al., 2005) and, second, a solution based on consistency of all thirty runs for each value of K (Kopelman et al., 2015).

All diagrams of the additional analysis see the Supplementary (Fig. S6). A consensus NeighborNet graph was constructed applying the software SplitsTree4 (Huson and Bryant, 2006), based on the Cavalli-Sforza distances calculated using Microsatellite Analyzer (Supplementary). We further carried out an autocorrelation analysis, see Supplementary for description.

Results

Soil seed bank

We detected a patchy seed distribution of *O. vulgare* in the soil. In 18.5% of the experimental 1x1 m sub-plots, no individuals of *O. vulgare* emerged from the soil samples. The number of seeds from the random soil sample was on average 4.55 per sampling area of 25.12 cm^2 in each experimental sub-plot. After correction for one square meter, the seed bank of *O. vulgare* contained on average 1,809 seeds/m^2 of *O. vulgare* and this number varied between the locations (Fig. S2b). The entire soil seed bank density (i.e. including all emerged seedlings irrespective of the species) was on average 10,354 seeds/m^2, and was highly variable between the locations as well (Fig. S2a). In the upper soil layer, *O. vulgare* counted totally 736 individuals, on average 147.2 per study location, the seed bank density was on average 1,465 seeds/m^2. In the lower soil layer, we counted in total 173 individuals, on average 34.6 per study location, and the seed bank density was 344 seeds/m^2, respectively. The difference between the two soil layers regarding the seed bank density was significant (p-value=0.02) (Fig. S2).

However, we detected no significant differences in genetic diversity between the aboveground and the seed bank populations, except for inbreeding index (Fis). For all available samples, the mean number of alleles per locus (Na) in the aboveground populations varied between 2.89 and 3.67, on average 3.42. In the seed bank (all available samples from pooled layers), the mean number of alleles per locus varied between 2.56 and 3.89, on average 3.40. Expected heterozygosity (He) in the aboveground populations varied between 0.36 and 0.57, with mean of 0.47, and, in the seed bank, it varied between 0.32 and 0.56 and had a mean of 0.47. Finally, inbreeding index (Fis) in the aboveground populations varied between -0.0007 and 0.06, on average 0.01. In the soil the values of Fis ranged between 0.05 and 0.14 with mean of 0.11. Here, we observed that the inbreeding index was slightly and significantly higher in the seed bank than in the aboveground population (p-value=0.04) (Tab.1).

We additionally compared genetic diversity in the two soil layers (upper, lower) and the aboveground population. We found a significant difference between the aboveground population and the lower soil layer (inbreeding index, p-value=0.02). In the above ground population, the inbreeding index (Fis) ranged between -0.19 and 0.08, mean -0.05. In the upper soil layers, Fis varied between -0.05 and 0.15, mean 0.05. In the lower soil layers, Fis ranged between -0.02 and 0.38, mean 0.21. The mean number of alleles per locus (Na) was slightly higher in both seed bank layers, and this difference was marginally significant (p-value=0.059). In the above ground population, Na ranged between 2.22 and

Table 1. Genetic diversity and characteristics within the vegetation and the seed bank populations of *Origanum vulgare*. Cohort, a membership in a particular group (veg, vegetation, upp, upper soil layer, low, lower soil layer), Ntot, total number of samples; %F, percentage of female plants in populations (measured for vegetation and seed bank populations); Na, mean number of alleles per locus; He, expected heterozygosity; Fis, inbreeding index; w, pairwise Wilcoxon test. All given results represent a mean ± standard deviation, across the five study locations; significant p-values are given in bold

Cohort	Ntot	%F	Na	He	Fis
All samples from the vegetation and seed bank with pooled layers					
veg total	37.8 ± 3.19	14.2 ± 5.4	3.42 ± 0.33	0.47 ± 0.07	0.01 ± 0.05
soil total	38.6 ± 8.99	15.6 ± 8.5	3.40 ± 0.51	0.47 ± 0.09	0.11 ± 0.04
t-test		p=0.70	p=0.90	p=0.71	**p=0.04**
Ten samples per vegetation and seed bank divided in the upper and lower layers					
veg	10	-	2.49 ± 0.20	0.40 ± 0.11	-0.05 ± 0.11
upp	10	-	3.00 ± 0.19	0.46 ± 0.03	0.05 ± 0.09
low	10	-	3.04 ± 0.21	0.49 ± 0.05	0.21 ± 0.16
t-test veg x upp		-	p=0.06 w	p=0.69	p=0.67
t-test veg x low		-	p=0.06 w	p=0.30	**p=0.02**
t-test upp x low		-	p=1.00 w	p=1.00	p=0.21

2.78, mean 2.49. In the upper soil layer, Na varied between 2.67 and 3.11, mean 3.00. In the lower soil layer, Na ranged between 2.67 and 3.22, mean 3.04. All other remaining indices of genetic diversity did not differ significantly and are given in the supplementary (Tab. S2).

Genetic differentiation and structure

The three-level-AMOVA detected zero vertical differentiation between the groups of the aboveground, the upper and the lower soil populations. Additionally, we performed the two-level-AMOVA for each location, between the vegetation and its corresponding seed bank populations from the upper and the lower soil layers. Overall, genetic differentiation was zero or nonsignificant. Furthermore, we inferred a horizontal genetic differentiation between the different study sites, using a two-way AMOVA. Here we detected moderate but significant levels of differentiation between the aboveground populations (Fst=0.14; p=0.001) and the seed bank populations (Fst=0.13; p=0.001) (Tab.2) from different sites.

Using PCoA, we constructed five diagrams related to particular study locations which included samples from the three cohorts. The first two axis explained accumulatively 28.76 % of variation (Aichahof), 30.64 % (Eitelberg), 30.69 % (Grabenhof), 35.38 % (Kühschlag), 40.47 % (Undorf). Here, we did not detect any apparent clustering according to the cohorts (Fig.2).

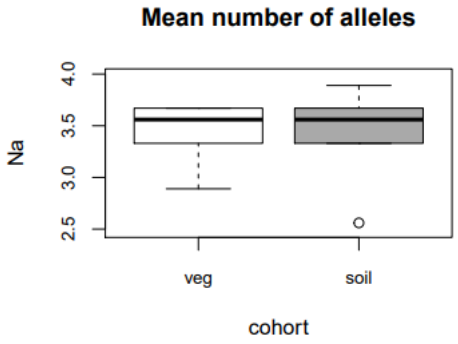
Further PCoA results were similar to the results of the Bayesian cluster analysis, the NeighborNet analysis and AMOVA and were included in the Supplementary (Fig. S4).

Table 2. Analyses of molecular variance (AMOVA) for all *Origanum vulgare* populations. Df indicates degrees of freedom; SS, sum of squares; MS, mean squares; % the proportion of genetic variability; Fst the level of genetic differentiation. Levels of significance are based on 999 iteration steps and are indicated by asterisks (*** p<0.001, ** p<0.01, *p<0.05). Significant values are given in bold.

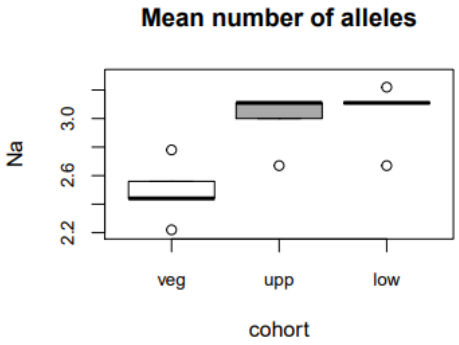
Source of molecular variation	df	SS	MS	%	F_{st}
All vegetation, upper and lower soil layer populations, pooled across all sites					
Between three groups of veg., upper and lower soil layers	2	5.5	2.7	0	
Between populations within the groups	12	240.8	20.1	14	**0.12*** **
Within populations	749	1630.6	2.2	86	
The vegetation and the corresponding seed bank populations, separately for each location					
Aichahof					
Between the aboveground and the seed bank population	1	3.2	3.2	1	0.006 n.s.
Within populations	174	377.4	2.2	99	
Eitelberg					
Between the aboveground and the seed bank population	1	3.7	3.7	1	0.008 n.s.
Within populations	150	347.9	2.3	99	
Grabenhof					
Between the aboveground and the seed bank population	1	2.2	2.2	0	0.00
Within populations	164	347.8	2.1	100	
Kühschlag					
Between the aboveground and the seed bank population	1	1.5	1.5	0	0.00
Within populations	154	394.9	2.6	100	
Undorf					
Between the aboveground and the seed bank population	1	0.8	0.8	0	0.00
Within populations	112	176.0	1.6	100	
The vegetation and the seed bank separately, five populations across sites					
Aboveground populations in the 5 study sites					
Between the aboveground populations	4	118.1	29.5	14	**0.14*** **
Within populations	373	806.9	2.2	86	
Seed bank populations in the 5 study sites					
Between the seed bank populations	4	112.5	28.1	13	**0.13*** **
Within populations	381	837.0	2.2	87	

Figure 1. Genetic diversity measures computed for vegetation (veg) and soil (soil). Soil seed bank is in b), d), f) divided in two layers, upper (upp) and lower (low). Genetic diversity was measured as: a, b) mean number of alleles per locus (Na); c, d) Expected heterozygosity (He); and e, f) Inbreeding index (Fis). We detected significantly lower inbreeding index Fis in the vegetation compared to the soil (over all loci for each population, p-value=0.04)

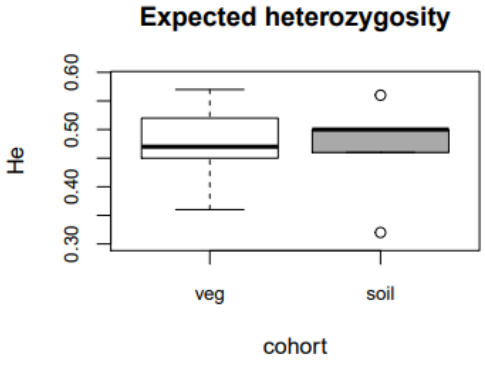
a)



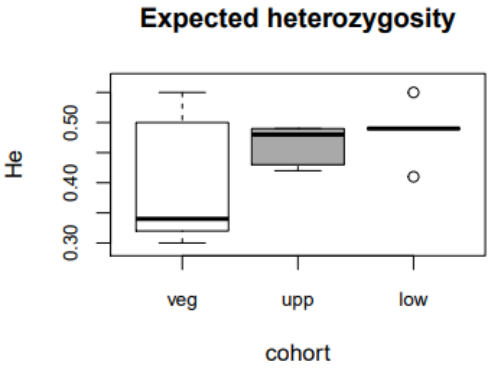
b)



c)



d)



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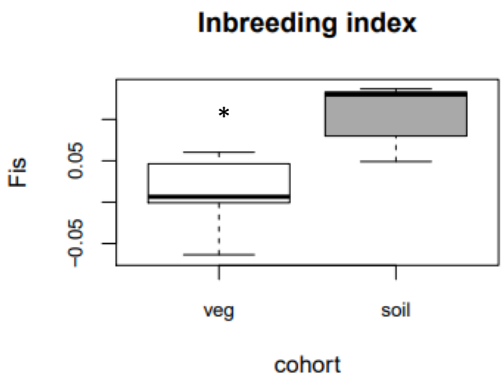


f)

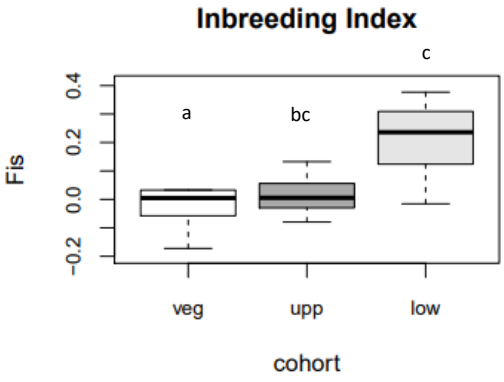


Figure 2. Principal coordinate analysis (PCoA) generated in GenAlEx based on the microsatellite data. Each diagram relates to one of the five study locations: a) Aichahof, b) Eitelberg; c) Grabenhof; d) Kühschlag; e) Undorf. Triangles correspond to individuals from the vegetation (veg), black circles to individuals from the upper soil layer (upp), and hollow circles to individuals from the lower soil layer (low), respectively. Samples from different cohorts are fully admixed, without any detectable separation.

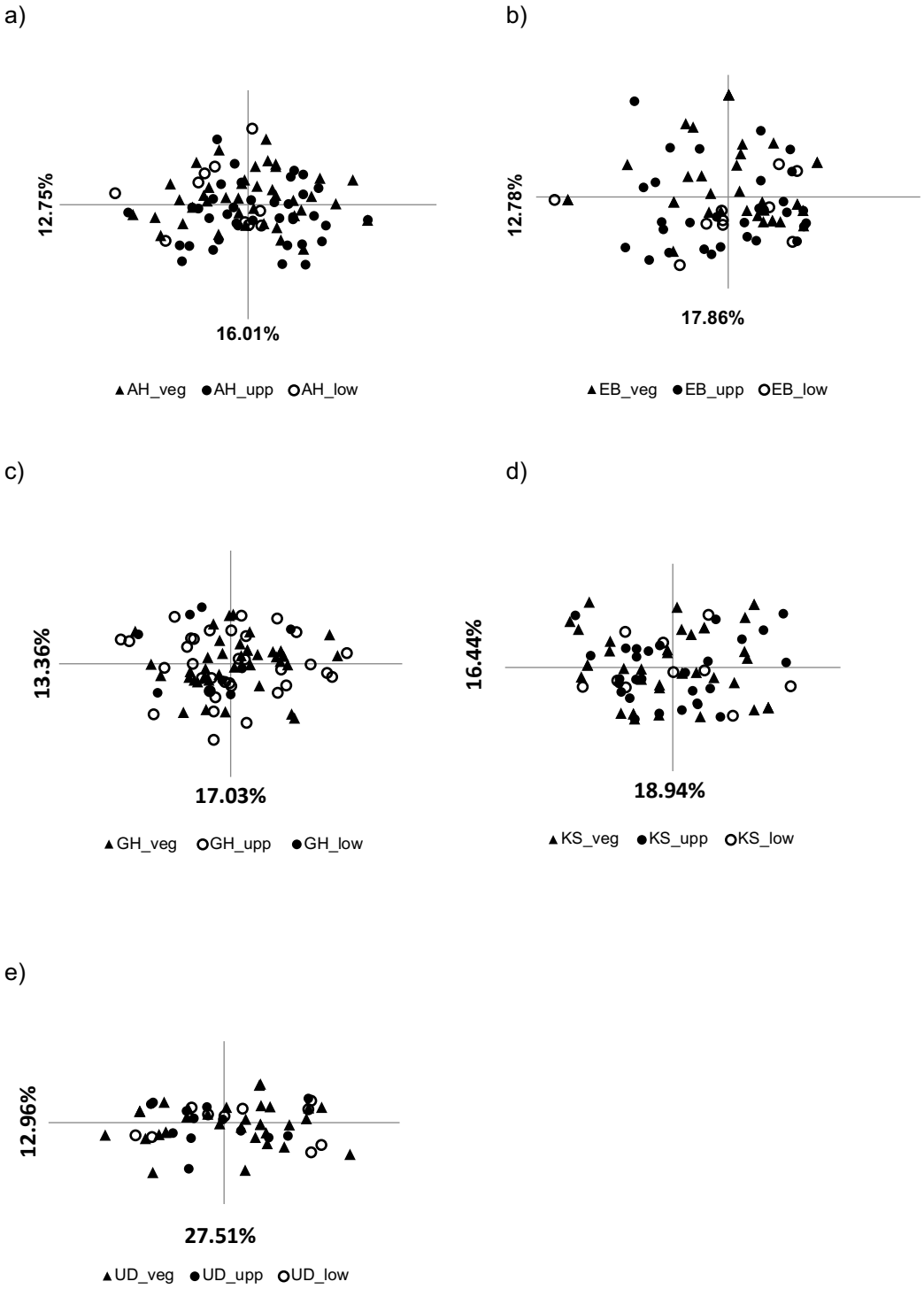
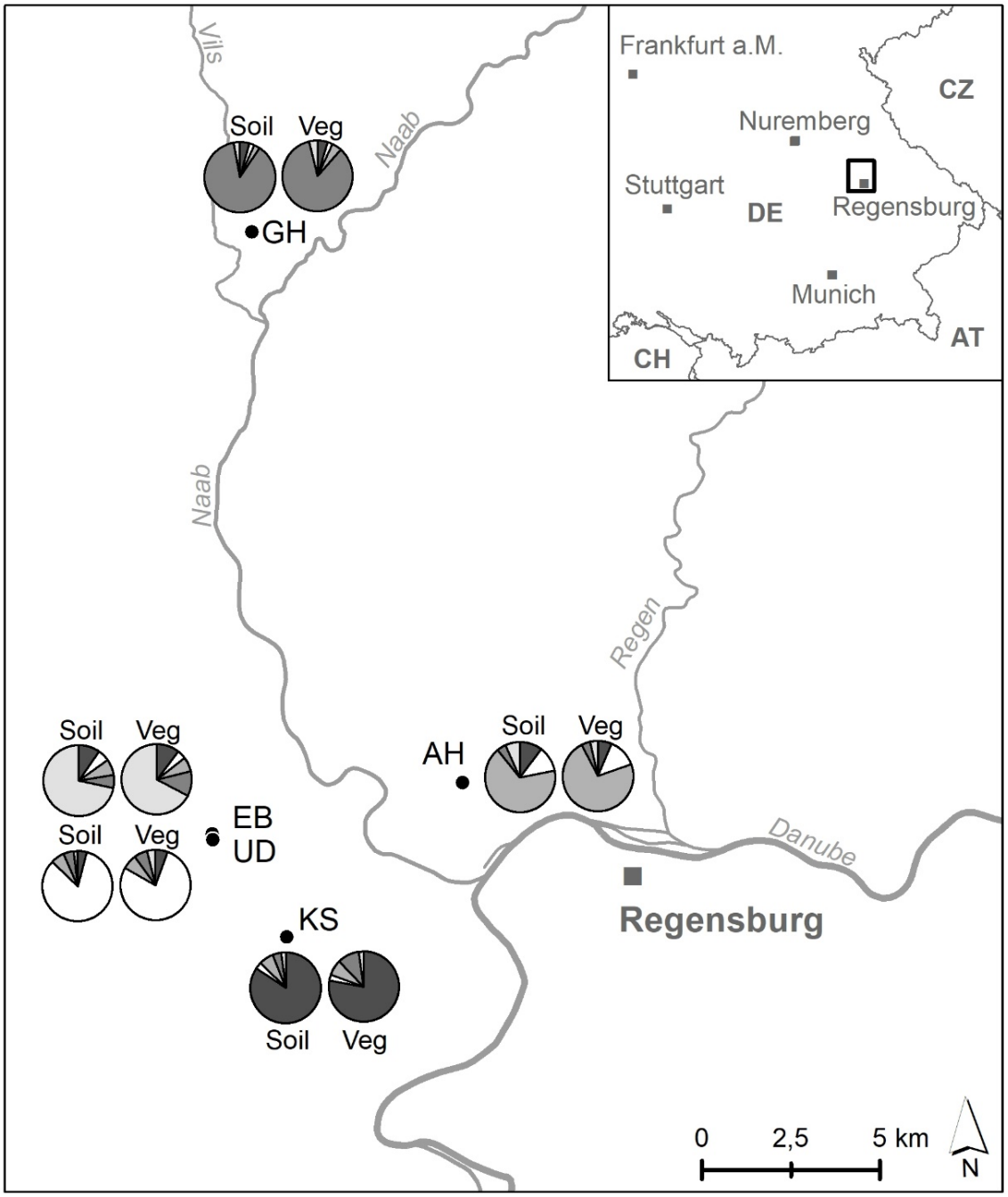


Figure 3. Map of the studied *Origanum vulgare* populations from two cohorts defined as soil (Soil) and vegetation (Veg). Pie diagram slices are equivalent to the population membership in five genetic groups inferred by the Bayesian cluster analysis STRUCTURE (Pritchard et al., 2000). Group 1, white, the site Undorf (UD); group 2, light grey, the site Eitelberg (EB); group 3, grey, the site Aichahof (AH); group 4, dark grey, the site Grabenhof (GH); group 5, black, the site Kühschlag (KS).



In the Bayesian cluster analysis, individuals from the whole dataset were assigned to five groups, reflecting the five study locations. At each study location, the aboveground and the seed bank populations were assigned to the same genetic cluster (Fig.3). This was the most probable solution for K, since ΔK reached the highest value (ΔK = 54.7) (Evanno et al., 2005). At the same time, the outputs of all thirty iterations for this value of K were identical (Kopelman et al., 2015). Additionally, at this level of K the mean logarithm of the probability of the data [LnP(X|K)] reached a plateau (Fig. S5), i.e. no additional information was gained from further increasing the number of clusters (Pritchard et al., 2000). We then carried out the Bayesian cluster analysis for each location separately, including all samples from the vegetation, the upper and the lower soil layers, partitioned according to the five study locations. These two approaches gave us the same information, implicating an absence of any apparent genetic structure between the three cohorts within each location (Fig. S6).

The NeighborNet diagram corroborated findings of previous analyses and displayed five groups corresponding to the five study sites, each containing three populations corresponding to the three cohorts. The diagram is given in the Supplementary (Fig. S3).

The spatial autocorrelation revealed no significant structure within our experimental plots, neither in the aboveground vegetation nor in the seed bank populations. More details are given in Supplementary (Fig. S7).

Discussion

Genetic diversity

The fundamental prerequisite of a seed bank, suitable for the restoration of aboveground populations is, that the genetic diversity of the seed bank must be higher, or at least as high, as in the aboveground population (Honnay et al., 2008). Overall, genetic diversity of *O. vulgare* was comparable to a pervious study (Helsen et al., 2013). Despite a relatively low seed bank density of *O. vulgare* in our study, levels of genetic diversity detected in the soil cohorts rival those detected in the aboveground populations. Virtually all indices of genetic diversity calculated for the total number of samples in the vegetation and the seed bank were similar or near equal. Only the inbreeding coefficient was slightly higher in the soil seed bank. The number of rare alleles in the aboveground populations and the corresponding seed banks was identical. However, low-frequency alleles tended to be more frequent in the soil seed bank (compare Honnay et al., 2008).

The slightly higher level of inbreeding we observed in the soil seed bank compared the aboveground population means that in the seed bank, ratio of homozygotes was higher than in the aboveground populations, and, consequently, that heterozygosity increases during the life cycle. This is in accordance with previous observations (Tonsor et al., 1993; Vitalis et al., 2004; Mandak et al., 2006a; Honnay et al., 2008). Three possible reasons may have caused this pattern. First, biparental inbreeding which might have caused elevated inbreeding levels relatively to random mating. However, this explanation is rather improbable, because we detected low inbreeding values in the aboveground

vegetation. Secondly, the potential consequences of the Wahlund effect may have caused differences (Wahlund, 1928; Garnier-Géré and Chikhi, 2013). The temporal – rather than spatial – Wahlund´s effect depends on the number of seasons represented in the seed bank as well as the among season variance in offspring allele frequencies. However, this assumption cannot be measured without a long-term data set. And, thirdly, the reason might involve a heterozygote advantage (Charlesworth et al., 1990). It seems that inbred seeds often fail to identify germination cues and, as a result, accumulate in the soil. Moreover, once germinated, due to the lower fitness less likely to survive afterword and to establish as adult plants (Kalisz, 1989; Tonsor et al., 1993). This factor may be the most probable reason for the gradual decrease of homozygosity in subsequent life stages from seeds to mature plants (Lesica and Allendorf, 1992; Vitalis et al., 2004; Mandak et al., 2006).

We additionally analysed the effect of a mediating variable, the soil sampling depth, on genetic differences between the seed bank and the aboveground population. Interestingly, we found that there was no significant difference in inbreeding index between the vegetation and the upper soil layer. On the other hand, the difference between lower soil layer and aboveground population was significant. This makes sense, since the seeds in deeper soil layers were buried earlier than those in the upper soil layers. Therefore, this is a more “ancient” genetic diversity representing the gentic diversity in the aboveground vegetation decades ago. In terms of homozygosity, this implicates higher similarity between the vegetation and the upper soil layer. Since more inbred individuals are less likely to perceive germination cues, this increases their dwelling time and accumulation in the soil (Tonsor et al., 1993). Moreover, *O. vulgare* is a species with light-induced germination and this trait could additionally reinforce the observed pattern (Mašková and Poschlod, 2021). Hence, we presume that higher inbreeding found in the seed bank would not necessarily mean its devaluation as a source for restoration purposes. For this reason, we would not doubt that the higher inbreeding index especially in the lower layers of the seed bank might jeopardize its beneficial role in restoration. Additionally, we presume that the post- and pre-germination selection would discriminate against homozygote individuals. Previous restoration results in *O. vulgare,* underpin this perception. Most probably, not only the successful colonization process, but also the seed bank prevents founder effect and genetic erosion, and contributes to a rapid build-up of genetic diversity in restored populations (Helsen et al., 2013).

In the analysis with partitioned soil layers, we found a marginally significant difference in the Mean number of alleles per locus (Na), which was higher in both soil layers than in the vegetation. Similar observations were done previously using comparisons of seedlings or mature plants with seed banks, in which allele frequencies displayed different patterns (Cabin, 1996; Schulz et al., 2018). Not quite contradictory, the meta-analysis done by Honnay et al. (2008) suggested a certain accumulation of rare alleles in the soil. However, there is most probably either direct or indirect local selection acting as a filter on alleles present in the soil. This selection filter seems to prevent at least some of them from germinating. Consequently, these

alleles do not appear in the aboveground populations and do not promote themselves in further generations (compare Cabin, 1996). It seems that the relationship between the seed bank and the aboveground population in the *O. vulgare* populations was most probably shaped by post- or pre-germination selection. Therefore, our findings imply that the soil seed bank may contribute to maintain the evolutionary potential especially in small and isolated populations as previously suggested (Stöcklin and Fischer, 1999; Piessens et al., 2005; Ayre et al., 2021).

Genetic Differentiation

Overall genetic differentiation among above ground populations of *O. vulgare* was higher than previously reported for this species (Helsen et al., 2013), most probably due to the higher distances among the studied populations and lower grassland connectivity in our experiment. Common species, especially outcrossing species, are demonstrably more dependent on gene flow between populations for maintaining genetic variation than selfing species, since the former require a pollen exchange between populations (Honnay and Jacquemyn, 2007). If populations shrink in size and number, connectivity becomes weaker. As a consequence, not only genetic diversity is prone to erosion, but also population genetic differentiation tends to increase (Honnay and Jacquemyn, 2007; Leimu et al., 2006; Willi et al., 2007). However, persistent seed banks may help to mitigate the impact of habitat fragmentation and shield species from genetic drift and populations genetic differentiation (Honnay et al., 2008) due to the beneficial allele supply from buried seeds, and due to enhanced effective population size (Del Castillo, 1994; Stöcklin and Fischer, 1999; Vitalis et al., 2004).

In our study, we detected a high similarity between the aboveground population and their underlying seedbanks, including both soil layers. AMOVA analyses at all levels, as well as PCoA and Bayesian cluster analysis, detected an absence of any apparent vertical genetic differentiation, i.e. between the aboveground population and its corresponding seed bank. This is in contrast to some previous observations (Cabin, 1996; Mandak et al., 2006b; Zaghloul et al., 2013). Not surprisingly, high levels of genetic differentiation were especially pronounced in environments coined by extreme climate conditions. Apparently, strong bottlenecks acting on aboveground populations – both natural and human-induced – may reinforce this differentiation paradigm (Zaghloul et al., 2013). However, similarly to Mahy et al. (1999) and partly Schulz et al. (2018), we did not observe this pattern. Mahy et al. (1999) suggested that this might be, inter alia, a result of a low number of sexual generations since the time of population founding. In our study system, the most probable reason corresponds to the absence of strong bottlenecks, disturbances and extreme climate conditions.

When comparing two soil depth layers, similar to Koch et al. (2003) for *Cardamine amara* in wet woodland, we detected that the two soil cohorts did not substantially differ from each other. This is not surprising, as this observation most likely reflects continuous seed supply from the surface population over the years and numerous generations. However, in a dynamic habitat of river banks and creeks, the two soil layers of *Cardamine amara* displayed clear genetic differences

(Koch et al., 2003), most probably driven by recurring disturbances in upper soil layers due to flooding events.

We have also conducted a horizontal analysis of genetic differentiation, i.e. between the aboveground populations at the study sites, and between the seed bank populations, respectively. The aboveground populations displayed a genetic differentiation of 14%, the seed bank populations of 13%, respectively. If analysed separately in the two soil layers, a previously described trend of lower differentiation in early life stages (i.e. seed populations) was even slightly reversed (differentiation of 16% among the populations from the lower soil layer). Similar patterns were observed in lower soil layers of wet meadows in *Cardamine amara* (Koch et al., 2003). This is in contrast to some previous observations suggesting that the partitioning genetic variation among populations increases with the population age, i.e. from soil seeds to mature plants (Cabin et al., 1998; Mandak et al., 2006b; Zaghloul et al., 2013). The possible reason might be the unequal severity of local selection pressures in aboveground vegetation. Thus, the persistent seed banks comprise and represent contributions of multiple generations of plants under different selection pressures in the face of environment fluctuations. The aboveground populations, in their turn, have to cope with aboveground environmental conditions and usually become more differentiated than the underlying seed pools (McCue and Holtsford, 1998; Zaghloul et al., random loss of alleles due to genetic drift, and slow down the increase of genetic differentiation resulting from the lack of gene flow between fragmented populations. Soil seed banks represent, therefore, a valuable source of genetic diversity for a recovery in case of population shrinkage or extinction. In line with the experience of practitioners, local perturbations may help to reactivate populations contained in a seed bank, and to 2013). Most probably, this is due to the genotype dependent local selection (Mandak et al., 2006b; Honnay et al., 2008). However, we did not observe any apparent expression of this pattern. This might be a natural consequence of lack of a strong selection pressures in the vegetation in our study system.

In accordance with previous analyses, we detected no apparent spatial genetic structure within our study plots. This is possibly due to (i) high outcrossing rates, (ii) insect pollination habit of the study species within the populations, which contributed to a random distribution of alleles within the boundaries of our experimental plot, (iii) due to the low levels or absence of clonality, and (iv) and due to the long-term persistent soil seed bank. An evidence from previous research suggests that clonality and/or a limited seed dispersal may bring about genetic correlation over short distances (Schnabel and Hamrick, 1990; Reisch et al., 2007; Listl and Reisch, 2012). In our study, most probably, the seed and pollen dispersal exceeded the spatial scale settings of our experiment.

Conclusions

In our study, we detected that soil seed banks contain levels of genetic variation comparable to the aboveground populations. Considering our observation in the context of the ongoing fragmentation of suitable habitats, we conclude that the genetic diversity stored in the soil seed bank may counteract detrimental effects of the fragmentation process, preventing from

reintroduce their genetic diversity into new aboveground populations.

However, our study sites are located in a relatively constant type of habitat. It would, therefore, be interesting to learn more about the seed bank of *O. vulgare* in contrasting environments under different selection regimes, e.g. along a habitat gradient toward alluvial sites under a stronger regime of disturbance.

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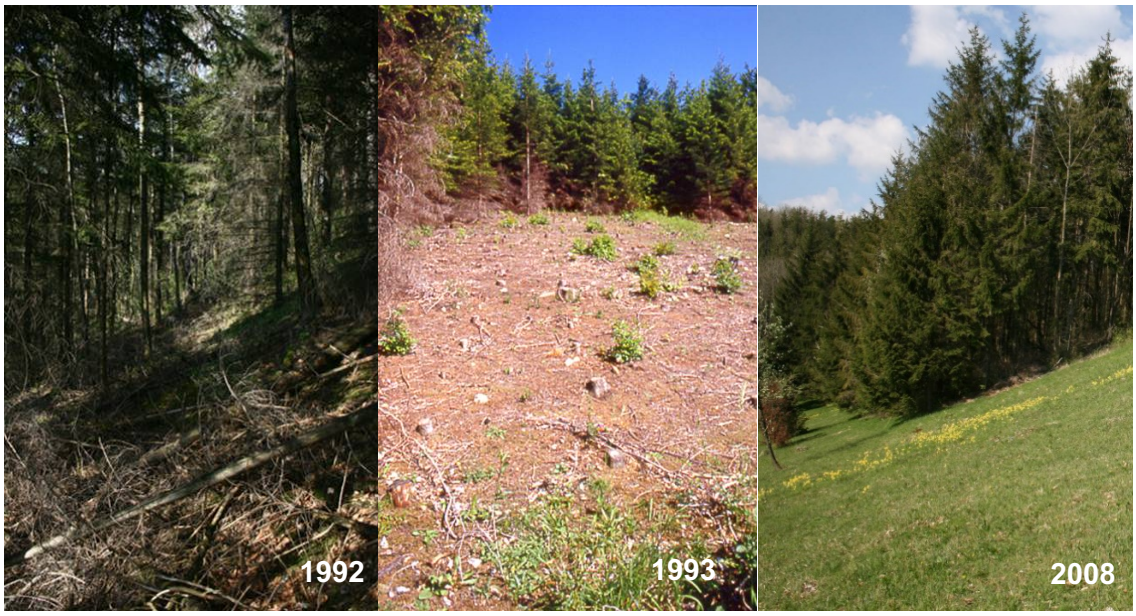
RESTORATION OF CALCAREOUS GRASSLANDS BY NATURAL RECOLONIZATION AFTER FOREST CLEARING AND ITS IMPACT ON THE GENETIC VARIATION OF THREE COMMON HERB SPECIES



The nature reserve Haarberg-Wasserberg, SW Germany.

The spruce forest planted after WW II, in 1992; the same spot after clear-cutting, in 1993;

a natural regeneration of the calcareous grassland fifteen years later, in 2008

Abstract

Species-rich calcareous grasslands in Europe strongly declined during the 20th century due to drastic land use changes. Many grasslands were converted into more productive pastures or are covered by shrubs or forests today, since they were overgrown after abandonment or afforested. Restoration of calcareous grasslands by shrub or forest clearing and subsequent recolonization of grassland species from adjacent grasslands is, therefore, an important conservation approach. Restored populations of calcareous grassland species may, however, differ from their source populations in genetic diversity and differentiation due to potential founder and bottleneck effects.

In our study we analysed, therefore, the impact of restoration by forest clearing and natural recolonization on the genetic variation of three common calcareous grassland species without persistent seed bank (*Agrimonia eupatoria*, *Campanula rotundifolia*, and *Knautia arvensis*) in South Western Germany. We used molecular markers AFLPs (Amplified fragment length polymorphisms) to compare genetic diversity within and differentiation between spontaneously recovered subpopulations with adjacent historically old, natural subpopulations at eight study sites. Restored parts of the grasslands have been re-established during the 1990s.

Molecular markers revealed broadly similar levels of genetic diversity in source and restored subpopulations of the study species. Only *A. eupatoria* exhibited slightly higher diversity in restored subpopulations, which may be explained by higher dispersal potential due to the hooky fruits of the species. Genetic differentiation between source and restored subpopulations was not significant, indicating strong gene flow between the subpopulations. Our study underlines, therefore, that restoration of calcareous grasslands by natural recolonization after forest clearing is an efficient method to re-establish genetically variable subpopulations comparable to their sources.

Keywords

Agrimonia eupatoria, calcareous grasslands, *Campanula rotundifolia*, genetic variation, *Knautia arvensis*, recolonization, restoration

Introduction

Semi-natural calcareous grasslands rank among the most species-rich, but also highly vulnerable and endangered ecosystems across Europe. Due to the tremendous changes of land-use practices during the 19th and 20th century, this habitat has rapidly declined in area and quality (Poschlod and WallisDeVries, 2002; Pullin et al., 2009; Huber et al., 2017). Calcareous grasslands were abandoned, afforested or converted into intensively managed agricultural land (WallisDeVries et al., 2002). Due to the extraordinary biodiversity of calcareous grasslands, they are an important target habitat for the biodiversity maintenance at the European scale (European Community, 1992). Since the 1990s, in some cases even earlier, numerous restoration schemes have been launched to re-establish species rich calcareous grassland communities.

Several methods of grassland restoration as well as techniques to enhance species richness have been frequently applied to recover near-natural grasslands following cropland, quarry and mining areas abandonment (Török et al., 2011) or forest felling (Poschlod et al., 1998a; Pärtel et al., 1998; Bisteau and Mahy, 2005). First, the common and widely used restoration practice is sowing regional seed mixtures (Höfner et al., 2021; Durka et al., 2019; Kaulfuß et al., 2022), either in high-diversity or low-diversity option (Kirmer et al., 2012). This method helps to restore grassland communities in a comparatively short time horizon (Kövendi-Jakó et al., 2019). It is especially vital in cases when the local species pool does not enable spontaneous grassland regeneration via natural diaspore input from the seed rain and seedbank (Willems and Bik, 1998) or, if effective dispersal vectors (DiLeo et al., 2017) are lacking, respectively. Second, translocation of plant material, e.g. green hay, raked litter, threshed seeds or barn chaff (Kaulfuß and Reisch, 2021a) have been applied to introduce target species and to enhance species richness in degraded grasslands, or to initiate new grasslands (Kiehl et al., 2010; Albert et al., 2019; Poschlod et al., 1997). Third, planting seedlings, mature plants or belowground parts, respectively, is a technique often applied to additionally enhance species richness and propagule availability in sites restored using other methods (Guerrant Jr and Kaye, 2007). This approach usually yields faster maturity and population establishment than seed sowing (Dalrymple et al., 2012) but requires more time and financial costs. Use of plants also implies multiple steps in a production process and may unintentionally cause erosion of genetic diversity (Basey et al., 2015).

Finally, spontaneous colonization on fallow lying old fields (review Rejmanek and van Katwyk, 2005), abandoned quarry areas (Ilves et al., 2015) or following scrub or woodland removal (Kiefer and Poschlod, 1996; Kiefer, 1998b; Blanckenhagen and Poschlod, 2005) imposes an important grassland restoration tool. This method is a natural and low cost way of grassland restoration (Prach and Hobbs, 2008), as it relies on spontaneous seed dispersal processes from locally available propagule sources (Redhead et al., 2014; Bakker et al., 1996c; Kirmer et al., 2008; Redhead et al., 2014). Natural colonization is usually combined with other techniques, e.g. sowing seed mixtures (Johanidesová et al., 2015; Kaulfuß et al., 2022), creation of artificial

biodiversity hotspots (Kiss et al., 2021), as well as topsoil removal (Johanidesová et al., 2015; Řehounková et al., 2021; Kiss et al., 2021). However, the occurrence of source grasslands in the nearby surrounding landscape plays a crucial role in colonization of restored grasslands by target species (Prach et al., 2015; Kiss et al., 2021; Aavik and Helm, 2018).

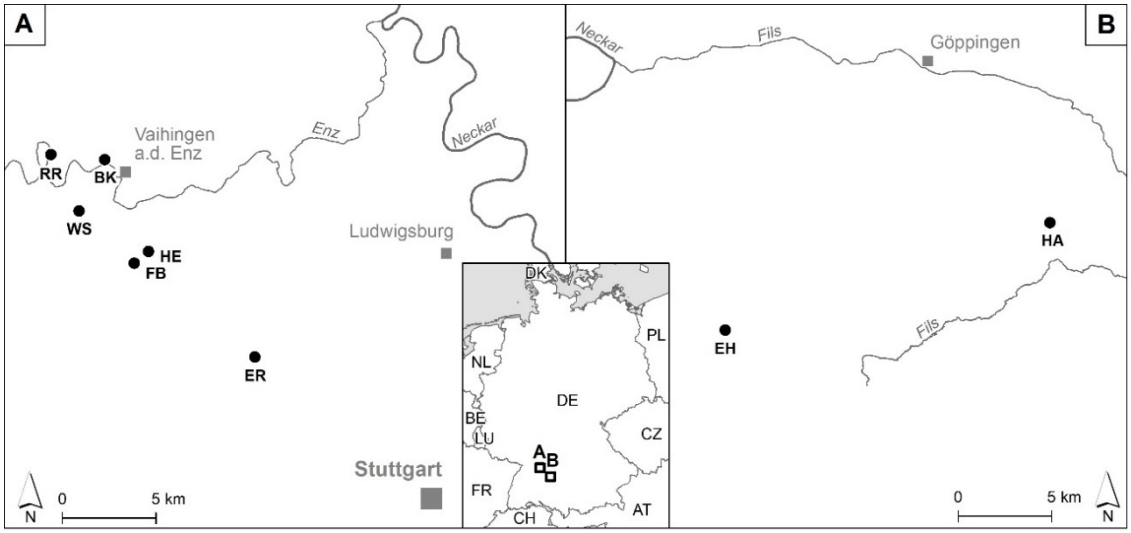
In comparison with other restoration methods, natural (re)colonization intrinsically involves the advantage of matching the gene pool of nearby populations, due to the seed rain from surrounding grasslands (McKay et al., 2005). For restoration purposes, the importance of regional seed material has been generally recommended (Mijnsbrugge et al., 2010) and thoroughly documented (Bucharova et al., 2019; Höfner et al., 2021). The local origin of diaspores is desirable to preserve patterns of genetic variation, because mixing strongly differing genotypes may lead to the outbreeding depression (Hufford and Mazer, 2003; Frankham et al., 2011). This may bring about decreased fitness and vitality in restored populations, since coadapted gene complexes could break down and local adaptations get lost (Montalvo and Ellstrand, 2001; Hufford and Mazer, 2003).

Regeneration process of restored sites always imposes several risks to genetic makeup of newly established populations. At the present time, especially genetic issues caused by bringing in propagules, particularly if those were bred specifically for this purpose have been studied rigorously (Durka et al., 2017; Höfner et al., 2021; Bucharova et al., 2022).

However, restoration by colonization from adjacent habitats may also impose the risk of genetic diversity loss, because founder groups may consist of just a limited number of individuals and comprise only a part of the source populations genetic diversity (Franklin and Frankham, 1998; Vandepitte et al., 2012). Further, the loss of alleles and changes in their frequencies may additionally occur due to random genetic drift in small populations (Franklin, 1980). Finally, founder events may not only lead to the locally decreased genetic diversity, but also to considerably enhanced genetic divergence between populations (Vandepitte et al., 2012). The magnitude of a founder effect and subsequent genetic differentiation as a consequence of an extinction and recolonization process, has been studied explicitly in the context of metapopulation theory (Slatkin, 1977a; Wade and McCauley, 1988; Whitlock and McCauley, 1990).

This theory predicts that the severity of a founder effect depends on two major parameters. First, the number of colonists arriving to a restored site, and their proportion in comparison with the number of migrants exchanged among extant populations. If the number of founders exceeds the number of migrants exchanged between established populations more than twice, then the founder effect is expected to be weak (Wade and McCauley, 1988). Second, the number (single or multiple) of source populations contributing to the formation of a colonist group is also determining regarding the founder effect magnitude (the “propagule pool” and the “migrant pool” model; Slatkin, 1977b; Whitlock and McCauley, 1990). The likelihood of founder effects will be reduced

Figure 1. Geographic location of the study sites in South Western Germany. Source and restored subpopulations of the study species *A. eupatoria*, *C. rotundifolia* and *K. arvensis* were investigated at six study sites each. *A. eupatoria* was sampled in EH, FB, HA, HE, RR and WS; *C. rotundifolia* was sampled in BK, EH, FB, HA, HE and RR; *K. arvensis* was sampled in ER, FB, HA, HE, RR and WS. BK, Botenklinge; EH, Eichhalde; ER, Eselrain; FB, Furtberg; HA, Haarberg; HE, Heulerberg; RR, Roter Rain; WS, Weiler-Schlätterle.



if more than one source populations appeared under seed sources (Slatkin, 1977a), and, further, if high migration rates into restored populations took place, enabled through spatial vicinity (Helsen et al., 2013), permeability and intrinsic species dispersal capacity, or availability of a suitable dispersal vector, respectively (DiLeo et al., 2017). Moreover, fast population growth following founding events also helps to rapidly recover population genetic diversity, owing to new mutations (Nei et al., 1975). The broad population genetic diversity is a necessary raw material for the population´s adaptability to changing environments. If levels of genetic diversity are reduced, then populations would be at risk in the long term due to the loss of their evolutionary potential (Franklin and Frankham, 1998; Bucharova et al., 2019).

Spontaneous colonization processes and their impact on genetic properties of founder populations were investigated in numerous studies, often in natural landscapes, e.g. new volcanic deposits (Bishop, 1996; Yang et al., 2008), glacier forelands (Raffl et al., 2006). And floodplains (van Looy et al., 2009a; Honnay et al., 2009). Vandepitte et al. (2007) and Jacquemyn et al. (2009) investigated the impact of gene flow, isolation and genetic drift on genetic variation in forest herbs with contrasting mating systems. Colonization of abandoned quarries and its consequences for genetic variation and fitness of a rare herb was studied by Ilves et al. (2015). However, only a few studies have highlighted the genetic con-sequences of colonization processes in temperate grasslands after scrub and tree removal. In addition, results of these studies were inconsistent, most probably due to different

configuration and initial number of source and restored populations, as well as due to differences in species traits. Thus, Vandepitte et al. (2012) found that a limited number of available remnant source populations led to the reduced genetic diversity as well as inflated genetic differentiation in founder populations. On the other hand, Helsen et al. (2013) stated that sufficient number of source populations occurring in the surroundings of colonized spots may result in neither de-creased genetic diversity, nor increased genetic differentiation between founder populations. However, this outcome was most probably also due to the gene flow and recruitment from the long-term seedbank.

A broader knowledge is thus still lacking in terms of genetic patterns in recolonized calcareous grasslands, without any contribution of viable seeds in soil. To address this gap, we highlight the impact of the recolonization on common grassland *species Agrimonia eupatoria, Campanula rotundifolia* and *Knautia arvensis*.

Specifically, we asked following questions: (i) what are possible inter-specific differences in genetic diversity between the study species relating to differences in their mating systems? (ii) Are levels of genetic diversity comparable in source and restored subpopulations? (iii) Is there a significant genetic differentiation between source and re-stored subpopulations? And, finally, (iv) is recolonization after clear-cutting an appropriate method to maintain local genetic variation pat-terns of common grassland species without persistent soil seed bank?

Materials and Methods

Study sites, study species and sampling design

The study is based on a restoration project situated in South-Western Germany, in the region of the Neckar basin and the Swabian Alb (Kiefer and Poschlod, 1996; Poschlod et al., 1998a). Here, former calcareous grasslands overgrown for at least one decade (Kiefer, 1998a) with trees and shrubs such as pine (*Pinus sylvestris*) and blackthorn (*Prunus spinosa*), or deliberately afforested with *Pinus sylvestris* and *Picea abies* were restored in the 1990s by clear-cutting. Trees, scrub, as well as the entire herbaceous vegetation layer were removed during the restoration. The sites were then left open for grassland recovery through spontaneous recolonization by species immigrating from adjacent well-preserved remnant grasslands and, to some extent, also from the soil seedbank (Blanckenhagen and Poschlod, 2005).

For our study, we selected eight study sites located in South Western Germany near Stuttgart city, on the hills of the Swabian Alb and its northern surroundings in the Neckar River basin (Fig.1). The altitude of the study sites ranges from 250 to 700 m above sea level. The climate in the study area is temperate; the mean precipitation varies between 650 mm in the Neckar basin and 900 mm in the Swabian Alb, respectively. The bedrock is formed by Malm series, which belongs to the upper Jurassic formation and consists mostly of the reef limestone. The prevailing soil type is Rendzina, sometimes developed as brown soil. In our study, we consequently applied a paired study design

in all study sites (source vs. restored subpopulations present at each study site). This means that we have strictly chosen sites where restored and source grasslands were simultaneously present. All subpopulation pairs in our study were adjacent, without any barriers between them. Zoochory was the most probable dispersal modus, though dispersal by gravity was also possible. Grazing occurred sporadically during regeneration and contributed to the seed dispersal. We consequently use the term subpopulation with regard to the ancient and restored parts of the grasslands. Recently, both the source and restored subpopulations build one single population occupying particular study locations.

In our study, we investigated not only one but three study species. Moreover, we selected taxa with different pollination strategies, thus enabling broader conclusions regarding impact on genetic variation. Suitable study species were selected according to the following criteria: first, widespread species which occur in each part (source and restored) of the grasslands. This enabled us to consequently

apply the paired design of source/restored grassland parts in all study sites. Second, taxa which do not regenerate from the soil seed bank, and, third, species which are likely to represent a gradient of genetic diversity, due to the different mating systems (mainly selfing, mixed-mating and outcrossing species). *Agrimonia eupatoria* and *Knautia arvensis* are known to only possess a transient soil seed bank. *Campanula rotundifolia* maintains a short-term persistent seed bank (Kleyer et al., 2008; Poschlod et al., 2003), i.e. the seeds should remain viable until at least the sixth germination season after dispersal (Walck et al., 2005). However, the restored sites were overgrown or afforested for at least ten years. Moreover, remaining individuals under the tree or scrub canopy did not contributed to the soil seed bank, because *C. rotundifolia* is a light demanding species (Ellenberg H. et al., 1991). This feature made it possible that exclusively genetic properties of subpopulations arisen from the recolonization process were assessed, without a contribution of propagules

from the soil seedbank (compare Helsen et al., 2013). *A. eupatoria* is a perennial, mainly selfing species (Chrtek, JR., 2018) occurring commonly in dry grasslands, mesic pastures and meadows, but also in the forest fringe communities, heathlands and scrub. Seed dispersal takes places predominantly through zoochory (Römermann et al., 2005; Fischer et al., 1996). *C. rotundifolia* is a perennial, insect-pollinated, predominantly outcrossing species. Self-incompatibility was also reported (Chrtek, JR., 2018). Seeds are dispersed mainly due to gravity, but zoochory is also possible especially by grazing sheep (Fischer et al., 1996). *C. rotundifolia* occurs in similar habitats as *A. eupatoria*, but also in the vegetation of screes and walls. *K. arvensis* is a perennial, mainly outcrossing species (Chrtek, JR., 2018), occurring in meadows and mesic pastures, as well as in acidophilous grasslands and pine forests. Seeds are dispersed by gravity, but zoochory, especially transport by sheep flocks plays an important role (Fischer et al., 1996; Poschlod et al., 1998; Chytrý et al., 2014-2018; Chytrý et al., 2021).

Each species was sampled at six study sites. At each site, we sampled subpopulations of study species in the historically old parts of grasslands which served as seed sources. Similarly, we evenly sampled restored subpopulations of the same species in grassland parts which developed after clearcutting. Geographic position was measured by means of global positioning system (GPS). In summer 2018, we collected fresh young leaves from eighteen individuals per subpopulation per species and dried them over silica gel. We collected the samples randomly within the whole subpopulation area, applying, if necessary, a threshold limit of three meters between the samples. We then compared the genetic variation of plants from subpopulations occurring in the ancient (source) and in the restored parts.

DNA ploidy level of the study species

Flow cytometrical analyses give information about the amount of DNA contained in nuclei of the study species. For all study species, polyploid complexes were described (Slavik, 2000). Since possible mating barrier between different ploidy levels may affect genetic variation and thus bias our results, we assessed DNA ploidy levels at all sites for all study species. Flow cytometrical analyses (Suda et al., 2006) were carried out according to the two-step protocol after Dolezel et al. (2007). We analyzed individuals in all six study sites per species, for each species separately. Nuclei from the leaf tissue were isolated from three individuals per subpopulation (both source and restored) in each study location. From each subpopulation, samples were randomly chosen for analyses. In total, we analyzed 132 individuals of three study species. In one site (Eichhalde), subpopulations of *A. eupatoria* contained individuals with a double amount of DNA, presumably caused by occurrence of a congener octoploid species *Agrimonia procera* at the same grassland. We therefore analyzed further twelve individuals from both subpopulations and then decided to exclude the Eichhalde study location from further analyses. In the remaining five study locations, *A. eupatoria* occurred as a tetraploid. In *C. rotundifolia,* we revealed the presence of diploids. For *K. arvensis*, we detected occurrence of tetraploids. Thus, we excluded co-occurrence of several ploidy levels in our study system, for all study species. For detailed description of the flow cytometric analyses see Supplementary.

AFLP analyses

The AFLPs yielded 149 analyzed individuals of *A. eupatoria*, 184 individuals of *C. rotundifolia*, and 175 individuals of *K. arvensis*, a total of 508 plants (Vos et al., 1995). This number is lower than that of sampled individuals due to the losses during the lab procedure. Genomic DNA for AFLPs was isolated from silica gel dried plant material following the CTAB protocol (Rogers and Bendich, 1994), using adaptations as described in previous studies (Reisch et al., 2005). Concentration of DNA stock solutions was detected using a nano-spectrophotometer and these were subsequently diluted with water to a concentration of 7.8 ng/µl. AFLP analyses were performed following the standardized protocol of Beckmann Coulter as described formerly (Bylebyl et al., 2008; Reisch, 2008). Selective amplifications were carried out using three primer combinations. For *A.*

eupatoria we used following primer combinations: MseI-CTG/EcoRI-AAC (D2), MseI-CTG/EcoRI-AAG (D3), MseI-CTC/EcoRI-ACT (D4), for *C. rotundifolia*: MseI-CAC/EcoRI-ACC (D2), MseI-CAT/EcoRI-AGG (D3), MseI-CAT/EcoRI-ACT (D4), and for *K. arvensis* MseI-ACC/EcoRI-CAG (D2), MseI-AGG/EcoRI-CTT (D3), MseI-ACT/EcoRI-CTT (D4), respectively. The fluorescence-labeled samples were separated by capillary gel electrophoresis on an automated sequencer (GenomeLab GeXP, Beckman Coulter). Raw data were examined applying the GeXP software (Beckman Coulter), exported as synthetic gel files (.crv files) and analyzed using the software Bionumerics 4.6 (Applied Maths). Across all samples, each band was scored as either present or absent. For quality control of the AFLP procedure, a genotyping error rate was calculated (Bonin et al., 2004), which was 0.18 % for *A. eupatoria*, 4.94 % for *C. rotundifolia* and 1.84 % for *K. arvensis*.

Statistical analyses

Based on the AFLP data, a binary matrix (0/1) was generated using the software Bionumerics 4.6 (Applied Maths). If present, fragments of a particular length were classified as 1 and in case of absence as 0. Using the 0/1 matrix, the percentage of polymorphic loci (PPL) was calculated across the whole dataset as a ratio n_i/N, where n_i is number of fragments containing polymorphism, N represents the full number of fragments (loci). Percentage of polymorphic loci (PPL), a frequency-based estimator of genetic diversity within subpopulations was computed using PopGene1.32 (Yeh et al., 1997). Genetic diversity was further estimated using the sample size independent formula based on the AMOVA measurements, SSWP/n-1 (sum of squares within subpopulations divided by the subpopulation sample size reduced by one). Thanks to the sample size-sensitivity of this genetic diversity measure, we were able to additionally compare subpopulations irrespective of whether or not the sample sizes were equal. We further calculated the level of rarity using the frequency-down-weighted marker values (DW), an index usually used as a measure of the rare fragment accumulation within a subpopulation (Schönswetter and Tribsch, 2005), which is an equivalent to range-down-weighted species values (Crisp et al., 2001). The number of occurrences of each AFLP marker in a subpopulation was divided by the number of occurrences of that particular marker in the entire dataset. These values were eventually summed.

Bayesian cluster analysis was performed using the program STRUCTURE 2.3.4. (Pritchard et al., 2000, Pritchard et al., 2009). The population structure of the whole data set was inferred based on the clustering of individuals. An admixture model with correlated allele frequencies was assumed. A number of Markov Chain Monte Carlo simulations was set to 100.000 and the burn-in period to 10.000 iterations. Number of clusters K was set to 13 for *C. rotundifolia* and *K. arvensis*, and 11 for *A. eupatoria*. This corresponds to the number of subpopulations plus one. Analyses were run independently 20 times for each K to assess the amount of variation of the likelihood for each value of K. The best estimate of K for the given data set was specified according to the model, which gave a consistent results for all 20 runs (Kopelman et al., 2015).

The program Harvester (Earl and vonHoldt, 2012b) was used to summarize results.

An estimate of the posterior probability of the data Pr (X|K) for a particular K was calculated (Pritchard et al., 2000). To identify the real number of clusters K, ad hoc statistic ΔK was used which was calculated as a second order rate of change of probability of the data Pr (X|K) with respect to K (Evanno et al., 2005). Genetic differentiation between subpopulations and regions (groups of subpopulations) was detected using analysis of molecular variance AMOVA (Excoffier et al., 1992) implemented in the program GenAlex 6.41 (Peakall and Smouse, 2006). Computations based on pairwise Euclidian distances between samples. Significance values related to variance components rest upon 999 permutations of individuals supposing no genetic structure. We tested partitioning of genetic variation within subpopulations, between subpopulations and between regions, applying two-level and three-level hierarchical AMOVAs.

A multivariate analysis (principal coordinate analysis, PCoA)) was calculated separately for each study species and plotted using GenAlEx 6.41 (Peakall and Smouse, 2006). Computations based on Jaccard similarities between individuals, $C_J = a / [a + b + c]$, where a is the number of fragments shared between two individuals and b and c are the numbers of fragments present in only one individual.

Results

Genetic diversity of source and restored subpopulations

For *A. eupatoria*, AFLP genotyping of 149 plants resulted in 146 fragments, of which 58% were polymorphic. The percentage of polymorphic loci (PPL) in source subpopulations ranged from 26.03% to 31.51% (mean 27.63%), in restored subpopulations between 27.40 and 32.88 (mean 30.03). The frequency-down-weighted marker value (DW) ranged between 7.93 and 8.86 (mean 8.44) in the source subpopulations, in restored subpopulations from 8.16 to 9.24 (mean 8.56). In the source subpopulations, SSWP/n-1 (sum of squares within a subpopulation divided by n-1, where n is a subpopulation size) ranged from 5.17 to 7.41 (mean 6.42). In the restored subpopulations, values of SSWP/n-1 were slightly but significantly higher (mean 7.14; p=0.03) (Table 1a). We observed, however, no significant differences in PPL and DW.

In *C. rotundifolia*, AFLP analysis of 184 plants yielded 179 fragments, of which 90.5% were polymorphic. The percentage of polymorphic loci in source subpopulations varied between 62.27% and 74.30% (mean 68.24%), in restored subpopulations between 68.16% and 73.74% (mean 70.67%). The frequency-down-weighted marker value (DW) in source subpopulations ranged between 12.61 and 14.16 (mean 13.39), in restored subpopulations between 13.40 and 14.04 (mean 13.61). SSWP/n-1 in source subpopulations ranged from 20.08 to 23.79 (mean 21.86), in restored subpopulations from 20.70 to 22.99 (mean 22.10). Genetic diversity in restored subpopulations of *C. rotundifolia* was slightly but not significantly higher than in source subpopulations (Table 1b).

Table 1. Genetic diversity of the study species (a) *A. eupatoria*, (b) *C. rotundifolia* and (c) *K. arvensis*. Given are number and name of the study sites, abbreviation with extension S (source subpopulation) or R (restored subpopulation), geographic location (latitude and longitude), a number of analyzed individuals per population (n) and genetic diversity as the sum of squares within populations divided by the number of individuals minus one (SSWP/n-1), frequency-down-weighted marker value (DW) and percentage of polymorphic loci (PPL). The results of the t-tests are also given; significant differences are marked with boldface.

Nr.	Study sites	Abb.	Latitude	Longi-	N	SSWP/n	DW	PPL%
	(a) *Agrimonia eupatoria*							
	Source subpopulations							
1	Furtberg	FB_S	48.889000	8.966417	17	7.29	8.66	30.82
2	Haarberg	HA_S	48.626583	9.735583	16	7.41	8.53	31.51
3	Heulerberg	HE_S	48.894694	8.976806	15	5.17	7.93	21.23
4	Roter Rain	RR_	48.941278	8.905667	11	6.69	8.86	26.71
5	Weiler-Schlät-	WS_	48.914194	8.926278	17	5.56	8.24	26.03
	Restored subpopulations							
6	Furtberg	FB_R	48.889000	8.966417	12	8.27	9.24	32.88
7	Haarberg	HA_	48.626583	9.735583	13	7.76	8.33	28.08
8	Heulerberg	HE_	48.894694	8.976806	14	6.46	8.54	32.88
9	Roter Rain	RR_	48.941278	8.905667	16	6.75	8.53	27.40
10	Weiler-Schlätterle	WS_	48.914194	8.926278	18	6.43	8.16	28.77
	mean ± SD source					6.42±1.01	8.44±0.37	27.63±3.8
	mean ± SD restored					7.14±0.83	8.56±0.41	30.03±2.3
	Paired t-test					**p=0.03**	p=0.59	p=0.33
	(b) *Campanula rotundifolia*							
	Source subpopulations							
1	Botenklinge	BK_S	48.938778	8.945028	18	21.40	13.55	70.95
2	Eichhalde	EH_S	48.576250	9.499528	15	23.79	13.68	74.30
3	Furtberg	FB_S	48.889000	8.966417	15	20.96	13.64	69.83
4	Haarberg	HA_S	48.626583	9.735583	14	20.08	14.16	62.57
5	Heulerberg	HE_S	48.894694	8.976806	16	21.84	12.61	62.27
6	Roter Rain	RR_	48.941278	8.905667	9	23.08	12.69	62.57
	Restored subpopulations							
7	Botenklinge	BK_R	48.938778	8.945028	18	20.70	13.40	68.16
8	Eichhalde	EH_	48.576250	9.499528	18	22.30	14.04	72.07
9	Furtberg	FB_R	48.889000	8.966417	16	22.63	13.24	73.74
10	Haarberg	HA_	48.626583	9.735583	15	22.59	13.52	70.39
11	Heulerberg	HE_	48.894694	8.976806	14	22.99	13.69	71.51
12	Roter Rain	RR_	48.941278	8.905667	16	21.40	13.79	68.16
	mean ± SD source					21.86±1.3	13.39±0.6	68.24±4.7
	mean ± SD restored					22.10±0.8	13.61±0.2	70.67±2.2
	Paired t-test					p=0.75	p=0.49	p=0.14
	(c) *Knautia arvensis*							
	Source subpopulations							
1	Eselrain	ER_S	48.843917	9.054417	15	13.78	9.75	56.16
2	Furtberg	FB_S	48.889000	8.966417	14	14.13	9.95	57.53
3	Haarberg	HA_S	48.626583	9.735583	15	12.03	10.85	47.95
4	Heulerberg	HE_S	48.894694	8.976806	13	13.00	10.19	50.00
5	Roter Rain	RR_	48.941278	8.905667	16	12.90	9.98	53.42
6	Weiler-Schlätterle	WS_	48.914194	8.926278	15	13.34	9.85	50.68

Table 1. (continued)

Nr.	Study sites	Abb.	Latitude	Longi-	N	SSWP/n	DW	PPL%
	(c) *Knautia arvensis*							
	Restored subpopulations							
7	Eselrain	ER_	48.843917	9.054417	15	11.52	9.85	50.68
8	Furtberg	FB_R	48.889000	8.966417	15	10.55	9.66	42.47
9	Haarberg	HA_	48.626583	9.735583	14	12.76	9.95	48.63
10	Heulerberg	HE_	48.894694	8.976806	15	12.21	9.85	47.95
11	Roter Rain	RR_	48.941278	8.905667	14	12.41	9.85	50.00
12	Weiler-Schlätterle	WS_	48.914194	8.926278	14	14.98	10.28	62.33
mean ± SD source						13.20±0.7	10.09±0.4	52.62±3.7
mean ± SD restored						12.41±1.4	9.91±0.21	50.34±6.5
Paired t-test						p=0.36	p=0.35	p=0.55

For *K. arvensis,* AFLP analysis of 175 plants yielded 146 fragments, 82% of them were polymorphic. The percentage of polymorphic loci in source subpopulations ranged between 47.95% and 57.53% (mean 52.62%), in restored subpopulations between 42.47% and 62.33% (mean 50.34%). The frequency-down-weighted marker value (DW) in source subpopulations varied between 9.75 and 10.85 (mean 10.09), in restored subpopulations between 9.66 and 10.28 (mean 9.91). SSWP/n-1 in source subpopulations ranged from 12.03 to 14.13 (mean 13.20) in restored subpopulations from 10.55 to 14.98 (mean 12.41). The difference between source and re-stored subpopulations was not significant (Table 1c).

Genetic differentiation between subpopulations

In the Bayesian cluster analysis, individuals of all three species were assigned to two groups. However, we did not detect any population grouping according to the study location or according to the feature source/restored (Figure 2). For each species, outputs for K=2 of all twenty iterations were equal. For *A. eupatoria,* Δ K was 170.68; for *C. rotundifolia,* Δ K was 563.89; for *K. arvensis,* Δ K was 110.71.

The PCoA did not reveal any separation of source and restored subpopulations for all study species *A. eupatoria, C. rotundifolia* and *K. arvensis.* Source and restored subpopulations were admixed and no groups could be identified (Figures 3a, b, c). Similarly, no grouping according to the study locations was detected.

Applying AMOVA, we detected low overall genetic differentiation between subpopulations of the study species. Genetic differentiation (Φ_{PT}) between all subpopulations of *A. eupatoria* was 0.08 (Tab.2a). Differentiation between all source subpopulations was 0.10, and between all restored subpopulations 0.06. Hierarchical partitioning of molecular variance revealed that its largest amount was comprised within subpopulations (92%). We detected only a weak variation between subpopulations within the groups of the source and the group of restored subpopulations (8%), and zero genetic variation between the two groups of the source and restored subpopulations

Table 2. Results of the analyses of molecular variance (AMOVA) for (a) *A. eupatoria*, (b) *C. rotundifolia* and (c) *K. arvensis.* Levels of significance are based on 999 iteration steps and are indicated by asterisks ($p<0.001$); df, degrees of freedom; SS, the sum of squares; MS, the mean squares; %, the proportion of genetic variability; Φ_{PT}, the level of genetic differentiation

Source of molecular variation	df	SS	MS	%	Φ_{PT}
(a) *Agrimonia eupatoria*					
All populations					
Among populations	9	133.65	14.85	8	0.08***
Within populations	139	934.75	6.73	92	
Natural subpopulations					
Among subpopulations	4	70.53	17.63	10	0.10***
Within subpopulations	71	456.03	6.42	90	
Restored subpopulations					
Among subpopulations	4	54.43	13.61	6	0.06***
Within subpopulations	68	478.73	7.04	94	
Natural and restored subpopulations					
Between natural and restored populations	1	8.67	8.67	0	0.08***
Among subpopulations	8	124.96	15.62	8	
Within subpopulations	139	934.75	6.73	92	
(b) *Campanula rotundifolia*					
All populations					
Among populations	11	397.53	36.14	4	0.04***
Within populations	172	3787.05	22.02	96	
Natural subpopulations					
Among subpopulations	5	175.58	35.12	4	0.04***
Within subpopulations	81	1780.56	21.98	96	
Restored subpopulations					
Among subpopulations	5	196.36	39.27	5	0.05***
Within subpopulations	91	2006.47	22.05	95	
Natural and restored subpopulations					
Between natural and restored populations	1	25.60	25.60	0	0.04***
Among subpopulations	10	371.93	37.19	4	
Within subpopulations	172	3787.05	22.01	96	
(c) *Knautia arvensis*					
All populations					
Among populations	11	207.78	18.89	3	0.03***
Within populations	163	2083.41	12.78	97	

Table 2. (continued)

Source of molecular variation	df	SS	MS	%	Φ_{PT}
Natural subpopulations					
Among subpopulations	5	89.70	17.94	2	0.02***
Within subpopulations	82	1081.41	13.19	98	
Restored subpopulations					
Among subpopulations	5	102.25	20.45	4	0.04***
Within subpopulations	81	1002.00	12.37	96	
Natural and restored subpopulations					
Between natural and restored populations	1	15.82	15.82	0	0.03***
Among subpopulations	10	191.96	19.20	3	
Within subpopulations	163	2083.41	12.78	97	

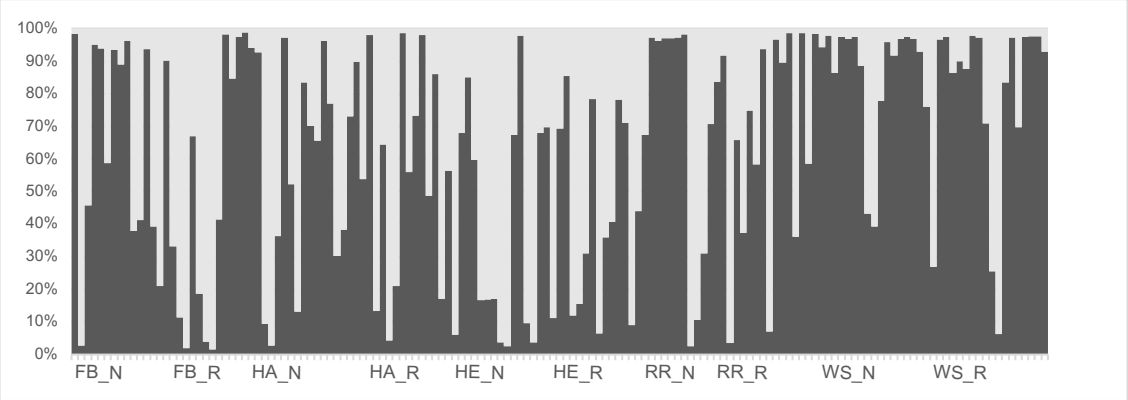
For *C. rotundifolia,* Φ_{PT} between all subpopulations was 0.04 (Tab. 2b). Differentiation between all source subpopulations was 0.04 and among all restored subpopulations 0.05. We detected no genetic differentiation between the groups of the source and restored subpopulations.
For *K. arvensis,* Φ_{PT} between all subpopulations was 0.03 (Tab. 2c). Genetic variation between all source subpopulations was 0.02 and between all restored subpopulations 0.04. Comparably to *A. eupatoria* and *C. rotundifolia,* no genetic variation between the two groups of the source and the restored subpopulations was be detected.

Discussion

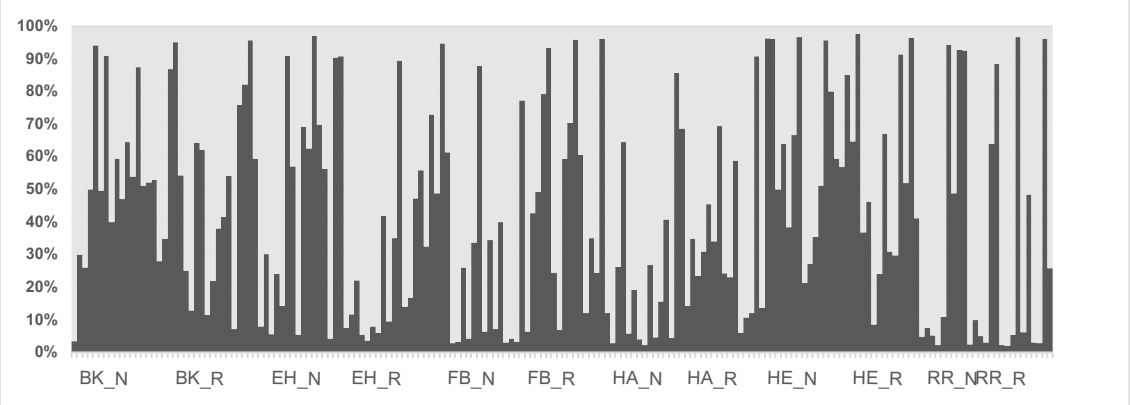
In our study, we detected apparent differences in genetic variation between the study species *A. eupatoria, C. rotundifolia* and *K. arvensis*. The species followed genetic variation patterns observed previously for outcrossing, mixed-mating and selfing taxa. The first, *A. eupatoria,* is a highly selfing species and showed the lowest levels of genetic diversity and the highest levels of genetic differentiations. The second, *C. rotundifolia,* which is predominantly outcrossing displayed the highest genetic diversity and low differentiation levels. The last, *K. arvensis,* is a mixed-mating but predominantly outcrossing species, which showed intermediate genetic diversity levels as well as low genetic differentiation (Tab.1, tab.2).
A significant, profound impact of the mating system on genetic variation within and among populations has been reported previously (Reisch and Bernhardt-Römermann, 2014). Outcrossing and mixed-mating species display considerably higher levels of variation within, and lower variation among populations. And vice versa, selfing species show conspicuously lower levels of genetic variation within populations, and stronger population differentiation than outcrossing or mixed-mating taxa. Thus, our results support the previous observation that mating system has a significant impact on the patterns of genetic variation in plant species.

Figure 2. Bayesian cluster analysis for (a) *A. eupatoria* based on 146 AFLP fragments. (b) *C. rotundifolia* based on 179 AFLP fragments. (c) *K. arvensis* based on 146 AFLP fragments. Populations of all three species were assigned to two groups.

a) *Agrimonia eupatoria*



b) *Campanula rotundifolia*



c) *Knautia arvensis*

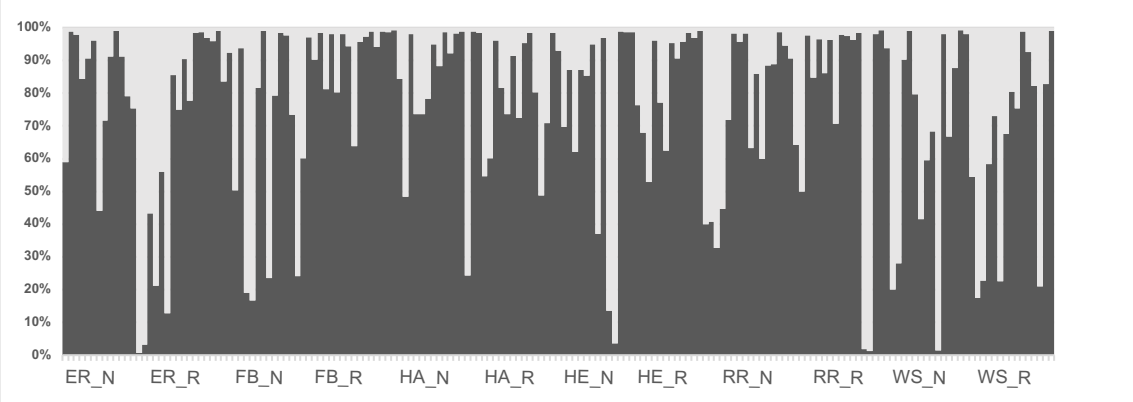
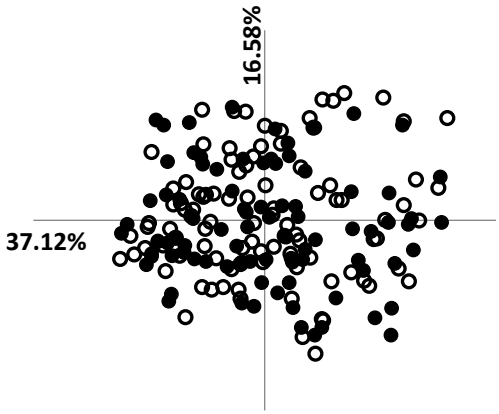
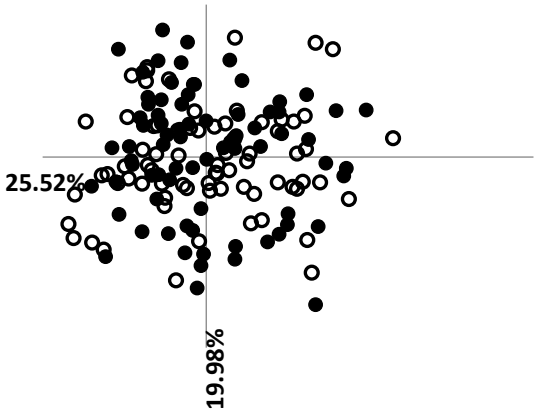


Figure 3. Results of the PCoA (principal coordinates analysis) based on Jaccard similarities. Black circles represent individuals of source subpopulations. Hollow circles show restored subpopulations. Individuals of source and restored subpopulations were mixed in all three species. (a) *A. eupatoria*: axis 1 explained 25.52% of variance in the data set. axis 2 explained 19.98%, respectively. (b) *C. rotundifolia*: axis 1 explained 37.12% of variance and axis 2 explained 16.58% of variance in the dataset. (c) *K. arvensis*: axis 1 explained 28.22% of variance, axis 2 explained 21.12%.

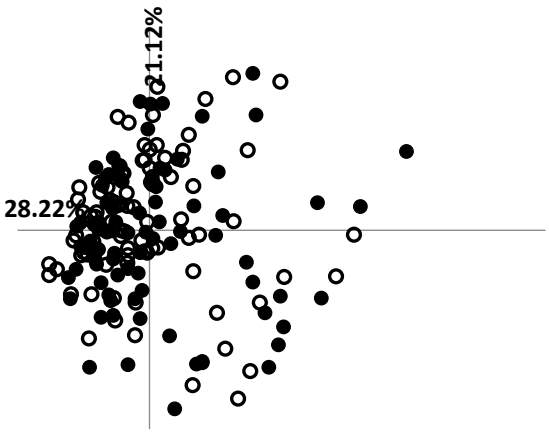
a) *Agrimonia eupatoria*



b) *Campanula rotundifolia*



c) *Knautia arvensis*



Apart from the mating system, genetic diversity and differentiation of populations can also strongly be affected by the restoration process, specifically by founder events during the colonization process plant (Vandepitte et al., 2012), because the number of propagules arriving in a restored site may be limited due to the restricted availability of source populations in the close vicinity (Poschlod et al., 2005; Willems and Bik, 1998; Prach et al., 2015b), or, by the lacking effective dispersal vector such as grazing sheep flocks (Fischer et al., 1996; Römermann et al., 2005; Fischer et al., 1996).

Moreover, only a limited number of propagules succeeds to establish as a mature plant for several reasons (lack of suitable microsites, biotic and abiotic conditions and their stochasticity, predation etc.) (Goldberg and Werner, 1983; Matos and Watkinson, 1998). Consequently, genetic diversity of restored populations may only partially represent the entire diversity of extant source stands. However, we detected no significant decrease of genetic diversity in restored sub-populations of *A. eupatoria*, *C. rotundofolia* and *K. arvensis*, in comparison with the source subpopulations. All measures of genetic diversity, i.e. SSWP, PPL as well as DW were marginal and not significant, or showed only negligible differences between the source and restored subpopulations, respectively (Tab.1).

Previous studies reported decreased genetic diversity in populations founded through natural colonization in comparison with long-standing, established populations (Bishop, 1996; Jacquemyn et al., 2009; Vandepitte et al., 2012), but this was not always the case. In accordance with our results, Vandepitte et al. (2007), van Looy et al. (2009b) as well as Ilves et al. (2015) and Helsen et al. (2013) revealed that genetic diversity in newly founded populations was not lowered. Two main factors may have played the major role in the process of enhancing genetic diversity and unification of the restored and source subpopulations. First, the spatial proximity of ancient grassland parts containing source subpopulations, which have served as a propagule and pollen donors. Occasionally grazing sheep flocks served additionally as mobile dispersal vectors accelerating the natural dispersal process via epi-zoochory (Lehmair et al., 2020; Rico et al., 2014). All three study species are insect pollinated, and, therefore, the physical closeness was a crucial factor for insects to reach newly established subpopulations in the restored parts (Zurbuchen et al., 2010).

Second, restored fragments not only directly adjoined ancient source grassland parts. Beyond that, several grassland fragments were detected in the close surroundings within a radius of 700 m from the restored grassland. These grasslands could have served as additional propagule sources which might have contributed to the unreduced genetic diversity in the restored subpopulations, due to gene flow (compare Wade and McCauley, 1988; Slatkin, 1987). Here, not only distance, but also possible dispersal barriers and dispersal vectors could have played a role; however, this would require a separate study.

Thus, the gene flow played a key role in the unification process between the source and restored subpopulations and contributed to the enhancement of genetic diversity in the restored stands (Dlugosch and Parker, 2008). This underpins our assessment that colonization from nearby sources can be a reasonable approach to restore populations without loss of local populations´ genetic diversity proper-ties.

Interestingly, we detected slightly but significantly higher genetic diversity levels in re-stored subpopulations in *A. eupatoria*, in comparison with its source subpopulations. This result was not massively surprising because of the seed morphology, i.e. hooks on the sur-face of A*. eupatoria* fruits, which can effectively be dispersed

by zoochory. One of the most common species found in the sheep fur were fruits of *A. eupatoria* (Fischer et al., 1996). Comparable results were reported for newly founded populations of *Geum urbanum*, which has a similar fruit morphology (Vandepitte et al., 2007). However, this fact alone cannot explain why K. arvensis, similarly to *A. eupatoria*, did not show the same pattern of higher genetic diversity in restored stands. According to Hintze et al. (2013), K. arvensis possess even higher dispersal abilities than *Agrimonia*. Most probably, numerous calcareous grass-land fragments in the close surroundings could have served as supplementary propagule sources (compare the "migrant pool" colonization model described by Wade and McCauley (1988)). Presumably both factors, i.e. good dispersal abilities and multiple seed sources in the close surroundings might have contributed to the increased genetic diversity levels in the restored subpopulations of *A. eupatoria*.

Restoration through colonization may not merely decrease genetic diversity within, but also enhance genetic divergence between populations. Low numbers of arriving propa-gules, and, generally, low levels of gene flow as well as limited establishment rates on re-stored sites may jointly increase genetic differentiation between source and restored grassland parts (Vandepitte et al., 2012). In this study, genetic differentiation between *A. eupatoria*, *C. rotundifolia* and *K. arvensis* sub-populations was, however, lower than previously reported for common species, and also lower than that detected for particular groups according to the mating system types (Reisch and Bernhardt-Römermann, 2014). Moreover, and this was of even greater importance, genetic differentiation between the group of the source and the restored subpopulations was lower than differentiation between the source subpopulations. Lower levels of differentiation between restored subpopulations and their genetic similarity to the source subpopulations can be attributed, first, to high levels and long-lasting gene flow between the source and re-stored grassland parts. Second, the entire vegetation layer removal in the course of restoration apparently reduced the density-dependent mortality of colonists in the re-stored sites and, thus, enabled population founding and growth, whereby the space in ancient (source) grasslands was already densely occupied (Helsen et al., 2013). Our study showed that any apparent and long-lasting founder effect as a consequence of the spontaneous recolonization process of the three common grassland species was avoid-ed.

Conclusions

The results of our study provide evidence that, at least under the settings of the underlying restoration project, grassland restoration through recolonization neither necessarily in-duces erosion of genetic diversity, nor enhanced levels of genetic differentiation be-tween the restored and source subpopulations. Hence, we can deduce that any apparent founder or bottleneck effect due to colonization events after clear-cutting were avoided. Restored subpopulations may further act as valuable diaspore sources in landscapes containing endangered calcareous grassland fragments.

Potentially, this approach may even induce an increase in genetic diversity relative to the nearby source stands. This supports our perception that scrub and woodland removal and subsequent recolonization are helpful tools to create genetically variable, non-differentiated restored subpopulations of common species, maintaining their local genetic patterns. These characteristics are necessary prerequisites for a long-term, sustainable persistence of the restored subpopulations in landscapes with calcareous grasslands.

We assume that for the restoration success, the direct spatial proximity was particularly important. This enabled the propagule availability from the sources and subsequent gene flow uniting the source and restored stands via further seed dispersal and pollen transfer. However, species with low dispersal abilities, as well as rare taxa should be analysed separately due to different levels of gene flow. Moreover, in future studies, it would definitely be interesting to look at the population genetics of natural regeneration sites that are not directly adjacent to source populations.

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GENERAL CONCLUSIONS

GENERAL CONCLUSIONS

Habitat and biodiversity loss at all levels has been creeping throughout the whole last century, but accelerated especially during the last few decades. The increasing human pressure on natural and semi-natural ecosystems, reinforced by the impact of climate change has taken a heavy toll, leaving behind huge areas of devastated landscapes. Meanwhile, the list of extinct species is getting longer and longer, whereby many of them were not even discovered and described. These trends might not only be threatening, but even detrimental to wellbeing and survival of man and nature.

Dealing with a very specific, “invisible” segment of the nature heritage – with the lowest rank of biodiversity, i.e. with the level of genes, is a fascinating journey into the core area of life.

At the same time, exactly this part of the whole biodiversity complex seems to be particularly vulnerable. In the context of nature conservation, it was a long journey to the recognition of its fundamental importance to the long-term survival capacity of species and populations.

In my PhD. thesis, I dealt with three restoration methods using tools of conservation genetics. This enabled me to assess, how efficient these approaches proved to be, with regard to their specific design. In the particular chapters of my PhD. thesis, I assessed what prerequisites contributed to the successful results – or, conversely, what kind of weaknesses in the process of restoration might be mitigated in future restoration schemes. I also outline possible prospects and lines of further research.

(Re)introductions

I dealt with a critically endangered pioneer species colonizing sand and gravel banks along Alpine streams – *Myricaria germanica*. Its abundance declined dramatically due to the changes in the natural flow regime of Alpine rivers. To rescue remaining populations along the middle and lower course of the River Isar, South-Eastern Germany, introduction measures took place during the 1990s. These efforts yielded several restored populations, planted along gravel pits in the floodplain of the River Isar. In these populations, I detected decreased genetic diversity, in comparison with the source. The most plausible explanation was the bad condition of the overaged source population, in which only a part of remaining individuals produced seeds. In highly dynamic habitats, such as floodplains of Alpine streams, in which long-distance dispersal and gene flow takes place, multiple seed sources would make sense to enhance genetic diversity and evolutionary potential of restored populations. In this type of habitat, this approach wound not pose a major risk to surrounding natural populations (if they are even present).

In future studies, genetic variation as well as performance of restored populations would make a good sense. Additional research to underpin – or to oppose the ´one catchment – one genepool´ hypothesis would further extend the knowledge horizon and our understanding of processes unfolding within dynamic river systems.

The pilot study investigating results of a 20-year-old practical restoration project have shown that there were no apparent differences between the populations founded using seeds or plants. Genetic variation was

similar in both groups of restored populations, as well as in the group of seeded & planted individuals. However, the groups of restored populations of a rare endangered herb *Armeria maritima* ssp. *elongata* contained on average higher levels of genetic diversity than the source populations. This is an important, positive feedback for conservation practitioners and their restoration efforts after decades of commitment and thoughtful activities to sustainably save the species despite the progressive climatic change. In future, a multispecies study using taxa with contrasting life history traits could deliver further insights in how different propagule type may influence the outcomes of restoration projects.

Recolonization

A widely applied restoration method, recolonization of calcareous grasslands after clear-cutting worked well also regarding genetic properties of restored populations. In all study species, any apparent founder effect and, consequently, decreased genetic diversity and inflated differentiation, could be excluded. In comparison with many previous studies on genetic consequences of colonization processes, which dealt with primary colonization (e.g., following eruptions, flood events, fire or landslides) or with colonization of anthropogenic areas (e.g., old fields, quarries and mines), I studied (re)colonization from genetic point of view, in a less explored context. My contribution to the current state of knowledge is, first, the study with a consequently applied paired design (source/restored grassland parts) in all study sites. Second, the study species represented a gradient of genetic diversity because of different pollination/mating strategies (predominantly selfing, mixed-mating and mainly outcrossing species). This enabled for more powerful inferences regarding genetic impact of the colonization process.

Further research of colonization in the context of different climate conditions (such as degree of continentality) or colonization in another habitat type (e.g., wet meadows, fens or psammophilous habitats) would be reasonable, to allow for broader conclusions regarding this restoration approach.
Moreover, historic DNA, if available, might be used, e.g., from herbarium specimens, to detect possible shifts in genetic diversity and structure in reference ancient grasslands.

Further, in our study system, the source and the restored sites were closely adjacent, without any barriers between them. This enabled pollen and seed dispersal to the restored sites. In future, it would definitely be interesting to look at the population genetics of natural regeneration sites that are not directly adjacent to source populations.

Moreover, future research might relate to the genetic issues in species with low colonization potential, as well as in rare species. Here, due to different levels of gene flow, a separate study would be necessary to assess the genetic impact of the colonization process.

Soil seed bank

Soil seed bank is a property of certain plant species, which enables them to survive under unpredictable environmental conditions. In restoration programs, soil seed banks could be applied as a part of a natural habitat recovery process in certain types of plant communities (e.g., heathlands in temperate climates). Alternatively, activated seed banks can be used as a tool to restore populations of target species (e.g., endangered

Pedicularis palustris in fens and waterlogged meadows, or rare weed communities which disappeared from the aboveground vegetation but still present as diaspores beneath recent grasslands). In this type of restoration approach, the self-evident prerequisite is that diaspores in the soil are still present and viable, i.e., a target plant community or species was recently present at the particular location. In a context of calcareous grassland restoration, approximately one third of target species were able to regenerate from the seed bank. This fact suggests that soil seed banks alone, without the seed rain from adjacent populations, are not fully sufficient as source for the adequate restoration of calcareous grasslands.

On the other hand, particular species that maintain long-time seed banks are able to regenerate and can help us to gain insights into processes that shape genetic makeup of populations recovered from the soil. In my study, I found apparent genetic similarities between the standing vegetation and the soil populations of *Origanum vulgare*, most probably due to the absence of severe disturbances and bottlenecks in this type of habitat. Only two measures differed between the seed bank and the vegetation. First, the number of alleles per locus (difference was marginally significant). This measure was higher in both soil layers than in the aboveground vegetation. Second, the inbreeding index was higher in the deeper layers of soil than in the upper layers and the vegetation, most probably due the pre- and post-germination selection filter acting against the homozygotes. These finding implicate, first, that evolutionary potential of the soil populations in *O. vulgare* is not reduced. The seed bank can be considered a good source for restoration of vital, genetically variable populations in case of extinction of aboveground populations. Second, most probably, a certain fraction of homozygote individuals in the soil are naturally eliminated in the stage of seed or seedlings, and do not reach the juvenile or even adult stage. Thus, they would not jeopardize a restoration success with higher levels of inbreeding.

Future multispecies genetic studies might focus on the role of the soil seed bank in the context of habitat fragmentation and possible mitigation of its consequences, as well as contrasting habitat types and their impact on genetic properties of soil seed banks.

Prospects

In my study, I investigated source and restored populations using indices of the neutral genetic diversity. However, in a conservation context, there is a vital need for a better understanding of adaptive genetic diversity and its role in survival prospects of populations. This is especially urgent as we are facing the ongoing climate change. To understand the genetic and genomic basis of complex trait variation, however, more studies in adaptive genetic diversity are needed. Therefore, until more knowledge of adaptive genetic diversity is available, conservation practitioners and scientists may further use nonadaptive genetic diversity to plan restoration strategies for rare and endangered taxa.

REFERENCES

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SCIENTIFIC LITERATURE

Aavik, T., Edwards, P., Holderegger, R., Graf, R., Billeter, R., 2012. Genetic consequences of using seed mixtures in restoration: A case study of a wetland plant Lychnis flos-cuculi. Biological Conservation 145, 195–204.

Aavik, T., Helm, A., 2018. Restoration of plant species and genetic diversity depends on landscape-scale dispersal. Restor Ecol, 92-102.

Abeli, T., Dalrymple, S., Godefroid, S., Mondoni, A., Müller, J.V., Rossi, G., Orsenigo, S., 2020. Ex situ collections and their potential for therestoration of extinct plants. Review. Conservation Biology, 303–313. https://conbio.onlinelibrary.wiley.com/doi/pdf/10.1111/cobi.13391.

Albert, Á.-J., Mudrák, O., Jongepierová, I., Fajmon, K., Frei, I., Ševčíková, M., Klimešová, J., Doležal, J., 2019. Grassland restoration on ex-arable land by transfer of brush-harvested propagules and green hay. Agriculture, Ecosystems & Environment, 74–82. https://www.sciencedirect.com/science/article/pii/S0167880918304626.

Albrecht, M., Maschinski, J., 2012. Influence of Founder Population Size, Propagule Stages, and Life History on the Survival of Reintroduced Plant Populations. In: Maschinski, J., Haskins, K., Raven, P. (Eds.), Plant Reintroduction in a Changing Climate. Promises and Perils. Island Press/Center for Resource Economics, Washington, DC.

Allendorf, F.W., Luikart, G., Aitken, S.N., 2013. Conservation and the genetics of populations, 2. ed. Wiley-Blackwell, Chichester.

Anderson, J.T., Wadgymar, S.M., 2020. Climate change disrupts local adaptation and favours upslope migration. Ecology letters, 181–192.

Andrén, H., 1994. Effects of Habitat Fragmentation on Birds and Mammals in Landscapes with Different Proportions of Suitable Habitat: A Review. Oikos, 355.

Ayre, D., Haynes, A., Gregory, D., 2021. Low Genetic Differentiation despite Fragmentation in an Endan-gered Fire-Sensitive Shrub. International Journal of Plant Sciences, 229–237.

Bakker, J., Bakker, E., Rosén, E., Verweij, G., Bekker, R., 1996a. Soil seed bank composition along a gradient from dry alvar grassland to Juniperus shrubland. Journal of Vegetation Science, 165–176. https://onlinelibrary.wiley.com/doi/pdfdirect/10.2307/3236316?download=true.

Bakker, J., Poschlod, P., Strykstra, R.J., Bekker, R.M., Thompson, K., 1996a. Seed banks and seed dispersal: important topics in restoration ecology. Acta botanica neerlandica, 461–490. file:///C:/Users/LOCALA~1/AppData/Local/Temp/ABN1996045004004-2.pdf.

Bakker, J.P., van Diggelen, R., Bekker, R.M., Marrs, R.H., 2012. Restoration of dry grasslands and heathlands. In: van Andel, J., Aronson, J. (Eds.), Restoration Ecology. The New Frontier, 2., Auflage. John Wiley & Sons, New York, NY, pp. 173–189.

Barnosky, A.D., Matzke, N., Tomiya, S., Wogan, G.O.U., Swartz, B., Quental, T.B., Marshall, C., McGuire, J.L., Lindsey, E.L., Maguire, K.C., Mersey, B., Ferrer, E.A., 2011. Has the Earth's sixth mass extinction already arrived? Nature, 51–57. https://www.nature.com/articles/nature09678.

Basey, A., Fant, J., Kramer, A., 2015. Producing native plant materials for restoration: 10 rules to collect and maintain genetic diversity. Native Plants Journal, 37–53. http://npj.uwpress.org/content/16/1/37.abstract.

Baskin, C.C., Baskin, J.M., 2011. Seeds. Ecology, biogeography, and evolution of dormancy and germination, [Repr.]. Academic Press, San Diego, Calif.

Bayerisches Staatsministerium für Umwelt, Gesundheit und Verbraucherschutz. Rote Liste der gefährdeten Tiere und Gefäßpflanzen Bayerns. 2005

Bekker, R.M., Bakker, J.P., Grandin, U., Kalamees, R., Milberg, P., Poschlod, P., Thompson, K., Willems, J.H., 1998. Seed size, shape and vertical distribution in the soil: indicators of seed longevity. Functional Ecology 12, 834–842.

Benayas, J., Newton, A., Diaz, A., Bullock, J., 2009. Enhancement of Biodiversity and Ecosystem Services by Ecological Restoration. A Meta-Analysis. Science, 1121–1124. http://science.sciencemag.org/content/sci/325/5944/1121.full.pdf.

Betz, C., Scheuerer, M., Reisch, C., 2013. Population reinforcement – A glimmer of hope for the conservation of the highly endangered Spring Pasque flower (Pulsatilla vernalis). Biological Conservation, 161–167.

Bill, H.-C., 2000. Besiedlungsdynamik und Populationsbiologie charakteristischer Pionierpflanzenarten nordalpiner Wildflüsse. Dissertation, Marburg.

Bischoff, A., Steinger, T., Müller-Schärer, H., 2010. The Importance of Plant Provenance and Genotypic Diversity of Seed Material Used for Ecological Restoration. Restor Ecol, 338–348.

Bischoff, A., Vonlanthen, B., Steinger, T., Müller-Schärer, H., 2006. Seed provenance matters — Effects on germination of four plant species used for ecological restoration. Basic and Applied Ecology, 347–359. https://www.sciencedirect.com/science/article/pii/S1439179105000915.

Bishop, J.G., 1996. Demographic and population genetic variation during colonization by the herb Lupinus lepidus on Mount St. Helens. https://www.proquest.com/openview/c3a4d3e7c59cd3c507920d3255e1cab8/1?pq-origsite=gscholar&cbl=18750&diss=y (12 May 2021).

Bisteau, E., Mahy, G., 2005. Vegetation and seed bank in a calcareous grassland restored from a Pinus-forest. Applied Vegetation Science, 167–174. https://onlinelibrary.wiley.com/doi/epdf/10.1111/j.1654-109X.2005.tb00642.x.

Blanckenhagen, B. von, Poschlod, P., 2005. Restoration of calcareous grasslands: the role of the soil seed bank and seed dispersal for recolonisation processes. Biotechnology, Agronomy, Society and Environment, 143–149. file:///C:/Users/LOCALA~1/AppData/Local/Temp/143.pdf.

Bonin, A., Bellemain, E., Eidesen, P., Pompanon, F., Brochmann, C., Taberlet, P., 2004. How to track and assess genotyping errors in population genetics studies. Molecular ecology 13, 3261–3273.

Bosbach, K., Hurka, H., Haase, R., 1982. The Soil Seed Bank of Capsella bursa-pastoris (Cruciferae): its Influ-ence on Population Variability. Flora, 47–56. https://www.sciencedirect.com/science/article/abs/pii/S0367253017313105.

Bossuyt, B., Honnay, O., 2008. Can the seed bank be used for ecological restoration? An overview of seed bank characteristics in European communities. Journal of Vegetation Science, 875–884.

Bowles, M.L., McBride, J.L., Bell T. J., 2015. Long-term processes affecting restoration and viabilityof the federal threatened Mead's milkweed (Asclepias meadii). Ecosphere, 1–22. https://esajournals.onlinelibrary.wiley.com/doi/pdf/10.1890/ES14-00240.1.

Breed, M.F., Stead, M.G., Ottewell, K.M., Gardner, M.G., Lowe, A.J., 2013. Which provenance and where? Seed sourcing strategies for revegetation in a changing environment. Conserv Genet, 1–10.

Broadhurst, L., Lowe, A., Coates, D., Cunningham, S., McDonald, M., Vesk, P., Yates, C., 2008. Seed supply for broadscale restoration. maximizing evolutionary potential. Evolutionary applications, 587–597.

Brown, A., Briggs, J., 1991. Sampling Stragies for Genetic Variation in Ex Situ Collections of Endangered Species. In: Falk, D., Holsinger, K., Falk, D.A., Holsinger, K.E. (Eds.), Genetics and Conservation of Rare Plants // Genetics and conservation of rare plants. Oxford Uni-versity Press, Oxford, pp. 99–119.

Brütting, C., Hensen, I., Wesche, K., 2013. Ex situ cultivation affects genetic structure and diversity in arable plants. Plant Biology, 505–513.

Bucharova, A., 2017. Assisted migration within species range ignores biotic interactions and lacks evidence. Restoration Ecology. https://onlinelibrary.wiley.com/doi/epdf/10.1111/rec.12457.

Bucharova, A., Bossdorf, O., Hoelzel, N., Kollman, J., Prasse, R., Durka, W., 2019. Mix and match: regional admixture provenancing strikes a balance among different seed-sourcing strategies for ecological restoration. Conserv Genet, 7–17. file:///C:/Users/LocalAdmin/AppData/Local/Microsoft/Windows/INetCache/IE/P44DLBI6/Bucharova2019_Article_MixAndMatchRegionalAdmixturePr.pdf.

Bucharova, A., Durka, W., Hoelzel, N., Kollman, J., Michalski, S., Bossdorf, O., 2017. Are local plants the best for ecosystem restoration? It depends on how you analyze the data. Ecology and evolution, 10683–10689. https://onlinelibrary.wiley.com/doi/epdf/10.1002/ece3.3585.

Bullock, J.M., Pywell, R.F., Walker, K.J., 2007. Long-term enhancement of agricultural production by restoration of biodiversity. J Appl Ecol, 6–12.

Burgarella, C., Navascués, M., Soto, Á., Lora, Á., Fici, S., 2007. Narrow genetic base in forest restoration with holm oak (Quercus ilex L.) in Sicily. Ann. For. Sci., 757–763.

Busch, V., Reisch, C., 2016. Population size and land use affect the genetic variation and performance of the endangered plant species Dianthus seguieri ssp. glaber. Conserv Genet, 425–436. https://link.springer.com/content/pdf/10.1007%2Fs10592-015-0794-1.pdf.

Bylebyl, K., Poschlod, P., Reisch, C., 2008. Genetic variation of Eryngium campestre L. (Apiaceae) in Central Europe. Molecular ecology, 3379–3388.

Cabin, R.J., 1996. Genetic comparisons of seed bank and seedling populations of the desert mustard Les-querella fendleri. Evolution; international journal of organic evolution, 1830–1841.

Cabin, R.J., Mitchell, R.J., Marshall, D.L., 1998. Do surface plant and soil seed bank populations differ genetically? a multipopulation study of the desert mustard Lesquerella fendleri (Brassicaceae). Am. J. Bot., 1098–1109.

Cardinale, B.J., Duffy, E., Gonzalez, A., Hooper, D.U., Perrings, C., Venail, P., Narwani, A., Mace, G., Tilman, D., Wardle, D.A., Kinzig, A.P., Daily, G.C., Loreau, M., Grace, J.B., Larigauderie, A., Srivastava, D., 2012. Biodiversity loss and its impact on humanity. Science, 59–67. https://pub.epsilon.slu.se/10240/7/wardle_d_etal_130415.pdf.

Carvalho, C.S., Forester, B.R., Mitre, S.K., Alves, R., Imperatriz-Fonseca, V.L., Ramos, S.J., Resende-Moreira, L.C., Siqueira, J.O., Trevelin, L.C., Caldeira, C.F., Gastauer, M., Jaffé, R., 2021. Combining genotype, phenotype, and environmental data to delineate site-adjusted provenance strategies for ecological restoration. Molecular Ecology Resources, 44–58.

Cavalli-Sforza, L.L., Edwards, A., 1967. Phylogenetic Analysis. Models and Estimation Procedures. AMERI-CAN JOURNAL OF HUMAN GENETICS, 233–257. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1706274/pdf/ajhg00393-0055.pdf.

CBD, 2018. Comprehensive and participatory process for the preparation of the post-2020 global biodiversity framework. CBD/COP/DEC/14/34, 9. https://www.cbd.int/doc/decisions/cop-14/cop-14-dec-34-en.pdf (18.10.22).

CBD, 2020. Post-2020 global biodiversity framework. Zero draft of the post-2020 global biodiversity framework. https://www.cbd.int/doc/c/2c69/df5a/01ee87752c3612d3ba7ec341/wg2020-02-03-add1-en.pdf (18.10.22).

CBD, 2022. Post-2020 global biodiversity framework. Draft recommendation submitted by the Co-Chairs. https://www.cbd.int/doc/c/079d/0d26/91af171843b6d4e9bee25086/wg2020-04-l-02-annex-en.pdf (18.20.22).

Charlesworth, D., Morgan, M.T., Charlesworth, B., 1990. Inbreeding depression, genetic load, and the evo-lution of outcrossing rates in a multilocus system with no linkage. Evolution; international journal of or-ganic evolution, 1469–1489.

Chase, J.M., Blowes, S.A., Knight, T.M., Gerstner, K., May, F., 2020. Ecosystem decay exacerbates biodiversity loss with habitat loss. Nature, 238–243. https://www.nature.com/articles/s41586-020-2531-2.

Chrtek, J., JR., 2018. Generative reproduction type. www.pladias.cz (22 December 2021).

Chytrý, Milan; Danihelka, Jiří; Kaplan, Zdeněk; Wild, Jan; Holubová, Dana; Novotný, Petr; Řezníčková, Marcela.2014-2018. Plant Diversity Analysis and Synthesis

Chytrý, M; Danihelka, J; Kaplan, Z; Wild, J; Holubová, D; Novotný, P; Řezníčková, M; Rohn, M; Dřevojan, P; Grulich, V; Klimešová, J; Lepš, J; Lososová, Z; Pergl, J; Sádlo, J; Šmarda, P; Štěpánková, P; Tichý, L; Axmanová, I; Bartušková, A; Blažek, P; Chrtek, J; Fischer, M.; Guo, W; Herben, T; Janovský, Z; Konečná, M; Kühn, I; Moravcová, L; Petřík, P; Pierce, S; Prach, K; Prokešová, H; Štech, M; Těšitel, J; Těšitelová, T; Večeřa, M; Zelený, D; Pyšek, P.2021. Pladias Database of the Czech flora and vegetation 93.

Clavel, J., Julliard, R., Devictor, V., 2011. Worldwide decline of specialist species: toward a global functional homogenization? Frontiers in Ecology and the Environment, 222–228.

Cockerham, C.C., Weir, B.S., 1993. Estimation of gene flow from f-statistics. Evolution; international journal of organic evolution, 855–863.

Coiffait-Gombault, C., Buisson, E., Dutoit, T., 2011. Hay Transfer Promotes Establishment of Mediterranean Steppe Vegetation on Soil Disturbed by Pipeline Construction. Restor Ecol, 214–222.

Cowie, R.H., Bouchet, P., Fontaine, B., 2022. The Sixth Mass Extinction: fact, fiction or speculation? Biological reviews of the Cambridge Philosophical Society, 640–663.

Crisp, M.D., Laffan, S., Linder, H.P., Monro, A., 2001. Endemism in the Australian Flora. Journal of Biogeography, 183–198. https://onlinelibrary.wiley.com/doi/pdf/10.1046/j.1365-2699.2001.00524.x.

Crow, J.F., Kimura, M., 1970. An introduction to population genetics theory. Haraper Row, New York.

Crutzen P.J., Stoermer E.F., 2000. The anthropocene. IGBP Global Change Newsletter, 17–18.

Dalrymple, S.E., Banks, E., Stewart, G.B., Pullin, A.S., 2012. A Meta-Analysis ofThreatened Plant Reintroductions from across the Globe. In: Maschinski, J., Haskins, K.E., Raven, P.H. (Eds.), Plant Reintroduction in a Changing Climate. Island Press/Center for Resource Economics, Washington, DC, pp. 31–50.

Darvill, B., Knight, M., Goulson, D., 2004. Use of genetic markers to quantify bumblebee foraging range and nest density. Oikos, 471–478.

Davies, A., Waite, S., 1998. The persistence of calcareous grassland species in the soil seed bank under developing and established scrub. Plant Ecol, 27–39. https://link.springer.com/content/pdf/10.1023/A:1009759227900.pdf.

DeFaveri, J., Viitaniemi, H., Leder, E., Merila, J., 2013. Characterizing genic and nongenic molecular markers:comparison of microsatellites and SNPs. Molecular Ecology Resources, 377–392. https://onlinelibrary.wiley.com/doi/pdf/10.1111/1755-0998.12071.

Del Castillo, R.F., 1994. Factors influencing the genetic structure of Phacelia dubia, a species with a seed bank and large fluctuations in population size. Heredity, 446–458. https://www.nature.com/articles/hdy199463.

Des Roches, S., Post, D.M., Turley, N.E., Bailey, J.K., Hendry, A.P., Kinnison, M.T., Schweitzer, J.A., Palkovacs, E.P., 2018. The ecological importance of intraspecific variation. Nat Ecol Evol, 57–64. https://www.nature.com/articles/s41559-017-0402-5/briefing/signup/?origin=nature&originreferralpoint=emailbanner.

Dieringer, D., Schlötterer, C., 2003. microsatellite analyser (MSA): a platform independent analysis tool for large microsatellite data sets. Mol Ecol Notes, 167–169.

DiLeo, M.F., Rico, Y., Boehmer, H.J., Wagner, H.H., 2017. An ecological connectivity network maintains genetic diversity of a flagship wildflower, Pulsatilla vulgaris. Biological Conservation, 12–21.

Dirzo, R., Young, H.S., Galetti, M., Ceballos, G., Isaac, N.J.B., Collen, B., 2014. Defaunation in the Anthropocene. Science (New York, N.Y.), 401–406.

Dittberner, H., Becker, C., Jiao, W.-B., Schneeberger, K., Hölzel, N., Tellier, A., Meaux, J. de, 2019. Strengths and potential pitfalls of hay transfer for ecological restoration revealed by RAD-seq analysis in floodplain Arabisspecies. Molecular ecology, 3887–3901. https://onlinelibrary.wiley.com/doi/pdf/10.1111/mec.15194.

Dlugosch, K.M., Parker, I.M., 2008. Founding events in species invasions: genetic variation, adaptive evolution, and the role of multiple introductions. Mol Ecol, 431–449.

Dolezel, J., Binarova, P., Lucretti, S., 1989. Analysis of Nuclear DNA Content in Plant Cells by Flow Cytometry. Biologia Plantarum (Praha), 113–120. file:///C:/Users/LocalAdmin/AppData/Local/Microsoft/Windows/INetCache/IE/5ALXG3DM/DpooleÅ¾el1989_Article_AnalysisOfNuclearDNAContentInP.pdf.

Dolezel, J., Greilhuber, J., Suda, J., 2007. Estimation of nuclear DNA content in plants using flow cytometry. Nature publishing Group, 2233–2244. https://www.nature.com/articles/nprot.2007.310.pdf?origin=ppub.

Dorogina, O. V.; Elisafenko, T. V.; Nechepurenko, S. B.; Achimova, A. A.; Yamtirov, M. B. Restoration's experience of populations of Hedysarum theinum (Fabaceae) in Altai Mountains. Ratitelnyj mir Aziatskoi Rossii. 2014 (3), 81-86

Dostálek, T., Münzbergová, Z., Plačková, I., 2010. Genetic diversity and its effect on fitness in an endangered plant species, Dracocephalum austriacum L. Conserv Genet, 773–783. https://link.springer.com/content/pdf/10.1007%2Fs10592-009-9879-z.pdf.

Drayton, B., Primack, R., 2000. Rates of success in the reintroduction by four methods of several perennial plant species in Eastern Massachusetts. Rhodora, 299–331.

Drayton, B., Primack, R., 2012. Success Rates for Reintroductions of Eight Perennial Plant Species after 15 Years. Restoration Ecology, 299–303. https://onlinelibrary.wiley.com/doi/epdf/10.1111/j.1526-100X.2011.00860.x.

Duchesne, P., Bernatches, L., 2002. AFLPOP: a computer program for simulated and real population allocation, based on AFLP data. Mol Ecol Notes, 380–383. https://onlinelibrary.wiley.com/doi/epdf/10.1046/j.1471-8286.2002.00251.x.

Durka, W., Bossdorf, O., Bucharova, A., Frenzel, M., Hermann, J.-M., Hoelzel, N., Kollmann, J., Michalski, S., 2019. Regionales Saatgut von Wiesenpflanzen: genetische Unterschiede, regionale Anpassung und Interaktion mit Insekten. Natur und Landschaft 94, 146–153. https://www.ufz.de/export/data/global/226129_Natur-und-Landschaft_4-2019_Durka.pdf.

Earl, D., vonHoldt, B., 2012. STRUCTURE HARVESTER. a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conservation Genet Resour, 359–361. https://link.springer.com/content/pdf/10.1007%2Fs12686-011-9548-7.pdf.

EC, 1992. Council Directive 92/43/EEC. https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:31992L0043 (19.10.22).

EC, 2020. Biodiversity strategy for 2030. https://environment.ec.europa.eu/strategy/biodiversity-strategy-2030_en (19.10.22).

EEA, 2020. State of Nature in the EU. Methodological paper Methodologies under the Nature Directives reporting 2013-2018 and analysis for the State of Nature 2000. https://www.researchgate.net/profile/Thomas-Ellmauer-2/publication/344648986_State_of_Nature_in_the_EU_-Methodological_paper_Methodologies_under_the_Nature_Directives_reporting_2013-2018_and_analysis_for_the_State_of_Nature_2000/links/5f86cdaa299bf1b53e263e43/State-of-Nature-in-the-EU-Methodological-paper-Methodologies-under-the-Nature-Directives-reporting-2013-2018-and-analysis-for-the-State-of-Nature-2000.pdf (19.10.22).

Egger, G., Angermann, K., Gruber, A., 2010. Wiederansiedlung der Deutschen Tamariske (Myricaria germanica (L.) Desv.) in Kärnten. Carinthia II, 393–418.

Egger, G., Baur, P., Lautsch, E., Stöhr, O., Brunner, Evelyn, Senfter, S., Lumasegger, M., Niedermayr, A., Schletterer, M., 2017. Habitate und Vorkommen der Deutschen Tamariske (Myricaria germanica (L.) Desv.). Vergleich zwischen Voll- und Restwasserstrecken. Carin-thia II, 401–430.

Ellenberg H., Weber H. E., Düll R., Wirth V., Werner W., Paulißen D., 1991. Zeigerwerte von Pflanzen in Mitteleuropa. Scripta geobotanica, 1–248.

Ellenberg, H., Leuschner, C., Dierschke, H., 2010. Vegetation Mitteleuropas mit den Alpen. in ökologischer, dynamischer und historischer Sicht, 6., vollst. neu bearb. und stark erw. Aufl. Ulmer, Stuttgart.

Ensslin, A., Godefroid, S., 2019. How the cultivation of wild plants in botanic gardens can change their genetic and phenotypic status and what this means for their conservation value. Sibbaldia: The journal of Botanic Garden Horticulture, 51-71. https://boris.unibe.ch/127313/1/Ensslin%26Godefroid_2019Sibbaldia.pdf.

Ensslin, A., Godefroid, S., 2020. Ex situ cultivation impacts on plant traits and drought stress response in a multi-species experiment. Biological Conservation, 1–9.

Enßlin, A., Sandner, T.M., Matthies, D., 2011. Consequences of ex situ cultivation of plants: Genetic diversity, fitness and adaptation of the monocarpic Cynoglossum officinale L. in botanic gardens. Biological Conservation, 272–278.

Ensslin, A., Tschöpe, O., Burkart, M., Joshi, J., 2015. Fitness decline and adaptation to novel environments in ex situ plant collections: Current knowledge and future perspectives. Biological Conservation, 394–401.

Ensslin, A., van de Vyver, A., Vanderborght, T., Godefroid, S., 2018. Ex situ cultivation entails high risk of seed dormancy loss on short-lived wild plant species. J Appl Ecol, 1145–1154.

Espeland, E., Emery, N., Mercer, K., Woolbright, S., Kettenring, K., Gepts, P., Etterson, J., 2017. Evolution of plant materials for ecological restoration: insights from the applied and basic literature. Journal of Applied Ecology, 102–115. https://besjournals.onlinelibrary.wiley.com/doi/pdf/10.1111/1365-2664.12739.

Estoup, A., Jarne, P., Cornuet, J.-M., 2002. Homoplasy and mutation model at microsatellite loci and their consequences for population genetics analysis. Mol Ecol, 1591–1604.

EU, 2011. The eu Biodiversity Strategy to 2020. https://ec.europa.eu/environment/nature/info/pubs/docs/brochures/2020%20Biod%20brochure%20final%20lowres.pdf (19.10.22).

European Commission, Directorate-General for Environment, Sundseth, K., 2008. Natura 2000. Protecting Europe's biodiversity. https://data.europa.eu/doi/10.2779/45963 (19.10.22).

European Community, 1992. The Habitats Directive. 92/43/92/43/EEC of the European Community. https://ec.europa.eu/environment/nature/legislation/habitatsdirective/index_en.htm (16 April 2021).

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.

Excoffier, L., Smouse, P., Quattro, J., 1992. Analysis of Molecular Variance Inferred From Metric Distances Among DNA Haplotypes: Application to Human Mitochondrial DNA Restriction Data. Genetics, 479–491. http://www.genetics.org/content/genetics/131/2/479.full.pdf.

Excoffier, L., Smouse, P., Quattro, J., 1992. Analysis of Molecular Variance Inferred From Metric Distances Among DNA Haplotypes: Application to Human Mitochondrial DNA Restriction Data. Genetics, 479–491. http://www.genetics.org/content/genetics/131/2/479.full.pdf.

Exposito-Alonso, M., Booker, T.R., Czech, L., Gillespie, L., Hateley, S., Kyriazis, C.C., Lang, P.L.M., Leventhal, L., Nogues-Bravo, D., Pagowski, V., Ruffley, M., Spence, J.P., Toro Arana, S.E., Weiß, C.L., Zess, E., 2022. Genetic diversity loss in the Anthropocene. Science, 1431–1435.

Falahati-Anbaran, M., Lundemo, S., Ågren, J., Stenøien, H.K., 2011. Genetic consequences of seed banks in the perennial herb Arabidopsis lyrata subsp. petraea (Brassicaceae). American journal of botany, 1475–1485.

Falush, D., Stephens, M., Pritchard, J., 2003. Inference of Population Structure Using Multilocus Genotype Data: Linked Loci and Correlated Allele Frequencies. Genetics, 1567–1587. http://www.genetics.org/content/genetics/164/4/1567.full.pdf.

Falush, D., Stephens, M., Pritchard, J., 2007. Inference of population structure using multi-locus genotype data: dominant markers and null alleles. Molecular ecology notes 7, 574–578.

Felipe-Lucia, M.R., Soliveres, S., Penone, C., Fischer, M., Ammer, C., Boch, S., Boeddinghaus, R.S., Bonkowski, M., Buscot, F., Fiore-Donno, A.M., Frank, K., Goldmann, K., Gossner, M.M., Hölzel, N., Jochum, M., Kandeler, E., Klaus, V.H., Kleinebecker, T., Leimer, S., Manning, P., Oelmann, Y., Saiz, H., Schalle, P., Schlotert, M., Schöningu, I., Schrumpfu, M., Sollyu, E.F., Stempfhubert, B., Weisserl, W.W., Wilckeq, W., Wubetb, T., Allan, E., 2020. Land-use intensity

alters networks between biodiversity, ecosystem functions, and services. Proceedings of the National Academy of Sciences, USA, 28140–28149. https://www.pnas.org/content/pnas/117/45/28140.full.pdf.

Fer, T., Hroudova, Z., 2009. Genetic diversity and dispersal of Phragmites australis in a small river system. Aquatic Botany, 165–171.

Fink, S., Lanz, T., Stecher, R., Scheidegger, C., 2017. Colonization potential of an endangered riparian shrub species. Biodivers Conserv 26, 2099–2114.

Fischer, S.F., Poschlod, P., Beinlich, B., 1996. Experimental studies on the dispersal of plants and animals on sheep in calcareous grasslands. Journal of Applied Ecology, 1206–1222. https://www.jstor.org/stable/2404699.

Flanagan, S.P., Jones, A.G., 2019. The future of parentage analysis: From microsatellites to SNPs and beyond. Mol Ecol, 544–567.

Frankham, R., 1996. Relationship of Genetic Variation to Population Size in Wildlife. Conservation Biology, 1500–1508. https://conbio.onlinelibrary.wiley.com/doi/pdf/10.1046/j.1523-1739.1996.10061500.x.

Frankham, R., Ballou, J., Eldridge, M., Lacy, R., Ralls, K., Dudash, M., Fenster, C., 2011. Predicting the Probability of Outbreeding Depression. Conservation Biology, 465–475. https://onlinelibrary.wiley.com/doi/epdf/10.1111/j.1523-1739.2011.01662.x.

Frankham, R., Ballou, J.D., Briscoe, D.A., 2010. Introduction to Conservation Genetics. Cambridge University Press, Cambridge.

Frankham, R., Ballou, J.D., Briscoe, D.A., 2015. Introduction to conservation genetics, 2nd ed., Reprint. Univ. Press, Cambridge.

Frankham, R., Bradshaw, C.J., Brook, B.W., 2014. Genetics in conservation management: Revised recommendations for the 50/500 rules, Red List criteria and population viability analyses. Biological Conservation, 56–63.

Franklin, I.R., 1980. Evolutionary change in small populations. In: Soulé, M.E., Wilcox, B.A. (Eds.), Conservation Biology, an Evoluitonary and Ecological Perspective. Sinauer Associates Inc., Sunderland, Massachusetts, pp. 135–150.

Franklin, I.R., Frankham, R., 1998. How large must populations be to retain evolutionary potential? Animal Conservation, 69–70.

Gabel, A.-R., Sattler, J., Reisch, C., 2017. Genetic variation and performance of the alpine plant species Dianthus callizonus differ in two elevational zones of the Carpathians. Alp Botany, 65–74.

García, D., Carleos, C., Parra, D., Cañón, J., 2002. Sib-parentage testing using molecular markers when parents are unknown. Animal Genetics, 364–371.

Garnier-Géré, P., Chikhi, L., 2013. Population Subdivision, Hardy–Weinberg Equilibrium and the Wahlund Effect. John Wiley & Sons, Ltd.

Godefroid, S., Pajolec Le, S., van Rossum, F., 2016. Pre-translocation considerations in rare plant reintroductions: implications for designing protocols. Plant Ecol, 169–182. file:///C:/Users/LocalAdmin/AppData/Local/Microsoft/Windows/INetCache/IE/P44DLBI6/Godefroid2016_Article_Pre-translocationConsideration.pdf.

Godefroid, S., Piazza, C., Rossi, G., Buord, S., Stevens, A.-D., Aguraiuja, R., Cowell, C., Weekley, C.W., Vogg, G., Iriondo, J.M., Johnson, I., Dixon, B., Gordon, D., Magnanon, S., Valentin, B., Bjureke, K., Koopman, R., Vicens, M., Virevaire, M., Vanderborght, T., 2011. How successful are plant species reintroductions? Biological Conservation 144, 672–682.

Goldberg, D.; Werner, P. 1983. The effects of size of opening in vegetation and litter cover on seedling establishment of goldenrods (Solidago spp.) Oecologia (Oecologia) 60, 149–155

Gostner, W., Paternolli, M., Schleiss, A.J., Scheidegger, C., Werth, S., 2017. Gravel bar inundation frequency: an important parameter for understanding riparian corridor dynamics. Aquat Sci 79, 825–839.

Grandin, U., Rydin, H., 1998. Attributes of the seed bank after a century of primary succession on islands in Lake Hjälmaren, Sweden. J Ecology, 293–303.

Guerrant Jr, E., 1996. Designing populations: Demographic, genetic, and horticultural dimen-sions. In: Falk, D., Millar, C., Olwell, M. (Eds.), Restoring diversity. Strategies for reintroduc-tion of endangered plants. Island Press, Washington, DC, pp. 171–207.

Guerrant Jr, E., Kaye, T., 2007. Reintroduction of rare and endangered plants: common factors, questions and approaches. Aust. J. Bot. 55, 362.

Hamilton, N., 2001. Is Local Provenance Important in Habitat Creation? A Reply. Journal of Applied Ecology, 1374–1376. https://www.jstor.org/stable/pdf/827307.pdf.

Hanin, N., Quaye, M., Westberg, E., Barazani, O., 2013. Soil seed bank and among-years genetic diversity in arid populations of Eruca sativa Miller (Brassicaceae). Journal of Arid Environments 91, 151–154.

Harzer, R., Mueller, N., Reich, M., Kollmann, Johannes, 2018. Potentialstudie zur Wiederan-siedlung von Wildflussarten. Im Auftrag des WWF Deutschland, Berlin. https://www.alpenflusslandschaften.de/files/downloads/aktuelles/Potentialstudie_Abschlussbericht_Harzer_et_al.pdf.

Hauser, S.S., Athrey, G., Leberg, P.L., 2021. Waste not, want not: Microsatellites remain an economical and informative technology for conservation genetics. Ecology and evolution, 15800–15814.

Havens, K., Vitt, P., Still, S., Kramer, A.T., Fant, J.B., Schatz, K., 2015. Seed Sourcing for Restoration in an Era of Climate Change. Natural Areas Journal 35, 122–133.

Heerdt, G.N.J. ter, Verweij, G.L., Bekker, R.M., Bakker, J.P., 1996. An Improved Method for Seed-Bank Analysis: Seedling Emergence After Removing the Soil by Sieving. Functional Ecology, 144–151.

Helsen, K., Jacquemyn, H., Hermy, M., Vandepitte, K., Honnay, O., 2013. Rapid Buildup of Genetic Diversity in Founder Populations of the Gynodioecious Plant Species Origanum vulgare after Semi-Natural Grassland Restoration. PloS one, 1-11.

Hermoso, V., Carvalho, S.B., Giakoumi, S., Goldsborough, D., Katsanevakis, S., Leontiou, S., Markantonatou, V., Rumes, B., Vogiatzakis, I.N., Yates, K.L., 2022. The EU Biodiversity Strategy for 2030: Opportunities and challenges on the path towards biodiversity recovery. Environmental Science & Policy, 263–271.

Hintze, C; Heydel, F; Hoppe, C; Cunze, S; König, A; Tackenberg, O. 2013. D3: The Dispersal and Diaspore Database – Baseline data and statistics on seed dispersal. Perspectives in Plant Ecology, Evolution and Systematics 15. 180–192.

Hoban, S., Bruford, M., D'Urban Jackson, J., Lopes-Fernandes, M., Heuertz, M., Hohenlohe, P.A., Paz-Vinas, I., Sjögren-Gulve, P., Segelbacher, G., Vernesi, C., Aitken, S., Bertola, L.D., Bloomer, P., Breed, M., Rodríguez-Correa, H., Funk, W.C., Grueber, C.E., Hunter, M.E., Jaffe, R., Liggins, L., Mergeay, J., Moharrek, F., O'Brien, D., Ogden, R., Palma-Silva, C., Pierson, J., Ramakrishnan, U., Simo-Droissart, M., Tani, N., Waits, L., Laikre, L., 2020. Genetic diversity targets and indicators in the CBD post-2020 Global Biodiversity Framework must be improved. Biological Conservation, 108654.

Höfner, J., Klein-Raufhake, T., Lampei, C., Mudrak, O., Bucharova, A., Durka, W., 2021. Populations restored using regional seed are genetically diverse and similar to natural populations in the region. J Appl Ecol, 1–11.

Holderegger, R., Balkenhol, N., Bolliger, J., Engler, J.O., Gugerli, F., Hochkirch, A., Nowak, C., Segelbacher, G., Widmer, A., Zachos, F.E., 2019. Conservation genetics: Linking science with practice. Mol Ecol, 3848–3856.

Hölzel, N., Buisson, E., Dutoit, T., 2012. Species introduction - a major topic in vegetation restoration. Appl Veg Sci 15, 161–165.

Honnay, O., Bossuyt, B., Jacquemyn, H., Shimono, A., Uchiyama, K., 2008. Can a seed bank maintain the genetic variation in the above ground plant population? Oikos, 1–5.

Honnay, O., Jacquemyn, H., 2007. Susceptibility of common and rare species to the genetic consequences of habitat fragmentation. Conservation Biology, 823–831. https://conbio.onlinelibrary.wiley.com/doi/epdf/10.1111/j.1523-1739.2006.00646.x.

Honnay, O., Jacquemyn, H., Bossuyt, B., Hermy, M., 2005. Forest fragmentation effects on patch occupan-cy and population viability of herbaceous plant species. New Phytol, 723–736.

Honnay, O., Jacquemyn, H., van Looy, K., Vandepitte, K., Breyne, P. 2009. Temporal and spatial genetic variation in a metapopulation of the annual Erysimum cheiranthoides on stony river banks. Journal of Ecology 97, 131–141

Honnay, O., Jacquemyn, H., Nackaerts, K., Breyne, P., van Looy, K., 2010. Patterns of population genetic diversity in riparian and aquatic plant species along rivers. Journal of Biogeog-raphy, 1730–1739. https://onlinelibrary.wiley.com/doi/epdf/10.1111/j.1365-2699.2010.02331.x.

Huber, S., Stahl, B., Schmid, S., Reisch, C. 2017. Species diversity of remnant calcareous grasslands in south eastern Germany depends on litter cover and landscape structure. Acta Oecologica (83), 48–55

Hudson, R.R., Slatkin, M., Maddison, W.P., 1992. Estimation of levels of gene flow from DNA sequence data. Genetics, 583–589.

Hufford, K.M., Mazer, S.J., 2003. Plant ecotypes: genetic differentiation in the age of ecological restoration. Trends in Ecology & Evolution 18, 147–155.

Hunter, M.E., Hoban, S.M., Bruford, M.W., Segelbacher, G., Bernatchez, L., 2018. Next-generation conservation genetics and biodiversity monitoring. Evolutionary applications, 1029–1034.

Huson, D., Bryant, D., 2006. Application of phylogenetic networks in evolutionary studies. Molecular biology and evolution 23, 254–267.

Hutchings, M.J., 1986. Plant population biology. In: Moore, P.D. (Ed.), Methods in plant ecology, 2. ed. Blackwell Scientific Publ, Oxford, pp. 377–435.

Iberl, K., Poschlod, P., Reisch, C., 2022. Restoration of calcareous grasslands by natural recolonization after forest clearing and its impact on the genetic variation of three common herb species. Biodivers Conserv, 671–690. https://link.springer.com/article/10.1007/s10531-022-02518-2.

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 Syst Evol, 1875–1886.

IPBES, 2019. Global assessment report on biodiversity and ecosystem services of the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services. Zenodo.

Isbell, F., Tilman, D., Reich, P.B., Clark, A.T., 2019. Deficits of biodiversity and productivity linger a century after agricultural abandonment. Nature ecology & evolution, 1533–1538.

IUCN/SSC, 2013. Guidelines for reintroductions and other conservation translocations (4 December 2020).

Jacquemyn, H., Vandepitte, K., Roldán-Ruiz, I., Honnay, O., 2009. Rapid loss of genetic variation in a founding population of Primula elatior (Primulaceae) after colonization. Ann Bot 103, 777–783.

Janovsky, Z., 2020. Pollinator spectrum. www.pladias.cz (22 December 2021).

Johanidesová, E., Fajmon, K., Jongepierová, I., Prach, K., 2015. Spontaneous colonization of restored dry grasslands by target species: restoration proceeds beyond sowing regional seed mixtures. Grass Forage Sci, 631–638.

Jones, L., Stevens, C., Rowe, E.C., Payne, R., Caporn, S., Evans, C.D., Field, C., Dale, S., 2017. Can on-site management mitigate nitrogen deposition impacts in non-wooded habitats? Biological Conservation, 464–475. https://www.sciencedirect.com/science/article/abs/pii/S0006320716302361.

Kalisz, S., 1989. Fitness consequences of mating sys- tem, seed weight, and emergence date in a winter annual, Collinsia verna. Evolution; international journal of organic evolution, 1263–1272.

Karlík, P., Poschlod, P., 2014. Soil seed-bank composition reveals the land-use history of calcareous grass-lands. Acta Oecologica, 22–34.

Kaulfuß, F., Reisch, C., 2017. Reintroduction of the endangered and endemic plant species Cochlearia bavarica-Implications from conservation genetics. Ecology and evolution, 11100–11112.

Kaulfuß, F., Reisch, C., 2021b. Restoration of species-rich grasslands by transfer of local plant material and its impact on species diversity and genetic variation-Findings of a practical restoration project in southeastern Germany. Ecology and evolution, 12816–12833.

Kaulfuß, F., Rosbakh, S., Reisch, C., 2022. Grassland restoration with local seed mixture. new evidence from a practical 15 years old study. (subm.). Applied Vegetation Science.

Keiper, F., McConchie, R., 2000. An analysis of genetic variation in natural populations of Sticherus flabellatus [R. Br. (St John)] using amplified fragment length polymorphism (AFLP) markers. Molecular ecology, 571–581. https://onlinelibrary.wiley.com/doi/pdf/10.1046/j.1365-294x.2000.00901.x.

Keller, L., Waller, D., 2002. Inbreeding effects in wild populations. Trends in Ecology & Evolu-tion, 230–241. https://ac.els-cdn.com/S0169534702024898/1-s2.0-S0169534702024898-main.pdf?_tid=aadd7d04-3bb1-42dd-ad8d-b9d2e7517681&acdnat=1543567326_7e9cea7657c0cbfaa355c02a10120715.

Kettenring, K.M., Mercer, K.L., Reinhardt Adams, C., Hines, J., 2014. EDITOR'S CHOICE: Application of genetic diversity-ecosystem function research to ecological restoration. J Appl Ecol, 339–348. https://besjournals.onlinelibrary.wiley.com/doi/full/10.1111/1365-2664.12202.

Kiefer, S., 1998. Wiederherstellung brachgefallener oder aufgeforsteter Kalkmagerrasen.

Kiefer, S., Poschlod, P., 1996. Restoration of Fallow or Afforested Calcareous Grasslands by Clear-Cutting. In: Settele J., Margules C., Poschlod P., Henle K. (Ed.), Species Survival in Fragmented Landscapes. Springer, Dordrecht, pp. 209–218.

Kiehl, K., Kirmer, A., Donath, T.W., Rasran, L., Hölzel, N., 2010. Species introduction in restoration projects – Evaluation of different techniques for the establishment of semi-natural grasslands in Central and Northwestern Europe. Basic and Applied Ecology, 285–299.

Kirmer, A., Baasch, A., Tischew, S., 2012. Sowing of low and high diversity seed mixtures in ecological restoration of surface mined-land. Appl Veg Sci 15, 198–207.

Kirmer, A., Tischew, S., Ozinga, W.A., Lampe, M. von, Baasch, A., van Groenendael, J.M., 2008. Importance of regional species pools and functional traits in colonization processes: predicting re-colonization after large-scale destruction of ecosystems. J Appl Ecol, 1523–1530.

Kiss, R., Deák, B., Tóthmérész, B., Miglécz, T., Tóth, K., Török, P., Lukács, K., Godó, L., Körmöczi, Z., Radócz, S., Kelemen, A., Sonkoly, J., Kirmer, A., Tischew, S., Švamberková, E., Valkó, O., 2021. Establishment gaps in species-poor grasslands: artificial biodiversity hotspots to support the colonization of target species. Restor Ecol, e13135.

Kiss, R., Valkó, O., Tóthmérész, B., Török, P., 2016. Seed bank research in Central-European grasslands - An overview. Nova Science Publisher.

Kleyer, M., Bekker, R., Knevel, I.C., Bakker, J., Thompson, K., Sonnenschein, M., Poschlod, P., Van Groenendael J.M., Klimes, L., Klimesova, J., Klotz, S., Rusch, G.M., Hermy, M., Adriaens, D., Boedeltje, G., Bossyut, B., Dannemann, A., Endels, P., Götzenberger, L., Hodgson, J.G., Jackel, A.-K., Kühn, I., Kunzmann, D., Ozinga, W.A., Römermann, C., Stadler, M., Schlegelmilch, J., Steendam, H.J., Tackenberg, O., Wilmann, B., Cornelissen, J., Eriksson, O., Garnier, E., Peco, B., 2008. A database of life-history traits of Northwest European flora. Journal of Ecology, 1266–1274. https://uol.de/f/5/inst/biologie/ag/landeco/download/LEDA/Data_files/seed_bank.txt.

Klotz, S., Kühn, I., Durka, W., 2002. BIOLFLOR. eine Datenbank mit biologisch-ökologischen Merkmalen zur Flora von Deutschland. BfN-Schriftenvertrieb im Landwirtschaftsverl., Münster.

Koch, M., Huthmann, M., Bernhardt, K.-G., 2003. Cardamine amara L. (Brassicaceae) in dynamic habitats: Genetic composition and diversity of seed bank and established populations. Basic and Applied Ecology, 339–348.

Konnert, M., Ruetz, W., 2003. Influence of nursery practices on the genetic structure of beech (Fagus sylvatica L.) seedling populations. Forest and Ecology Management, 193–200.

Kopelman, N., Mayzel, J., Jakobsson, M., Rosenberg, N., Mayrose, I., 2015. CLUMPAK: a program for identifying clustering modes and packaging population structure inference s across K. Molecular Ecology Resources, 1179–1191. https://onlinelibrary.wiley.com/doi/epdf/10.1111/1755-0998.12387.

Kovanda, M., 1990. Armeria (DC.) Willd. – trávnička. In: Hejný S., Slavík B., Hrouda L., Skalický V. (Eds.), Květena České republiky. Academia, Praha, pp. 370–372.

Kövendi-Jakó, A., Halassy, M., Csecserits, A., Hülber, K., Szitár, K., Wrbka, T., Török, K., 2019. Three years of vegetation development worth 30 years of secondary succession in urban-industrial grassland restoration. Appl Veg Sci, 138–149.

Kubitzki, K., Bayer, C. (Eds.), 2003. The Families and Genera of Vascular Plants. Flowering plants, Dicotyledons. Malvales, Capparales and non-betalain Caryophyllales. Springer, Ber-lin.

Kucera, K.F., Fant, J., Jensen, S., Landeen, M., Orr, E., Kramer, A.T., 2021. Genetic variation and structure change when producing and using mixed-source seed lots for. Restor Ecol, 1–13. https://onlinelibrary.wiley.com/doi/epdf/10.1111/rec.13521.

Kudrnovsky, H., 2013. Alpine rivers and their ligneous vegetation with Myricaria germanica and riverine land- scape diversity in the Eastern Alps. proposing the Isel river system for the Natura 2000 network. eco.mont, 5–18. https://www.austriaca.at/0xc1aa5576_0x002e4f3c.pdf.

Ladouceur, E., Jiménez-Alfaro, B., Marin, M., Vitis, M. de, Abbandonato, H., Iannetta, P.P.M., Bonomi, C., Pritchard, H.W., 2018. Native Seed Supply and the Restoration Species Pool. Conservation letters, e12381.

Laikre, L., Hoban, S., Bruford, M.W., Segelbacher, G., Allendorf, F.W., Gajardo, G., Rodríguez, A.G., Hedrick, P.W., Heuertz, M., Hohenlohe, P.A., Jaffé, R., Johannesson, K., Liggins, L., MacDonald, A.J., OrozcoterWengel, P., Reusch, T.B.H., Rodríguez-Correa, H., Russo, I.-R.M., Ryman, N., Vernesi, C., 2020. Post-2020 goals overlook genetic diversity. Science (New York, N.Y.), 1083–1085.

Lamont, B.B., Enright, N.J., 2000. Adaptive advantages of aerial seed banks. Plant Species Biology, 157–166.

Larsson, T.-B., 2001. Biodiversity evaluation tools for European forests. Enckell, Lund.

Latzin, S., Schratt-Ehrendorfer, L., 2005. Wiederansiedlung der Deutschen Tamariske (Myricaria germanica) im Nationalpark Donau-Auen. Endbericht, Wien.

Lauterbach, D., Burkart, M., Gemeinholzer, B., 2012. Rapid genetic differentiation between ex situ and their in situ source populations: an example of the endangered Silene otites (Caryophyllaceae). Botanical Journal of the Linnean Society, 64–75. https://onlinelibrary.wiley.com/doi/epdf/10.1111/j.1095-8339.2011.01185.x.

Lauterbach, S., Jungandreas, A., Blanke, J., Lehsten, V., Mühlner, S., Kühn, I., Volk, M., 2017. Trade-offs between plant species richness and carbon storage in thecontext of afforestation – Examples from afforestation scenarios inthe Mulde Basin, Germany. Ecological Indicators, 139–155.

Lefebvre, C., 1970. Self-Fertility in Maritime and Zinc Mine Populations of Armeria maritima (Mill.) Willd. Evolution, 571–577. https://www.jstor.org/stable/pdf/2406837.pdf?refreqid=excelsior%3A0e1849a5133e6fd050a943c3ae1548c9.

Lefebvre, C., Chandler-Mortimer, A., 1984a. Demographic Characteristics of the Perennial Herb Armeria maritima on Zilc Lead Mine Wastes. Journal of Applied Ecology, 255–264. https://www.jstor.org/stable/pdf/2403051.pdf?refreqid=excelsior%3A2407477c784053cf6a0bc2d0119b1249.

Lehmair, T.A., Pagel, E., Poschlod, P., Reisch, C., 2020. Surrounding landscape structures, rather than habitat age, drive genetic variation of typical calcareous grassland plant species. Landscape Ecol, 2881–2893. https://link.springer.com/article/10.1007/s10980-020-01120-7.

Leimu, R., Fischer, M., 2008. A meta-analysis of local adaptation in plants. PloS one 3, e4010.

Leimu, R., Mutikaien, P., KORICHEVA, J., Fischer, M., 2006. How general are positive relationships between plant population size, fitness and genetic variation? Journal of Ecology 94, 942–952.

Lener, F., Egger, G., Karrer, G., 2013. Sprossaufbau und Entwicklung der Deutschen Tamariske (Myricaria germanica) an der Oberen Drau (Kärnten, Österreich). Carinthia II, 515–552.

Lesica, P., Allendorf, F., 1992. Are Small Populations of Plants Worth Preserving? Conservation Biology, 135–139.

Lesica, P., Allendorf, F., 1999. Ecological Genetics and the Restoration ofPlant Communities:Mix or Match? Restor Ecol, 42–50. https://onlinelibrary.wiley.com/doi/pdf/10.1046/j.1526-100X.1999.07105.x.

Lewis, S.L., Maslin, M.A., 2015. Defining the anthropocene. Nature, 171–180. https://www.nature.com/articles/nature14258.

Li, Y.-Y., Chen, X.-Y., Zhang, X., Wu, T.-Y., Lu, H.-P., Cai, Y.-W., 2005. Genetic Differences between Wild and Artificial Populations of Metasequoia glyptostroboides: Implications for Species Recovery. Conservation Biology, 224–231. https://conbio.onlinelibrary.wiley.com/doi/epdf/10.1111/j.1523-1739.2005.00025.x.

Listl, D., Reisch, C., 2012. Spatial Genetic Structure of the Sedge Carex Nigra Reflects Hydrological Condi-tions in an Alpine Fen. Arctic, Antarctic, and Alpine Research, 350–358.

Liu, M.-H., Chen, X.-Y., Xin, Z., Shen, D.-W., 2008. A population genetic evaluation of ecological restoration with the case study on Cyclobalanopsis myrsinaefolia (Fagaceae). Plant Ecol, 31–41. https://link.springer.com/content/pdf/10.1007/s11258-007-9357-y.pdf.

Liu, Y., Wang, Y., Huang, H., 2006. High interpopulation genetic differentiation and unidirec-tional linear migration patterns in Myricaria laxiflora (Tamaricaceae), an endemic riparian plant in the

Three Gorges Valley of the Yangtze River. American journal of botany, 206–215. https://bsapubs.onlinelibrary.wiley.com/doi/epdf/10.3732/ajb.93.2.206.

Loiselle, B.A., Sork, V.L., Nason, J., Graham, C., 1995. Spatial genetic structure of a tropical understory shrub, Psychotria officinalis (Rubiaceae). Am. J. Bot., 1420–1425.

Lundemo, S., Falahati-Anbaran, M., Stenøien, H.K., 2009. Seed banks cause elevated generation times and effective population sizes of Arabidopsis thaliana in northern Europe. Mol Ecol, 2798–2811.

Lynch, M., Milligan, B.G., 1994. Analysis of population genetic structure with RAPD markers. Molecular ecology, 91–99. http://www.indiana.edu/~lynchlab/PDF/Lynch63.pdf.

Mace, G.M., Barrett, M., Burgess, N.D., Cornell, S.E., Freeman, R., Grooten, M., Purvis, A., 2018. Aiming higher to bend the curve of biodiversity loss. Nat Sustain 1, 448–451. https://www.nature.com/articles/s41893-018-0130-0.

Mahy, Vekemans, Jacquemart, 1999. Patterns of allozymic variation within calluna vulgaris populations at seed bank and adult stages. Heredity, 432–440. https://www.nature.com/articles/6884990.

Mandak, B., Bimova, K., Mahelka, V., Plackova, I., 2006. How much genetic variation is stored in the seed bank? A study of Atriplex tatarica (Chenopodiaceae). Molecular ecology, 2653–2663. https://onlinelibrary.wiley.com/doi/pdf/10.1111/j.1365-294X.2006.02953.x.

Mandak, B., Zakravsky, P., Mahelka, V., Plackova, I., 2012. Can Soil Seed Banks Serve as Genetic Memory? A Study of Three Species with Contrasting Life History Strategies. PloS one, 1–12. https://journals.plos.org/plosone/article/file?id=10.1371/journal.pone.0049471&type=printable.

Mantel, N., 1967. The Detection of Disease Clustering and a Generalized Regression Ap-proach. Cancer Research, 209–220. http://cancerres.aacrjournals.org/content/canres/27/2_Part_1/209.full.pdf#zoom=75.

Marinov, Y., Dimitrov, D., Gussev, C., Pachedjieva, K., 2017. Current Status, Distribution and Habitat of the Threatened Species Myricaria germanica (Tamaricaceae) in Bulgaria. Bulletin of the Natural History Museum - Plovdiv, 21–28. http://rnhm.org/upload/bnhmp-17105.pdf.

Mašková, T., Poschlod, P., 2021. Soil Seed Bank Persistence Across Time and Burial Depth in Calcareous Grassland Habitats. Front. Plant Sci., 790867. https://www.frontiersin.org/articles/10.3389/fpls.2021.790867/full.

Matos, S; Watkinson, A. 1998. The Fecundity, Seed, and Seedling Ecology of the Edible Palm Euterpe edulis in Southeastern Brazil. Biotropica 30, 595–603

McCue, K.A., Holtsford, T.P., 1998. Seed bank influences on genetic diversity in the rare annual Clarkia springvillensis (Onagraceae). Am. J. Bot., 30–36.

McDonald, T., Gann, G.D., Jonson, J., Dixon, K., 2016. International Standards for the Practice of Ecological Restoration. Including principles and key concepts. https://www.riversedgewest.org/sites/default/files/resource-center-documents/SER_International_Standards.pdf (6 September 2022).

McKay, J., Christian, C., Harrison, S., Rice, K., 2005. ‘‘How Local Is Local?’’. A Review of Practical and Conceptual Issues in the Genetics of Restoration. Restor Ecol, 432–440. https://onlinelibrary.wiley.com/doi/epdf/10.1111/j.1526-100X.2005.00058.x.

Meirmans, P.G., Hedrick, P.W., 2011. Assessing population structure: F(ST) and related measures. Molecular Ecology Resources, 5–18.

Meusel, E., Rauschert, S., Weinert, E., 1978. Vergleichende Chorologie der Zentraleuropaischen Flora. Karten. Fischer Verlag, Jena.

Mijangos, J., Pacioni, C., Spencer, P., Craig, M., 2015. Contribution of genetics to ecological restoration. Molecular ecology, 22–37. https://onlinelibrary.wiley.com/doi/epdf/10.1111/mec.12995.

Mijnsbrugge, K.V., Bischoff, A., Smith, B., 2010. A question of origin: Where and how to collect seed for ecological restoration. Basic and Applied Ecology, 300–311.

Millar, M., Anthony, J., Coates, D., Byrne, M., Krauss, S., Williams, M., Hopper, S., 2019a. Genetic Diversity, Mating System, and Reproductive Output of Restored Melaleuca acuminata Populations are Comparable to Natural Remnant Populations. Ecological Restoration, 222–232. http://er.uwpress.org/content/37/4/222.abstract.

Millar, M., Coates, D., Byrne, M., Krauss, S., Jonson, J., Hopper, S., 2019. Assessment of genetic diversity and mating system of Acacia cyclops restoration and remnant populations. Restoration Ecology, 1327–1338. https://onlinelibrary.wiley.com/doi/pdf/10.1111/rec.13007.

Mitchley, J., Jongepierová, I., Fajmon, K., 2012. Regional seed mixtures for the re-creation of species-rich meadows in the White Carpathian Mountains: results of a 10-yr experiment. Appl Veg Sci 15, 253–263.

Montalvo, A., Ellstrand, N., 2001. Nonlocal transplantation and outbreeding depression in the subshrub Lotus scoparius (Fabaceae). Am. J. Bot., 258–269. https://bsapubs.onlinelibrary.wiley.com/doi/pdf/10.2307/2657017.

Moravec, J., Balátová, E., Blažková, D., Hadač, E., Hejný, S., Husák, Š., Jeník, J., Kolbek, J., Krahulec, F., Kropáč, Z., Neuhäusel, R., Rybníček, K., Řehořek, V., Vicherek, J., 1995. Rostlinná společenstva České republiky a jejich ohrožení. Red list of plant communities of the Czech Republic and their endangerment.

Morris, A., Baucom, R., Cruzan, M., 2002. Stratified Analysis of the Soil Seed Bank in the Cedar Glade Endemic Atragallus bibullatus. Evidence for Historical Changes in Genetic Structure. American journal of botany, 29–36. https://bsapubs.onlinelibrary.wiley.com/doi/epdf/10.3732/ajb.89.1.29.

Nagel, R., Durka, W., Bossdorf, O., Bucharova, A., 2019. Rapid evolution in native plants cultivated for ecological restoration: not a general pattern. Plant Biology, 551–558.

Narum, S.R., Banks, M., Beacham, T.D., Bellinger, M.R., Campbell, M.R., Dekoning, J., Elz, A., Guthrie, C.M., Kozfkay, C., Miller, K.M., Moran, P., Phillips, R., Seeb, L.W., Smith, C.T., Warheit, K., Young, S.F., Garza, J.C., 2008. Differentiating salmon populations at broad and fine geographical scales with microsatellites and single nucleotide polymorphisms. Mol Ecol, 3464–3477.

Nei, M., 1973. Analysis of Gene Diversity in Subdivided Populations. (population structure/ genetic variability/heterozygosity/gene differentiation). Proceedings of the National Academy of Sciences, USA, 3321–3323. https://www.pnas.org/content/pnas/70/12/3321.full.pdf.

Nei, M., Maruyama, T., Chakraborty, R., 1975. The bottleneck effect and genetic variability in populations. Evolution, 1–10. https://onlinelibrary.wiley.com/doi/epdf/10.1111/j.1558-5646.1975.tb00807.x.

Ngeve, M., van der Stocken, T., Sierens, T., Koedam, N., Triest, L., 2017. Bidirectional gene flow on a mangrove river landscape and between-catchment dispersal of Rhizophora rac-emosa (Rhizophoraceae). Hydrobiologia, 93–108. https://link.springer.com/content/pdf/10.1007/s10750-016-3021-2.pdf.

Novak, J., Lukas, B., Bolzer, K., Grausgruber-Gröger, S., Degenhardt, J., 2008. Identification and characteri-zation of simple sequence repeat markers from a glandular Origanum vulgare expressed sequence tag. Molecular Ecology Resouces, 599–601. https://onlinelibrary.wiley.com/doi/epdf/10.1111/j.1471-8286.2007.02059.x.

Nybom, H., Bartish, I., 2000. Effects of life history traits and sampling strategies on genetic diversity estimates obtained with RAPD markers in plants. Perspectives in Plant Ecology, Evolution and Systematics 3, 93–114. https://ac.els-cdn.com/S1433831904700311/1-s2.0-S1433831904700311-main.pdf?_tid=d0936438-bcc5-4422-a9f5-7a9355df6869&acdnat=1532681036_09684267587c03e258257b4a48c60b19.

Oberdorfer, E., 2001. Pflanzensoziologische Exkursionsflora für Deutschland und angrenzende Gebiete, 8., stark überarb. u. erg. Aufl. Ulmer, Stuttgart.

Ohta, T., Kimura, M., 1973. A model of mutation appropriate to estimate the number of electrophoretically detectable alleles in a finite population. Genet. Res., 201–204.

Ottewell, K.M., Bickerton, D., Lowe, A.J., 2011. Can a seed bank provide demographic and genetic rescue in a declining population of the endangered shrub Acacia pinguifolia? Conserv Genet, 669–678. https://link.springer.com/article/10.1007/s10592-010-0173-x.

Pärtel, M., Kalamees, R., Zobel, M., Rosén, E., 1998. Restoration of species-rich limestone grassland communities from overgrown land: the importance of propagule availability. Ecological Engineering, 275–286. https://www.sciencedirect.com/science/article/pii/S0925857498000147.

Peakall, R., Smouse, P., 2006. genalex 6: genetic analysis in Excel. Population genetic software for teaching and research. Mol Ecol Notes, 288–295. https://onlinelibrary.wiley.com/doi/epdf/10.1111/j.1471-8286.2005.01155.x.

Philipp, M., Madsen, H., Siegismund, H., 1992. Gene flow and population structure in A rmeria maritima. Heredity, 32–42. https://www.nature.com/articles/hdy199291.pdf.

Pierson, S., Keiffer, C., McCarthy, B., Rogstad, S., 2007. Limited Reintroduction Does Not Always Lead toRapid Loss of Genetic Diversity: An Example fromthe American Chestnut (Castanea dentata; Fagaceae). Restor Ecol, 420–429. https://onlinelibrary.wiley.com/doi/pdf/10.1111/j.1526-100X.2007.00238.x.

Piessens, K., Honnay, O., Hermy, M., 2005. The role of fragment area and isolation in the conservation of heathland species. Biological Conservation, 61–69.

Piñeiro, R., Fuertes Aguilar, J., Menezes de Sequeira, M., Nieto Feliner, G., 2009. Low Genetic Diversity in the Rare Madeiran Endemic Armeria maderensis (Plumbagina-ceae). Folia Geobot, 65–81. https://link.springer.com/article/10.1007/s12224-009-9030-4.

Piñeiro, R., Fuertes Aguilar, J., Munt, D.D., Nieto Feliner, G., 2007. Ecology matters: Atlantic-Mediterranean disjunction in the sand-dune shrub Armeria pungens (Plum-baginaceae). Mol Ecol, 2155–2171.

Plue, J., Vandepitte, K., Honnay, O., Cousins, S.A.O., 2017. Does the seed bank contribute to the build-up of a genetic extinction debt in the grassland perennial Campanula rotundifolia? Annals of botany 120, 373–385.

Pollux, B., LUTEIJN, A., Van Groenendael J.M., Ouborg, J., 2009. Gene flow and genetic structure of the aquatic macrophyte Sparganium emersum in a linear unidirectional river. Freshwater Biology 54, 64–76.

Poschlod, P., Deffner, A., Beier, B., Grunicke, U., 1991. Untersuchungen zur Diasporenbank von Samen-pflanzen auf beweideten, gemähten, brachgefallenen und aufgeforsteten Kalkmagerrasenstandorten, 893–904. https://www.researchgate.net/profile/peter-poschlod/publication/291784724_untersuchungen_zur_diasporenbank_von_samenpflanzen_auf_beweideten_gemahten_brachgefallenen_und_aufgeforsteten_kalkmagerrasenstandorten/links/6034bed24585158939c2c2f2/untersuchungen-zur-diasporenbank-von-samenpflanzen-auf-beweideten-gemaehten-brachgefallenen-und-aufgeforsteten-kalkmagerrasenstandorten.pdf.

Poschlod, P., Jackel, A.-K., 1993. Untersuchungen zur Dynamik von generativen Diasporenbanken von Sa-menpflanzen in Kalkmagerrasen. Jahreszeitliche Dynamik des Diasporenregens und der Diasporenbank auf zwei Kalkmagerrasenstandorten der Schwäbischen Alb. Flora, 49–71.

Poschlod, P., Kiefer, S., Traenkle, U., Bonn, S., 1998. Plant species richness in calcareous grasslandsas affected by dispersability in space and time. Applied Vegetation Science, 75–91. https://onlinelibrary.wiley.com/doi/pdf/10.2307/1479087.

Poschlod, P., Kiefer, S., Tränkle, U., Fischer, S., Bonn, S., 1998. Plant species richness in calcareous grass-lands as affected by dispersability in space and time. Appl Veg Sci, 75–91.

Poschlod, P., Kleyer, M., Jackel, A.-K., Dannemann, A., Tackenberg, O., 2003. BIOPOP – a database of plant traits and internet application for nature conservation. Folia Geobotanica et Phytotaxonomica, 263–271. https://link.springer.com/content/pdf/10.1007/BF02803198.pdf#page=1&zoom=auto,-277,690.

Poschlod, P., Rosbakh, S., 2018. Mudflat species: Threatened or hidden? An extensive seed bank survey of 108 fish ponds in Southern Germany. Biological Conservation, 154–163.

Poschlod, P., Tackenberg, O., Bonn, S., 2005. Plant dispersal potential and its relation to species frequency and coexistence. In: van der Maarel, E. (Ed.), Vegeation Ecology. John Wiley & Sons, pp. 147–171.

Poschlod, P., Tränkle, U., Böhmer, J., Rahmann, H., 1997. Steinbrüche und Naturschutz. Sukzession und Renaturierung. ecomed, Landsberg.

Poschlod, P., WallisDeVries, M.F., 2002. The historical and socioeconomic perspective of calcareous grasslands—lessons from the distant and recent past. Biological Conservation, 361–376.

Powolny, M., Poschlod, P., Reisch, C. 2006. Genetic variation in Silene acaulis increases with population age. Botany 4, 241–247

Prach, K., Fajmon, K., Jongepierová, I., Řehounková, K., 2015. Landscape context in colonization of restored dry grasslands by target species. Appl Veg Sci, 181–189.

Prach, K., Hobbs, R.J., 2008. Spontaneous Succession versus Technical Reclamation in the Restoration of Disturbed Sites. Restor Ecol, 363–366. https://onlinelibrary.wiley.com/doi/epdf/10.1111/j.1526-100X.2008.00412.x.

Prach, K., Karešová, P., Jírová, A., Dvořáková, H., Konvalinková, P., Řehounková, K., 2015. Do not neglect surroundings in restoration of disturbed sites. Restor Ecol, 310–314.

Prentis, P., Vesey, A., Meyers, N.M., Mather, P., 2005. Genetic structuring of the stream lily Helmholtzia glaberrima within Toolona Creek, south-east Queensland. Chapter 2. http://eprints.qut.edu.au/16441/1/Peter_Prentis_Thesis.pdf#page=36 (8 November 2018).

Pritchard, J., Stephens, M., Donnelly, P., 2000. Inference of Population Structure Using Multilocus Genotype Data. Genetics, 945–959. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1461096/pdf/10835412.pdf.

Pritchard, J., Wen, X., Falush, D., 2009. Documentation for structure software. Version 2.3. http://web.stanford.edu/group/pritchardlab/software/structure_v.2.3.1/documentation.pdf.

Prober, S.M., Byrne, M., McLean, E.H., Steane, D.A., Potts, B.M., Vaillancourt, R.E., Stock, W.D., 2015. Climate-adjusted provenancing: a strategy for climate-resilient ecological restoration. Front. Ecol. Evol.

Puckett, E.E., 2017. Variability in total project and per sample genotyping costs under varying study designs including with microsatellites or SNPs to answer conservation genetic questions. Conservation Genet Resour, 289–304. https://link.springer.com/article/10.1007/s12686-016-0643-7.

Pullin, A.S., Báldi, A., Can, O.E., Dieterich, M., Kati, V., Livoreil, B., Lövei, G., Mihók, B., Nevin, O., Selva, N., Sousa-Pinto, I., 2009. Conservation focus on Europe: major conservation policy issues that need to be informed by conservation science. Conservation biology : the journal of the Society for Conservation Biology, 818–824.

R Core Team, 2014. R: A language and environment for statistical computing. www.r-project.org (12 May 2020).

Raffl, C., Schönswetter, P., Erschbamer, B., 2006. 'Sax-sess' - genetics of primary succession in a pioneer species on two parallel glacier forelands. Molecular ecology, 2433–2440. http://citeseerx.ist.psu.edu/viewdoc/download?doi=10.1.1.689.2604&rep=rep1&type=pdf.

Redhead, J.W., Sheail, J., Bullock, J.M., Ferreruela, A., Walker, K.J., Pywell, R.F., 2014. The natural regeneration of calcareous grassland at a landscape scale: 150 years of plant community re-assembly on Salisbury Plain, UK. Appl Veg Sci, 408–418.

Řehounková, K., Jongepierová, I., Šebelíková, L., Vítovcová, K., Prach, K., 2021. Topsoil removal in degraded open sandy grasslands: can we restore threatened vegetation fast? Restor Ecol, e13188.

Reisch, C., 2007. Genetic structure of Saxifraga tridactylites (Saxifragaceae) from natural and man-made habitats. Conserv Genet, 893–902. https://link.springer.com/content/pdf/10.1007%2Fs10592-006-9244-4.pdf.

Reisch, C., Anke, A., Röhl, M., 2005. Molecular variation within and between ten populations of Primula farinosa (Primulaceae) along an altitudinal gradient in the northern Alps. Basic and Applied Ecology 6, 35–45.

Reisch, C., Bernhardt-Römermann, M., 2014. The impact of study design and life history traits on genetic variation of plants determined with AFLPs. Plant Ecol, 1493–1511.

Reisch, C., Poschlod, P., 2009. Land use affects flowering time: seasonal and genetic differentiation in the grassland plant Scabiosa columbaria. Evolutionary Biology, 753–764. https://link.springer.com/article/10.1007/s10682-008-9270-4.

Reisch, C., Schurm, S., Poschlod, P., 2007. Spatial genetic structure and clonal diversity in an alpine popula-tion of Salix herbacea (Salicaceae). Ann Bot, 647–651.

Rejmanek, M., van Katwyk, K.P., 2005. Old field succession: A bibliographic review (1901–1991).

Reusch, T., Ehlers, A., Haemmerli, A., Worm, B., 2005. Ecosystem recovery after climatic extremes enhanced by genotypic diversity. Proceedings of the National Academy of Sciences, USA, 2826–2831. https://www.pnas.org/content/pnas/102/8/2826.full.pdf.

Reynolds, L., Waycott, M., McGlathery, K., Orth, R., Zieman, J., 2012. Eelgrass restoration by seed maintains genetic diversity: case study from a coastal bay system. Marine Ecology Progress Series, 223–233. https://www.int-res.com/articles/theme/m448p223.pdf.

Richard, G.-F., Kerrest, A., Dujon, B., 2008. Comparative genomics and molecular dynamics of DNA repeats in eukaryotes. Microbiology and molecular biology reviews : MMBR, 686–727.

Rico, Y., Holderegger, R., Boehmer, H.J., Wagner, H.H., 2014. Directed dispersal by rotational shepherding supports landscape genetic connectivity in a calcareous grassland plant. Mol Ecol, 832–842.

Riehl, S., Zehm, A., 2018. Wie gelingt die Wiederansiedlung der Deutschen Tamariske? Anliegen Natur, 17–21. https://www.anl.bayern.de/publikationen/anliegen/doc/an40102riehl_et_al_2018_tamariske.pdf.

Ritchie, A.L., Krauss, S.L., 2012. A Genetic Assessment of Ecological Restoration Success in Banksia attenuata. Restoration Ecology 20, 441–449.

Robichaux, R., Friar, E., Mount, D., 1997. Molecular Genetic Consequences of a Population Bottleneck Associated with Reintroduction of the Mauna Kea Silversword (Argyroxiphium sandwicense ssp. sandwicense [Asteraceae]). Conservation Biology, 1140–1146.

Rogers, S., Bendich, A., 1994. Extraction of total cellular DNA from plants, algae and fungi. In: Gelvin, S., Schilperoort, R. (Eds.), Plant Molecular Biology Manual, Second edition. Springer, Dordrecht, pp. 183–190.

Römermann, C., Tackenberg, O., Poschlod, P., 2005. How to predict attachment potential of seeds to sheep and cattle coat from simple morphological seed traits. Oikos, 219–230. https://onlinelibrary.wiley.com/doi/epdf/10.1111/j.0030-1299.2005.13911.x.

Rousset, F., 1996. Equilibrium values of measures of population subdivision for stepwise mutation processes. Genetics 142, 1357–1362.

Rousset, F., 2008. Genepop'007: a complete reimplementation of the Genepop software for Windows and Linux. Mol. Ecol. Resources, 103–106.

Rucinska, A., Puchalski, J., 2011. Comparative molecular studies on the genetic diversity of an ex situ garden collection and its source population of the critically endangered polish endemic plant Cochlearia polonica E. Froehlich. Biodivers Conserv, 401–413. https://link.springer.com/content/pdf/10.1007/s10531-010-9965-z.pdf.

Sánchez-Bayo, F., Wyckhuys, K.A., 2019. Worldwide decline of the entomofauna: A review of its drivers. Biological Conservation, 8–27.

Scheuerer, M., Ahlmer, W., 2003. Rote Liste gefährdeter Gefäßpflanzen Bayerns mit regionalisierter Florenliste. Schriftenreihe des Bayerischen Landesamtes für Umweltschutz, 1–372.

Schnabel, A., Hamrick, J.L., 1990. Organization of genetic diversity within and among population of Gleditsia tria- canthos (Leguminosae). Am. J. Bot., 1060–1069.

Schönswetter, P., Tribsch, A., 2005. Vicariance and dispersal in the alpine perennial Bupleurum stellatum L. (Apiaceae). Taxon, 725–732. https://onlinelibrary.wiley.com/doi/epdf/10.2307/25065429.

Schroder, R., Graf, M.D., Jochum, J., Rode, G., Schemmel, J., Thimm, I., 2013. Testing the Effects of a Regionalized Seed Production on the Germination Behavior of Wild Plant Species. Ecological Restoration, 295–301.

Schröder, R., Prasse, R., 2013. Cultivation and Hybridization Alter the Germination Behavior of Native Plants Used in Revegetation and Restoration. Restor Ecol, 793–800.

Schulz, B., Durka, W., Danihelka, J., Eckstein, R.L., 2018. Differential role of a persistent seed bank for genetic variation in early vs. late successional stages. PloS one, e0209840.

Seglias, A.,, Williams E.,, Bilge, E., Kramer, A. 2018. Plos One (13), e0191931.

Selkoe, K.A., Toonen, R.J., 2006. Microsatellites for ecologists: a practical guide to using and evaluating microsatellite markers. Ecology letters, 615–629.

Shafer, A.B.A., Wolf, J.B.W., Alves, P.C., Bergström, L., Bruford, M.W., Brännström, I., Colling, G., Dalén, L., Meester, L. de, Ekblom, R., Fawcett, K.D., Fior, S., Hajibabaei, M., Hill, J.A., Hoezel, A.R., Höglund, J., Jensen, E.L., Krause, J., Kristensen, T.N., Krützen, M., McKay, J.K., Norman, A.J., Ogden, R., Österling, E.M., Ouborg, N.J., Piccolo, J., Popović, D., Primmer, C.R., Reed, F.A., Roumet, M., Salmona, J., Schenekar, T., Schwartz, M.K., Segelbacher, G., Senn, H., Thaulow, J., Valtonen, M., Veale, A., Vergeer, P., Vijay, N., Vilà, C., Weissensteiner, M., Wennerström, L., Wheat, C.W., Zieliński, P., 2015. Genomics and the challenging translation into conservation practice. Trends in Ecology & Evolution, 78–87.

Sih, A., Jonsson, B.G., Luikart, G., 2000. Habitat loss: ecological, evolutionary and genetic consequences. Trends in Ecology & Evolution, 132–134.

Singh, P., Ekrtová, E., Holá, E., Štechová, T., Grill, S., Hájek, M., 2021. Restoration of rare bryophytes in degraded rich fens: The effect of sod-and-moss removal. Journal for Nature Conservation, 125928.

Sitzia, T., Michielon, B., Iacopino, S., Kotze, J., 2016. Population dynamics of the endangered shrub Myricaria germanica in a regulated Alpine river is influenced by active channel width and distance to check dams. Ecological Engineering, 828–838. https://www.sciencedirect.com/science/article/pii/S0925857416303913.

Slatkin, M., 1977. Gene flow and genetic drift in a species subject to frequent local extinctions. Theoretical Population Biology, 253–262.

Slatkin, M., 1987. Gene Flow and the Geographic Structure of Natural Populations. Science 236, 787–792.

Slavik, B. (Ed.), 2000. Květena České Republiky = Flora of the Czech Republic, Vyd. 1. Academia, Praha.

Snaydon, R., 1978. Genetic change in pasture population. In: Wilson, J.R. (Ed.), Plant relations in pastures. (symposium; Brisbane, May, 1976). Commonwealth scientific and industrial research Organization, Melbourne.

St. Clair, A.B., Dunwiddie, P.W., Fant, J.B., Kaye, T.N., Kramer, A.T., 2020. Mixing source populations increases genetic diversity of restored rare plant populations. Restoration Ecology, 583–593. https://onlinelibrary.wiley.com/doi/epdf/10.1111/rec.13131.

Stöcklin, J., Fischer, M., 1999. Plants with longer-lived seeds have lower local extinction rates in grassland remnants 1950-1985. Oecologia, 539–543.

Suda, J., Krahulcova, A., Travnicek, P., Krahulec, F., 2006. Ploidy level versus DNA ploidy level: an appeal for consistent terminology. Taxon, 447–450. https://onlinelibrary.wiley.com/doi/epdf/10.2307/25065591.

Suding, K.N., 2011. Toward an Era of Restoration in Ecology: Successes, Failures, and Opportunities Ahead. Annu. Rev. Ecol. Evol. Syst. 42, 465–487.

Summers, J.L., Bernik, B., Saunders, C.J., McLachlan, J.S., Blum, M.J., 2018. A century of genetic variation inferred from a persistent soil-stored seed bank. Evolutionary applications, 1715–1731.

Tangley, L., 1985. A New Plan to Conserve the Earth's Biota. BioScience, 334–336. https://www.jstor.org/stable/1309899#metadata_info_tab_contents.

Tautz, D., 1989. Hypervariability of simple sequences as a general source for polymorphic DNA markers. Nucl Acids Res, 6463–6471.

Teixeira, J.C., Huber, C.D., 2021. The inflated significance of neutral genetic diversity in conservation genetics. Proceedings of the National Academy of Sciences of the United States of America.

Templeton, A.R., Levin, D.A., 1979. Evolutionary Consequences of Seed Pools. The American Naturalist, 232–249.

Tero, N., Aspi, J., Siikamaki, P., Jakalaniemi, A., Tuomi, J., 2003. Genetic structure and gene flow in a metapopulation of an endangered plant species, Silene tatarica. Mol Ecol, 2073–2085.

Thompson, S., Chisholm, R., Rosindell, J. Characterising extinction debt following habitat fragmentationusing neutral theory. Ecology Letters, (2019)22: 2087–2096

Tonsor, S.J., Kalisz, S., Fisher, J., Holtsford, T.P., 1993. A life-history based study of population genetic struc-ture: seed bank to adults in plantago lanceolata. Evolution; international journal of organic evolution, 833–843.

Török, P., Vida, E., Deak, B., Lengyel ,Tothmeresz, B. 2011. Grassland restoration on former croplands in Europe: an assessment of applicability of techniques and costs. Biodivers Conserv (Biodiversity and Conservation) 20. 2311–2332

Tutin, T.G., 1968. Rosaceae to Umbelliferae. Cambridge Univ. Press, Cambridge.

Váchová, M., Májovský, J., 1978. IOPB chromosome number reports. LXI. Taxon, 375–392.

van Andel, J., Aronson, J., 2012. Restoration Ecology. The New Frontier. John Wiley & Sons, Oxford.

van Looy, K., Jacquemyn, H., Breyne, P., Honnay, O., 2009. Effects of flood events on the genetic structure of riparian populations of the grassland plant Origanum vulgare. Biological Conservation, 870–878.

van OOSTERHOUT, C., HUTCHINSON, W.F., WILLS, D.P.M., SHIPLEY, P., 2004. micro-checker: software for identifying and correcting genotyping errors in microsatellite data. Mol Ecol Notes, 535–538.

van Rossum, F., Hardy, O.J., Le Pajolec, S., Raspé, O., 2020. Genetic monitoring of translocated plant populations in practice. Mol Ecol, 4040–4058.

Vandepitte, K., Gristina, A.S., Hert, K. de, Meekers, T., Roldán-Ruiz, I., Honnay, O., 2012. Recolonization after habitat restoration leads to decreased genetic variation in populations of a terrestrial orchid. Molecular ecology 21, 4206–4215.

Vandepitte, K., Jacquemyn, H., Roldán-Ruiz, I., Honnay, O., 2007. Landscape genetics of the self-compatible forest herb Geum urbanum: effects of habitat age, fragmentation and local environment. Mol Ecol, 4171–4179.

Vekemans, X., 2002. AFLP-SURV 1.0. A program for genetic diversity analysis with AFLP (and RAPD) population data. http://www.ulb.ac.be/sciences/lagev/fichiers/manual_AFLPsurv.pdf.

Vekemans, X., Lambert, A., Lefebvre, C., 1992. Isozyme variation at the Populational Level in Armeria maritima. Belgian Journal of Botany, 270–275. https://www.jstor.org/stable/pdf/20794274.pdf?refreqid=excelsior%3A09a5303581b243a05608455ffe8d0c1c.

Vellend, M., 2003. Habitat Loss Inhibits Recovery of Plant Diversityas Forests Regrow. Ecology, 1158–1164.

Vitalis, R., Glémin, S., Olivieri, I., 2004. When genes go to sleep: the population genetic consequences of seed dormancy and monocarpic perenniality. The American Naturalist, 295–311.

Vitt, P., Havens, K., Kramer, A.T., Sollenberger, D., Yates, E., 2010. Assisted migration of plants: Changes in latitudes, changes in attitudes. Biological Conservation, 18–27.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Friters, A., Pot, J., Paleman, J., Kuiper, M., Zabeau, M., 1995. AFLP: a new technique for DNA fingerprinting. Nucl Acids Res 23, 4407–4414. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC307397/pdf/nar00021-0189.pdf.

Wade, M.J., McCauley, D., 1988. Extinction and recolonization: their effects on the genetic differentiation of local populations. Evolution, 995–1005. https://onlinelibrary.wiley.com/doi/epdf/10.1111/j.1558-5646.1988.tb02518.x.

Wagner, M., Fagan, K.C., Jefferson, R.G., Marrs, R.H., Mortimer, S.R., Bullock, J.M., Pywell, R.F., 2019. Species indicators for naturally-regenerating and old calcareous grassland in southern England. Ecological Indicators, 804–812.

Wagner, M., Hulmes, S., Hulmes, L., Redhead, J.W., Nowakowski, M., Pywell, R.F., 2021. Green hay transfer for grassland restoration: species capture and establishment. Restor Ecol, e13259.

Wahlund, S., 1928. Zusammensetzung von Populationen und Korrelationserscheinungen vom Standpunkt der Vererbungslehre aus betrachtet. Hereditas, 65–106.

Wakshum, S., Sebsebe, D., Tamrat, B., 2018. Ecology of soil seed banks: Implications for conservation and restoration of natural vegetation: A review. IJBC, 380–393.

Walck, J.; Baskin, J; Baskin, C.; Hidayati, S. 2005. Defining transient and persistent seed banks in species with pronounced seasonal dormancy and germination patterns. Seed Science Research 15, 189–196.

WallisDeVries, M.F., Poschlod, P., Willems, J.H., 2002. Challenges for the conservation of calcareous grasslands in northwestern Europe: integrating the requirements of flora and fauna. Biological Conservation, 265–273. file:///C:/Users/LOCALA~1/AppData/Local/Temp/1-s2.0-S0006320701001914-main.pdf.

Wan, Q.-H., Wu, H., Fujihara, T., Fang, S.-G., 2004. Which genetic marker for which conservation genetics issue? Electrophoresis, 2165–2176.

Wayne, R.K., Morin, P.A., 2004. Conservation genetics in the new molecular age. Frontiers in Ecology and the Environment, 89–97.

Weidema, I., Siegismund, H., Philipp, M., 1996. Distribution of genetic variation within and among Danish populations of Armeria maritima, with special reference to the effects of population size. Hereditas, 121–129. https://onlinelibrary.wiley.com/doi/epdf/10.1111/j.1601-5223.1996.00121.x.

Werth, S., Scheidegger, C., 2014. Gene Flow within and between Catchments in the Threat-ened Riparian Plant Myricaria germanica. PloS one, 1–13. https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0099400.

Werth, S., Schödl, M., Scheidegger, C., 2014. Dams and canyons disrupt gene flow among populations of a threatened riparian plant. Freshw Biol 59, 2502–2515.

Whitlock, M.C., McCauley, D.E., 1990. Some population genetic consequences of colony formation and extinction. genetic correlations within founding groups. Evolution, 1717–1724. https://onlinelibrary.wiley.com/doi/pdf/10.1111/j.1558-5646.1990.tb05243.x.

Wiedmer, A., Scheidegger, C., 2014. Genetische Untersuchung zur Deutschen Tamariske in Tirol. https://www.tirol.gv.at/fileadmin/themen/umwelt/naturschutz/downloads/Tamariske/Bericht_Tirol_WSL_20141212.pdf (30 October 2018).

Willems, J.H., Bik, L., 1998. Restoration of high species density in calcareous grassland: the role of seed rain and soil seed bank. Applied Vegetation Science, 91–100. https://onlinelibrary.wiley.com/doi/epdf/10.2307/1479088.

Willi, Y., Kristensen, T.N., Sgrò, C.M., Weeks, A.R., Ørsted, M., Hoffmann, A.A., 2022. Conservation genetics as a management tool: The five best-supported paradigms to assist the management of threatened species. Proceedings of the National Academy of Sciences of the United States of America.

Willi, Y., van Buskirk, J., Schmid, B., Fischer, M., 2007. Genetic isolation of fragmented populations is exac-erbated by drift and selection. Journal of evolutionary biology, 534–542.

Wilson, E.O. (Ed.), 2011. Biodiversity. National Academy Press, Washington, D.C.

Wilson, E.O., 2010. The diversity of life, 1. Harvard Univ. Press paperb. ed. Belknap Press of Harvard Univ. Press, Cambridge, Mass.

Woellner, R., Scheidegger, C., Fink, S., 2021. Gene flow in a highly dynamic habitat and a single founder event: Proof from a plant population on a relocated river site. Global Ecology and Conservation, e01686.

Woodell, S., Dale, A., 1993. Armeria maritima (Mill.) Willd. (Statice Armeria L.; S. maritima Mill.). Journal of Ecology, 573–588. https://www.jstor.org/stable/pdf/2261536.pdf?refreqid=excelsior%3Ae59a09ae54966a6ec31d4bf201511c64.

Yang, X., Baskin, C.C., Baskin, J.M., Pakeman, R.J., Huang, Z., Gao, R., Cornelissen, J.H.C., 2021. Global patterns of potential future plant diversity hidden in soil seed banks. Nature communications, 7023.

Yeh, F., Yang, R.-C., Boyle, T., Ye, Z.H., Mao, J.X., 1997. POPGENE, the user-friendly shareware for population genetic analysis. Biology and Biotechnology Centre, University of Atlanta in Edmonton, Canada.

Yeh, F., Yang, R.C., Boyle, T.B., Ye, Z., Xiyan, J.M., Yang, R., Boyle, T.J., 2000. PopGene32. Microsoft windows-based freeware for population genetic analysis.

Young, A., Boyle, T., Brown, T., 1996. The population genetic consequences of habitat fragmentation for plants. Trends in Ecology & Evolution, 413–418.

Zaghloul, M., Reisch, C., Poschlod, P., 2013. Soil seed bank contributes significantly to genetic variation of Hypericum sinaicum in a changing environment. Plant Syst Evol, 1819–1828.

Zhivotovsky, L., 1999. Estimating population structure in diploids with multilocus dominant DNA markers. Molecular ecology, 907–913. https://onlinelibrary.wiley.com/doi/epdf/10.1046/j.1365-294x.1999.00620.x.

Zucchi, M.I., Sujii, P., Mori, G., Viana, J., Grando, C., Aguiar Silvestre, E. de, Schwarz, K., Macrini, C., Bajay, M., Araujo, F., Siqueira, M., Alves-Pereira, A., Souza, A. de, Pinheiro, J., Rodrigues, R., Brancalion, P., 2018. Genetic diversity of reintroduced tree populations in res-toration plantations of the Brazilian Atlantic Forest. Restor Ecol, 694–701. https://onlinelibrary.wiley.com/doi/epdf/10.1111/rec.12620.

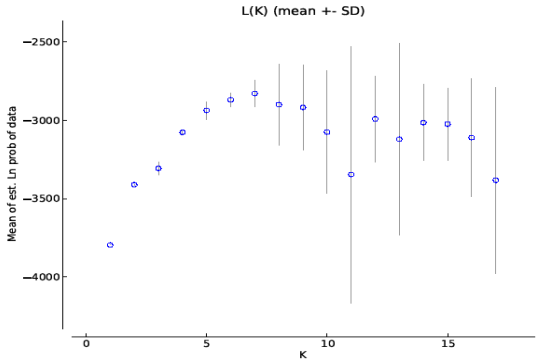
Zurbuchen, A., Landert, L., Klaiber, J., Müller, A., Hein, S., Dorn, S., 2010. Maximum foraging ranges in solitary bees: only few individuals have the capability to cover long foraging distances. Biological Conservation, 669–676. https://www.sciencedirect.com/science/article/pii/S0006320709005114

SUPPLEMENTARY

SUPPLEMENTARY MATERIAL – CHAPTER TWO

Figure S1b. Mean posterior probability plot of STRUCTURE over 20 runs for each value of K. a) the graph shows the mean ln Pr (X|K) over 20 iterations and standard deviation for each value of K. b) the graph shows *ad hoc* statistic ΔK (Evanno et al., 2005), for each of the tested values of K.

a)



b)

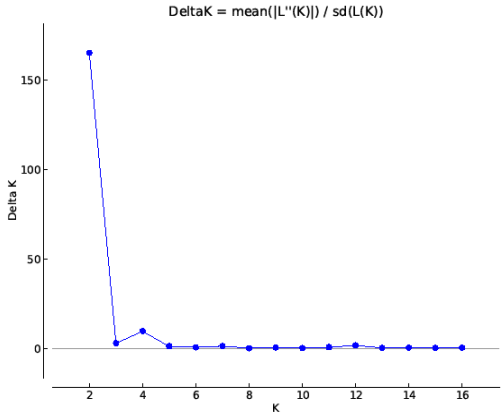


Table S1. Pairwise PhiPT values between all populations of *M. germanica* calculated employing AMOVA. Below diagonal: pairwise PhiPT values, above diagonal: probability values based on 999 permutations, non-significant values were highlighted in bold.

	IS1	**IS2**	**IS3**	**IS4**	**IS5**	**IS6**	**AM**	**LE1**	**HL2**	**HL3**	**LE2**	**LE3**	**RI**	**RII**	**RIII**	**F2**
IS1	0.000	0.001	0.032	0.001	0.003	0.001	0.001	0.002	0.001	0.001	0.001	0.002	0.004	0.026	0.312	0.300
IS2	0.33	0.000	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
IS3	0.09	0.28	0.000	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
IS4	0.22	0.32	0.13	0.000	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
IS5	0.18	0.24	0.17	0.20	0.000	**0.140**	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
IS6	0.19	0.24	0.20	0.23	0.02	0.000	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
AM	0.53	0.59	0.48	0.59	0.47	0.51	0.000	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
LE1	0.21	0.16	0.22	0.22	0.10	0.10	0.43	0.000	0.001	0.001	**0.100**	0.001	0.001	0.001	0.001	0.001
HL2	0.34	0.30	0.31	0.30	0.27	0.24	0.53	0.11	0.000	0.001	0.001	0.001	0.001	0.001	0.001	0.001
HL3	0.45	0.32	0.39	0.49	0.34	0.37	0.55	0.23	0.27	0.000	0.001	0.001	0.001	0.001	0.001	0.001
LE2	0.37	0.23	0.33	0.36	0.15	0.15	0.59	0.02	0.23	0.33	0.000	0.001	0.001	0.001	0.001	0.001
LE3	0.26	0.39	0.22	0.21	0.31	0.32	0.62	0.25	0.39	0.53	0.43	0.000	0.001	0.001	0.001	0.001
RI	0.16	0.37	0.24	0.30	0.29	0.33	0.62	0.31	0.47	0.54	0.44	0.39	0.000	0.101	0.038	0.001
RII	0.13	0.39	0.22	0.29	0.29	0.34	0.61	0.32	0.48	0.55	0.44	0.39	0.04	0.000	0.131	0.008
RIII	0.01	0.30	0.18	0.20	0.21	0.26	0.56	0.26	0.39	0.47	0.38	0.31	0.06	0.04	0.000	0.312
F2	0.02	0.28	0.19	0.27	0.23	0.27	0.55	0.28	0.42	0.47	0.37	0.31	0.13	0.08	0.01	0.000

Table S2. Results of assignment tests for 178 *Myricaria germanica* individuals from natural populations. For the allocation, three various levels of minimal log-likelihood difference (MLD) were employed, 0, 0.3, 0.5. Numerals in bold show numbers of correctly allocated individuals in each population. CA, number of correctly allocated; MA, mismatched allocated; NA, non-allocated individuals (further details see Materials&Methods).

	To											
From	**IS1**	**IS2**	**IS3**	**IS4**	**IS5**	**IS6**	**AM**	**LE1**	**HL2**	**HL3**	**LE2**	**LE3**
IS1	**1, 1, 1**	0, 0, 0	3, 2, 1	0, 0, 0	0, 0, 0	1, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
IS2	0, 0, 0	**12, 11, 11**	0, 0, 0	0, 0, 0	0, 0, 0	1, 1, 1	0, 0, 0	0, 0, 0	1, 1, 1	0, 0, 0	0, 0, 0	0, 0, 0
IS3	0, 0, 0	2, 2, 2	**11, 10, 10**	1, 1, 0	2, 1, 1	0, 0, 0	0, 0, 0	1, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	1, 1, 1
IS4	0, 0, 0	0, 0, 0	1, 1, 0	**11, 11, 11**	0, 0, 0	0, 0, 0	0, 0, 0	1, 1, 1	2, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
IS5	0, 0, 0	0, 0, 0	0, 0, 0	1, 0, 0	**7, 1, 1**	6, 3, 2	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
IS6	2, 1, 1	0, 0, 0	0, 0, 0	0, 0, 0	5, 4, 3	**6, 4, 3**	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
AM	1, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	1, 1, 1	**13, 13, 13**	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
LE1	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	**6, 3, 2**	1, 0, 0	0, 0, 0	3, 1, 1	0, 0, 0
HL2	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	**10, 10, 9**	1, 1, 0	1, 0, 0	0, 0, 0
HL3	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	**14, 13, 13**	0, 0, 0	0, 0, 0
LE2	0, 0, 0	1, 1, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	6, 5, 5	1, 0, 0	0, 0, 0	**12, 2, 1**	0, 0, 0
LE3	1, 1, 1	0, 0, 0	1, 0, 0	3, 3, 3	0, 0, 0	1, 1, 1	0, 0, 0	1, 1, 1	0, 0, 0	0, 0, 0	0, 0, 0	**13, 11, 10**
Summary												
n	**5**	**15**	**16**	**16**	**14**	**16**	**13**	**15**	**15**	**15**	**16**	**14**
CA (n)	1, 1, 1	12, 11, 11	11, 10, 10	11, 11, 11	7, 1, 1	6, 4, 3	13, 13, 13	6, 3, 2	10, 10, 9	14, 13, 13	12, 2, 1	13, 11, 10
MA (n)	4, 2, 2	3, 2, 2	5, 3, 1	5, 5, 4	7, 5, 4	10, 6, 5	0, 0, 0	9, 7, 7	5, 1, 1	1, 1, 0	4, 1, 1	1, 1, 1
NA (n)	0, 2, 2	0, 1, 2	0, 3, 5	0, 0, 1	0, 8, 9	0, 6, 8	0, 0, 0	0, 5, 6	0, 4, 5	0, 1, 2	0, 11, 13	0, 2, 3

SUPPLEMENTARY MATERIAL – CHAPTER FOUR

Table S1. Location name, abbreviation and geographic position (Latitude N, Longitude E) for the five study sites of *O. vulgare*

Location	**Abbreviation**	**Latitude N**	**Longitude E**
Aichahof	AH	N 49°02'26.2"	E 12°01'31.4"
Eitelberg	EB	N 49°1'48.7"	E 11°55'43.4"
Grabenhof	GH	N 49°10'53.4"	E 11°57'10.2"
Kühschlag	KS	N 49°00'12.3"	E 11°57'20.5"
Undorf	UD	N 49°1'43.3"	E 11°55'43.3"

Table S2. Population name, abbreviation, cohort, number of samples and genetic characteristics of *Origanum vulgare* populations. Population, the name of the site; Abb, abbreviation; Cohort, membership in a particular group (veg, vegetation, upp, upper soil layer, low, lower soil layer), Ntot, total number of samples; %F, percentage of female plants in populations; Na, mean number of alleles per locus; SI, Shannon´s information index; He, expected heterozygosity; Fis, inbreeding index; w, pairwise Wilcoxon test.

Population	Abb.	Cohort	Ntot	%F	Na	SI	He	Fis
Aichahof	AH	veg	40	21.4	3.33	0.79	0.45	0.06
Eitelberg	EB	veg	36	7.7	3.67	0.93	0.52	-0.0007
Grabenhof	GH	veg	40	16.7	3.56	0.83	0.47	-0.06
Kühschlag	KS	veg	40	15.2	3.67	0.97	0.57	0.05
Undorf	UD	veg	33	10.1	2.89	0.64	0.36	0.01
Mean			**37.8**	**14.2**	**3.42**	**0.83**	**0.47**	**0.01**
SD			3.19	5.4	0.33	0.13	0.07	0.05
Aichahof	AH	soil	48	21.4	3.89	0.90	0.50	0.08
Eitelberg	EB	soil	40	19.0	3.67	0.92	0.50	0.05
Grabenhof	GH	soil	43	23.9	3.56	0.81	0.46	0.13
Kühschlag	KS	soil	38	9.8	3.33	0.92	0.56	0.13
Undorf	UD	soil	24	3.7	2.56	0.55	0.32	0.14
Mean			**38.6**	**15.6**	**3.40**	**0.82**	**0.47**	**0.11**
SD			8.99	8.5	0.51	0.16	0.09	0.04
T-test				p=0.7	p=0.90	p=0.75	p=0.71	**p=0.04**
Aichahof	AH	veg	10	-	2.56	0.78	0.50	0.04
Eitelberg	EB	veg	10	-	2.78	0.88	0.55	0.08
Grabenhof	GH	veg	10	-	2.44	0.58	0.34	-0.06
Kühschlag	KS	veg	10	-	2.22	0.50	0.30	-0.19
Undorf	UD	veg	10	-	2.44	0.54	0.32	-0.10
Mean					**2.49**	**0.66**	**0.40**	**-0.05**
SD					±0.20	±0.17	±0.11	0.11
Aichahof	AH	upp	10	-	3.11	0.76	0.43	0.05
Eitelberg	EB	upp	10	-	3.00	0.85	0.49	0.15
Grabenhof	GH	upp	10	-	2.67	0.71	0.42	-0.05
Kühschlag	KS	upp	10	-	3.11	0.85	0.49	0.14
Undorf	UD	upp	10	-	3.11	0.83	0.48	-0.02
Mean					**3.00**	**0.80**	**0.46**	**0.05**
SD					±0.19	±0.06	±0.03	0.09
Aichahof	AH	low	10	-	3.22	0.87	0.49	0.12
Eitelberg	EB	low	10	-	3.11	0.85	0.49	-0.02
Grabenhof	GH	low	10	-	2.67	0.70	0.41	0.31
Kühschlag	KS	low	10	-	3.11	0.86	0.49	0.24
Undorf	UD	low	10	-	3.11	0.91	0.55	0.38
Mean					**3.04**	**0.84**	**0.49**	**0.21**
SD					±0.21	±0.08	±0.05	0.16
T-test VxU					p=0.06w	p=0.88w	p=0.69	p=0.67
T-test VxL					p=0.06w	p=0.45w	p=0.30	**p=0.02**
T-test UxL					p=1.00w	p=0.61w	p=1.00	p=0.21

Table S3. Rare alleles detected in the vegetation and the seed bank Names of locations are given using abbreviations of the study site names; cohorts are given as the vegetation (veg) and the upper soil layer (upp); further, locus names, allele size and allele frequencies are specified.

Location	Cohort	Locus	Allele	Frequency
AH	veg	OR13	137	0.013
GH	veg	OR14	79	0.013
UD	veg	OR77	119	0.015
AH	upp	OR77	122	0.013
EB	upp	OR10	110	0.033
EB	upp	OR44	142	0.017

Figure S1. Design of plot with subplots applying a chessboard pattern. Numbers indicate subplots included in analyses, i.e. from which vegetation and soil samples were collected.

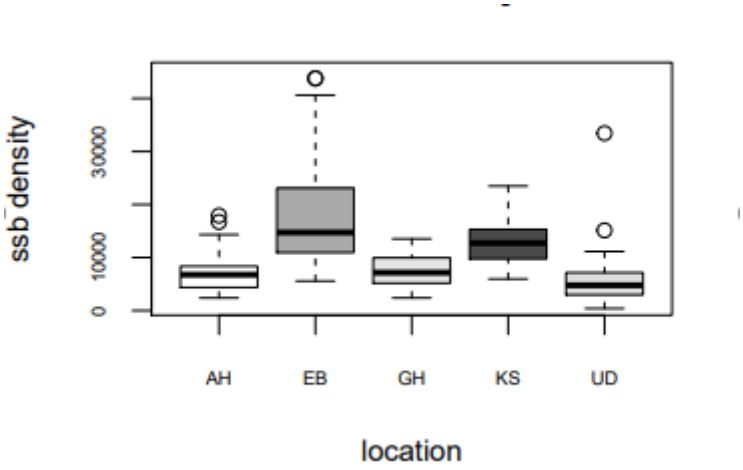
8m

10 m

1		2		3		4	
	5		6		7		8
9		10		11		12	
	13		14		15		16
17		18		19		20	
	21		22		23		24
25		26		27		28	
	29		30		31		32
33		34		35		36	
	37		38		39		40

Figure S2. Seed bank density given as a number of seeds/m2, related to a) all seedlings which emerged in course of our germination experiment, comprising the full scope of species; b) all emerged seedlings of *Origanum vulgare*. Seedlings were counted from the pooled soil layers. Names of locations are given as abbreviations: AH, Aichahof; EB, Eitelberg; GH, Grabenhof; KS, Kühschlag; UD, Undorf. Boxes represent means and standard errors; whiskers show standard deviations (0.95 confidence interval).

a) Seed bank density – all species



b) Seed bank density – *Origanum vulgare*

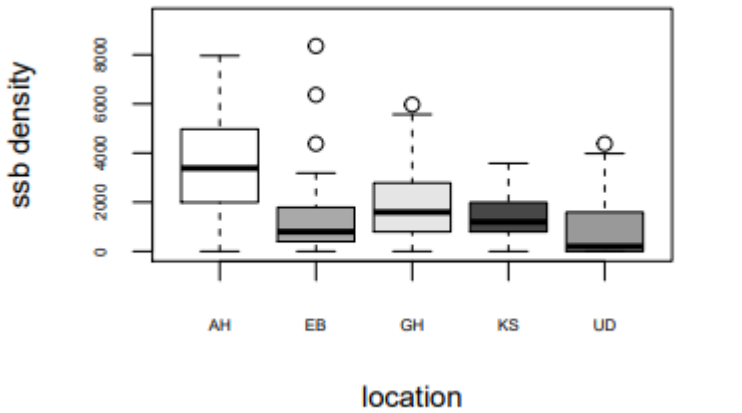


Figure S3. NeighborNet diagram constructed using SplitsTree4 software (Huson and Bryant, 2006). Five detectable clusters correspond to the five study locations (AH, EB, GH, KS, UD). The three cohorts (vegetation, veg; upper soil layer, upp; and lower soil layer, low) related to a particular study location (AH, EB, GH, KS, UD) were assigned to the same cluster.

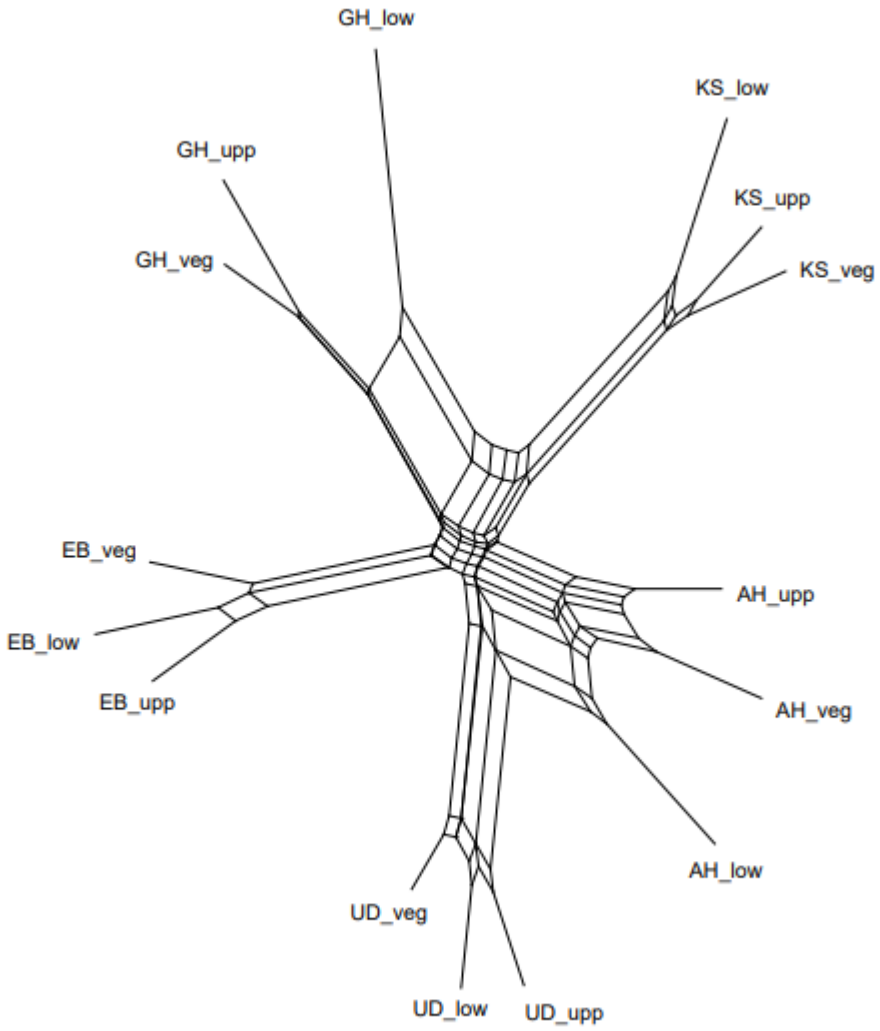
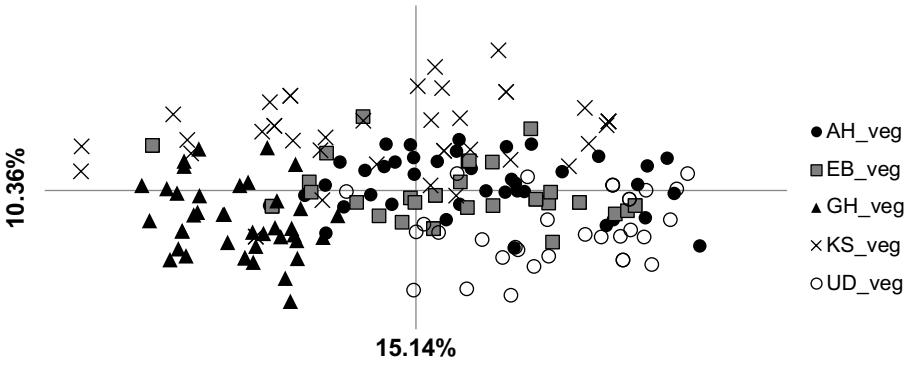
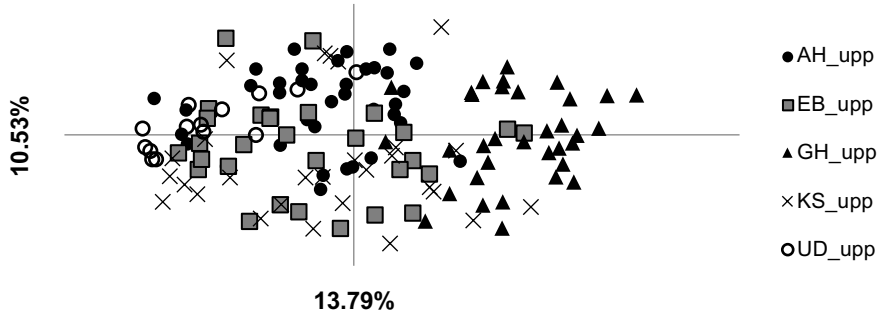


Figure S4. Principal coordinate analysis (PCoA) generated in GenAlEx based on the microsatellite data. Each diagram included samples from all locations (AH, EB, GH, KS, UD) corresponding to one of the three cohorts, a) vegetation (veg), b) upper soil layer(upp), c) lower soil layer (low). The first two axes explained accumulatively 25.5 % (a, vegetation), 24.43 % (b, upper soil layer) and 28.86 % (c, lower soil ölayer) of variation. We observed only a very slight grouping according to the locations but no distinct groups.

a)



b)



c)

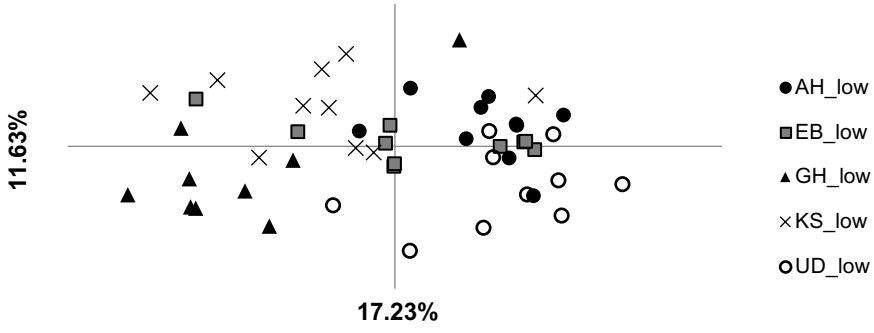


Figure S5. Mean posterior probability plot of STRUCTURE over 30 runs for each value of K=11 computed for the whole dataset. The graph shows the mean ln Pr (X|K) over 30 iterations and the standard deviation for each value of K

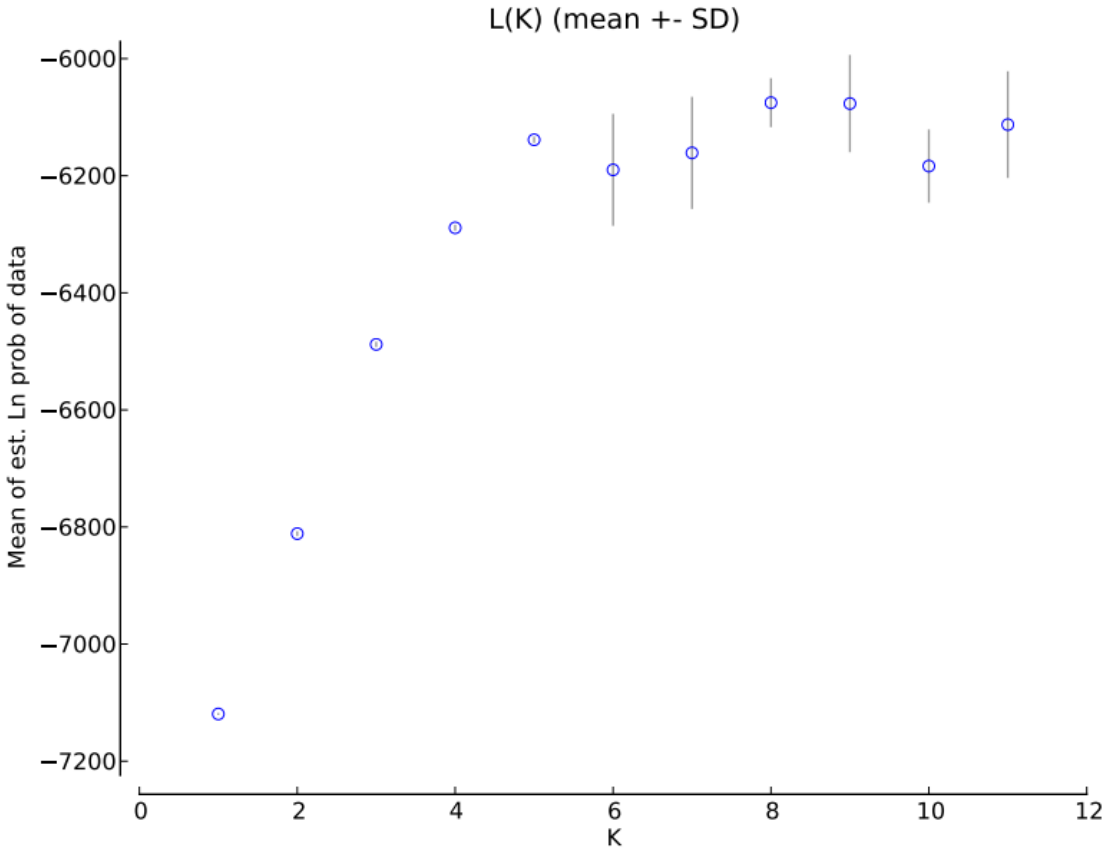
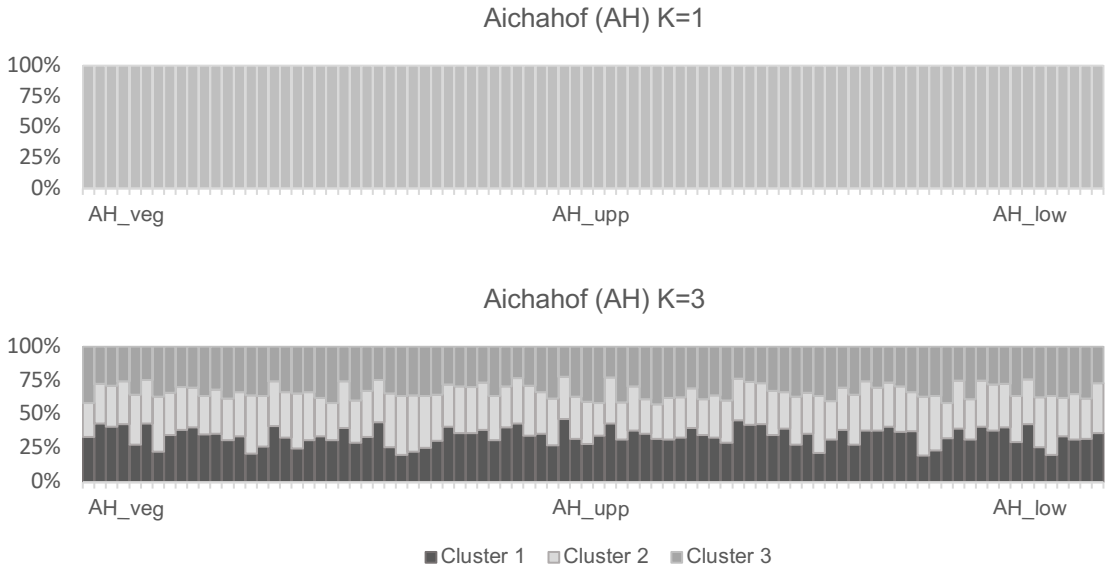
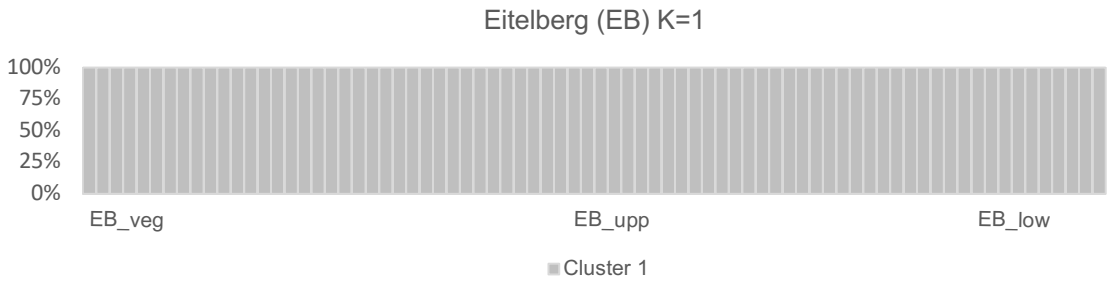


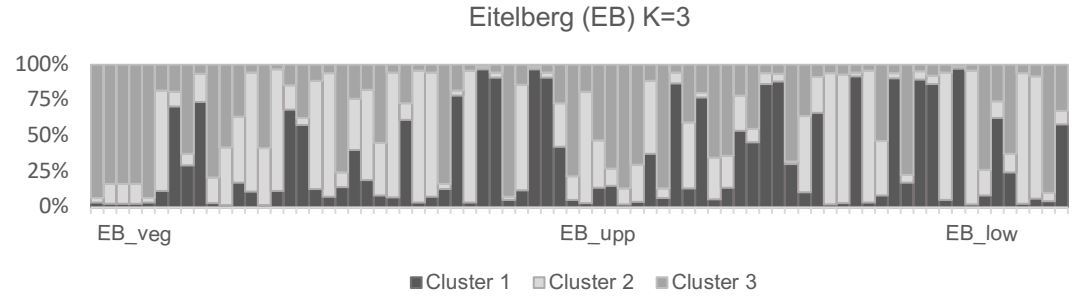
Figure S6. Bayesian cluster analysis (STRUCTURE, Pritchard et al., 2000) for all individuals of *Origanum vulgare* from the three cohorts, vegetation (veg), upper (upp) and lower (low) soil layers, partitioned according to the five study locations. We included, first, the most probable solutions for K which gave the highest value of ΔK after Evanno method (Evanno et al., 2005), and, second, the solutions which gave identical results over 30 iteration for each value of K (Kopelman et al., 2015); a) Aichahof (AH); b) Eitelberg (EB); c) Grabenhof (GH); d) Kühschlg (KS); e) Undorf (UD). Each bar corresponds to one individual.

a)

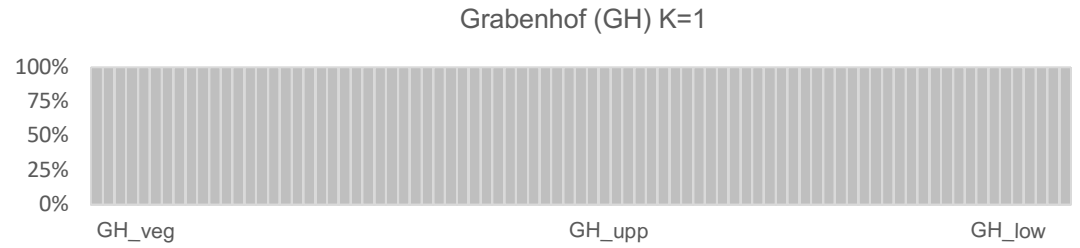


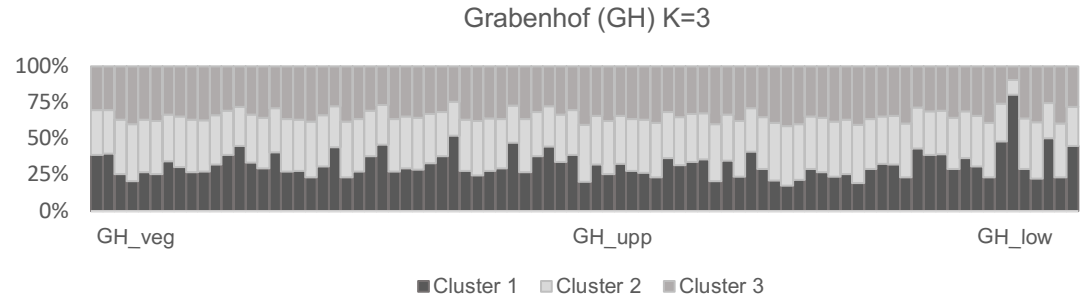
b)



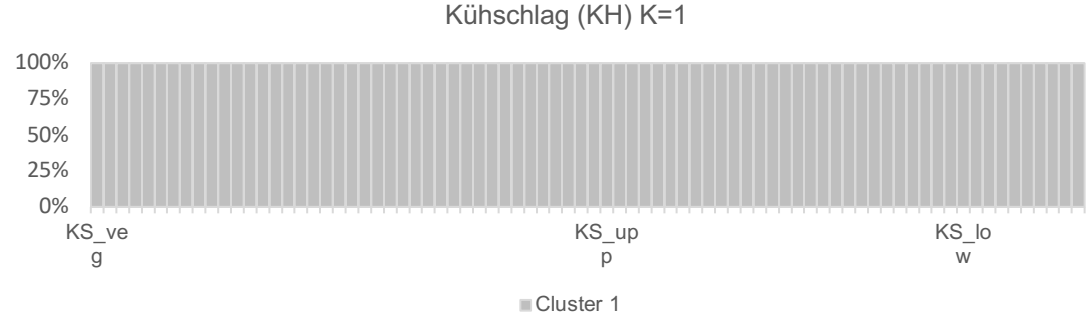

Eitelberg (EB) K=3
100%
75%
50%
25%
0%
EB_veg
EB_upp
EB_low
Cluster 1
Cluster 2
Cluster 3

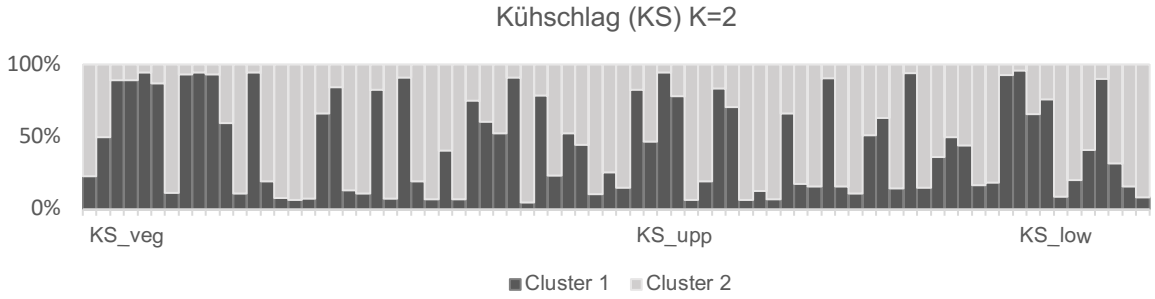
c)


Grabenhof (GH) K=1
100%
75%
50%
25%
0%
GH_veg
GH_upp
GH_low

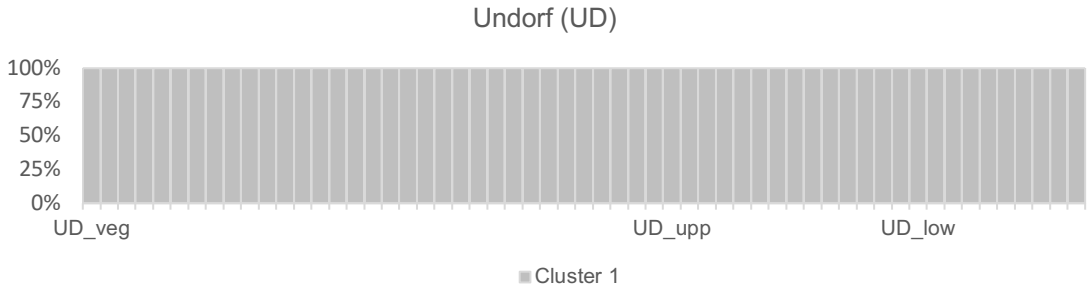

Grabenhof (GH) K=3
100%
75%
50%
25%
0%
GH_veg
GH_upp
GH_low
Cluster 1
Cluster 2
Cluster 3

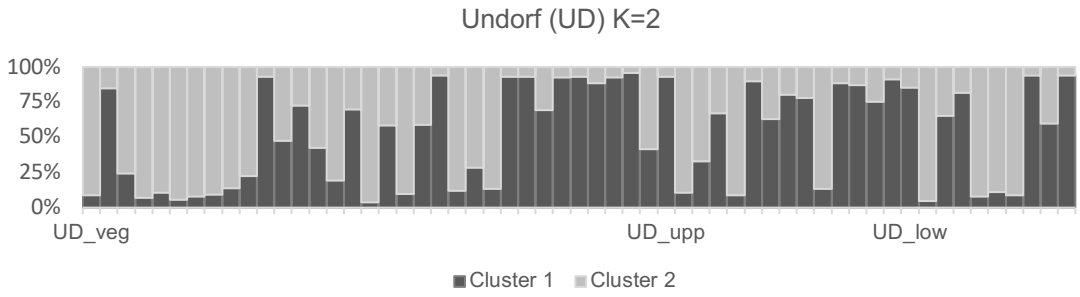
d)


Kühschlag (KH) K=1
100%
75%
50%
25%
0%
KS_veg
KS_up
p
KS_low
Cluster 1


Kühschlag (KS) K=2
100%
50%
0%
KS_veg
KS_upp
KS_low
Cluster 1
Cluster 2

e)


Undorf (UD)
100%
75%
50%
25%
0%
UD_veg
UD_upp
UD_low
Cluster 1


Undorf (UD) K=2
100%
75%
50%
25%
0%
UD_veg
UD_upp
UD_low
Cluster 1
Cluster 2

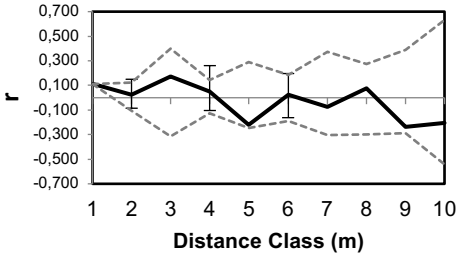
Autocorrelation analysis

Materials & Methods. Fine-scale spatial genetic structure was assessed in a spatial autocorrelation analysis using the same software (GenAlEx 6, Peakall and Smouse, 2006). For this purpose, we used all available samples from the lower soil cohort, as well as samples from the remaining two cohorts originating from the identical 1x1 m^2 plots. Random permutations allowed us to generate a distribution of permuted values under the assumption of no spatial structure, by the random mixing of all individuals among the positions in the experimental blocks. From 999 such random permutations, the values of the 25th and 975th ranked rp-values were taken to define the upper and lower bounds of the 95% confidence interval. If the calculated r-value fell outside of this confidence interval, then significant spatial genetic structure was detected. Bootstrap estimates allowed us to place a confidence interval around the observed estimate of r by drawing from within the set of pairwise comparisons for a specific distance class. For each of 999 bootstrap trials, the bootstrap autocorrelation coefficient was calculated for each distance class. The 25th and 975th ranked autocorrelation coefficient were then taken to define the 95% confidence interval. When the bootstrap confidence interval did not exceed r = 0, significant spatial genetic structure was inferred. We used even distance classes, whereby the distance class size was one meter.

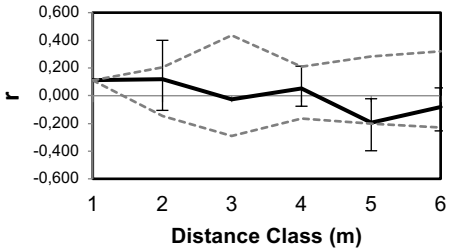
Results&Discussion. Correlograms were constructed with the correlation coefficient r as a function of distance. Distance classes were equivalent to one meter. Nonsignificant spatial structures corresponded to the full black line (r) which did not cross the upper and lower confidence limits (grey dashed lines). Upper and lower confidence limits bounded the 95% confidence interval about the null hypothesis of no spatial structure for the data set as determined by permutation. The values of the coefficients r, U and L were adjusted by the correction factor. Correlograms were constructed separately for each location, for each single cohort (vegetation, upper and lower soil layers). In accordance with Fst analysis, we detected no apparent spatial genetic structure within the experimental 8 x 10 m-plots (Fig. S6). This was possibly due to (i) high outcrossing rates, (ii) insect pollination habit of the study species, which contributed to a random distribution of alleles within the boundaries of our experimental block, and (iii) due to the low levels or absence of clonality. An evidence from previous research suggests that clonality and/or a limited seed dispersal may bring about genetic correlation over short distances (Reisch et al., 2007; Listl and Reisch, 2012), even in predominantly perennial outcrossing species in which pollen transfer is presumably effective (Schnabel and Hamrick, 1990; Loiselle et al., 1995). In our study, most probably, the seed and pollen dispersal exceeded the spatial scale settings of our experiment.

Figure S7. Correlogram with the correlation coefficient r as a function of distance. Distance classes are given in meters. Nonsignificant spatial structures correspond to the full black line (r) which does not cross the upper and lower confidence limits (grey dashed lines). Upper and lower confidence limits bound the 95% confidence interval about the null hypothesis of no spatial structure for the data set as determined by permutation. The coefficient r, U and L values were adjusted by the correction factor. Correlograms were constructed separately for each location, for the three cohorts (vegetation, upper and lower soil layers)

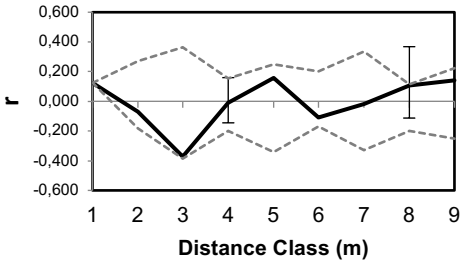
a) Vegetation – Aichahof



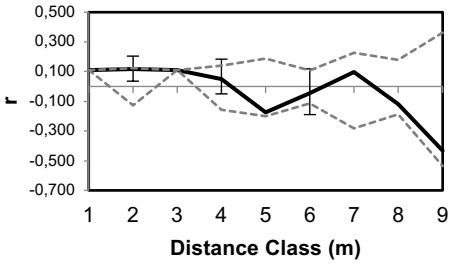
b) Vegetation – Eitelberg



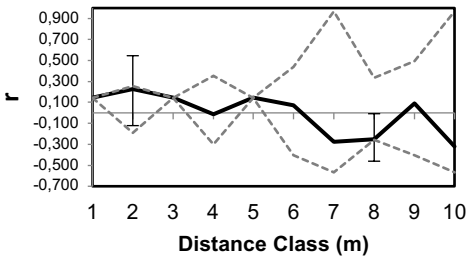
c) Vegetation - Grabenhof



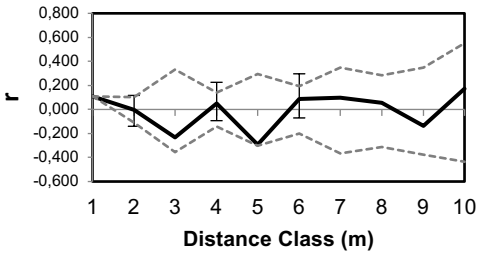
d) Vegetation - Kühschlag



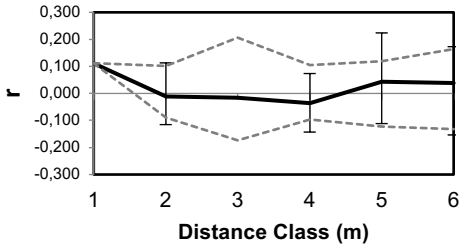
e) Vegetation - Undorf



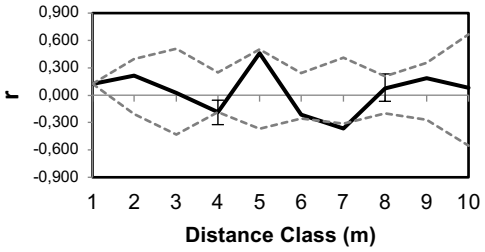
a) Upper soil layer - Aichahof



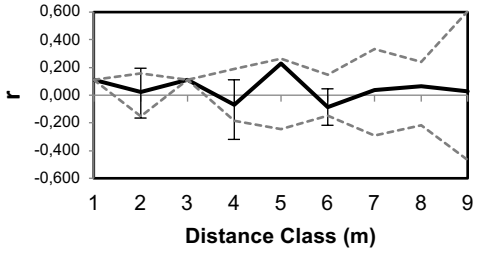
b) Upper soil layer – Eitelberg



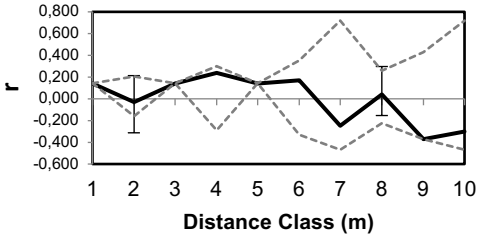
c) Upper soil layer – Grabenhof



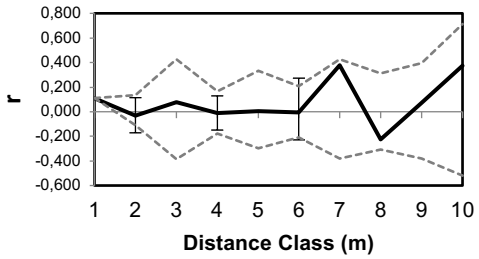
d) Upper soil layer - Kühschlag



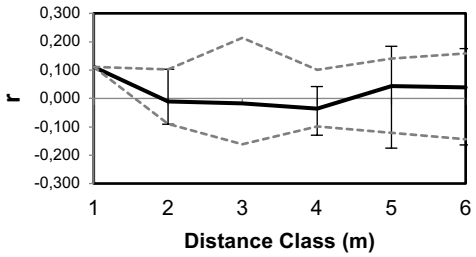
e) Upper soil layer - Undorf



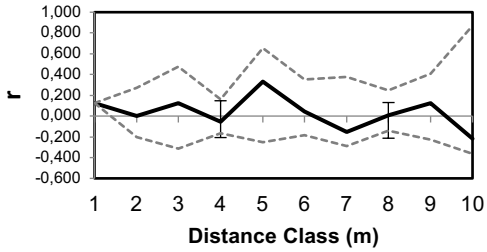
a) Lower soil layer – Aichahof



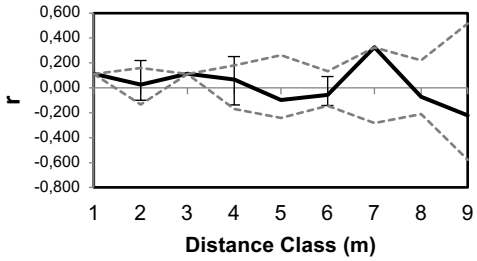
b) Lower soil layer - Eitelberg



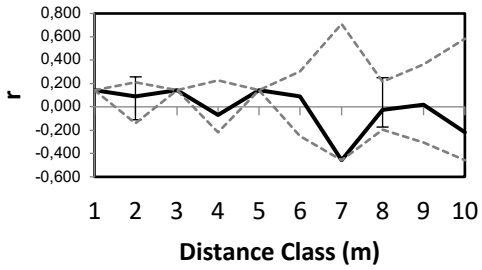
c) Lower soil layer – Grabenhof



d) Lower soil layer - Kühschag



e) Lower soil layer - Undorf



SUPPLEMENTARY MATERIAL – CHAPTER FIVE

Flow cytometrical analysis

For each sample, 1-2 cm2 leaf tissue together with circa 0.5 cm2 internal standard (Pisum sativum L. `Ctirad`, 2C=9.09 pg) was chopped with a sharp razor blade for 30-60 seconds in a Petri dish, and the plant tissue material was washed several times with citric-acid-Triton buffer (0.2 M citric acid, 0.5% triton X). Then, the nuclei suspension was filtered through the 50µm CellTrics filter into 1.5ml glass tubes on ice and subsequently centrifuged at 1400 RPM and 4°C. The supernatant was removed leaving 20-50 µl of it with remaining nuclei. The pellet was dissolved in ice-cold LB01 buffer (Dolezel et al., 1989) containing DAPI staining solution (4´, 6-diamidino-2´-phenylindole, dihydrochloride; Carl Roth, Karlsruhe, Germany) and incubated at least 10 minutes in the dark. DAPI is a dye that fluoresces blue (455 nm) when bound to double-stranded DNA and excited by exposure to 345 nm light. Sheath fluid was prepared using one litre Millipore water, 10ml 2% NaN3, and 200 µl Tween 20. The samples were excitated employing UV-LED and a sensitive blue photo-multiplier tube detecting fluorescent light between 435 and 560 nm. Measurements were carried out using Cyflow® Ploidy Analyser (Sysmex, Norderstedt, Germany). Acquisition was cut off when the standard peak reached 8000 nuclei. The DNA content of the study species was calculated by referencing of the Pisum sativum standard peak.

Figure S1 (a). Mean posterior probability plot of Structure for *Agrimonia eupatoria*.

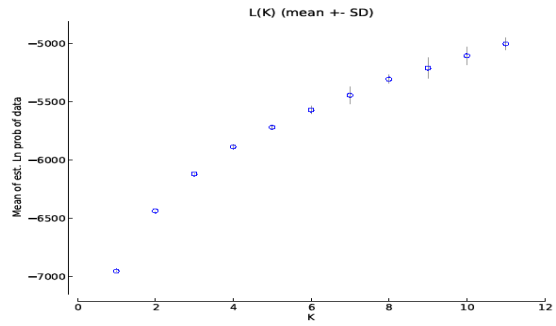


Figure S1 (b). Mean posterior probability plot of Structure for *Campanula rotundifolia*.

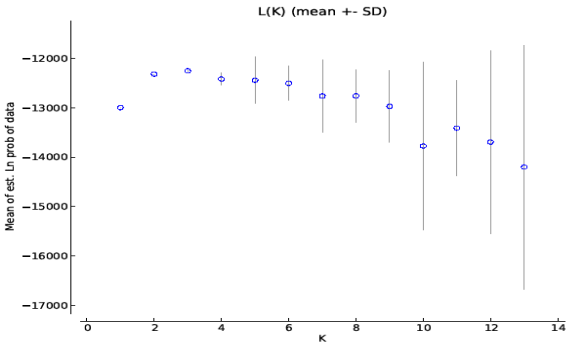


Figure S1 (c). Mean posterior probability plot of Structure for *Knautia arvensis.*