RESTORATION OF PLANT POPULATIONS AND COMMUNITIES - INSIGHTS FROM ECOLOGY AND GENETICS FOR CONSERVATION

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“The world is a fine place and worth fighting for.”

Ernest Hemingway

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Published manuscripts included within this thesis:

Chapter Two was published with the thesis' author as lead author:

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SUMMARY

Biodiversity is defined as the variety of life on earth, comprising genetic diversity, species diversity and ecosystem diversity. It is crucial for human life since it is the foundation of functioning ecosystems, which provide necessary ecosystem services. In the past centuries, human activities have altered the world's ecosystems and led to massive losses in biodiversity. To counteract the ongoing decrease in biological diversity therefore is a key issue in conservation. The reintroduction of native plant species is a common and useful practice in restoration and approaches range from reintroduction of plant populations to the creation of whole plant communities.

Chapter One addresses the research question of this thesis and introduces the scientific context. The importance of biodiversity and its ongoing loss is described. Further, plant species reintroduction as an effective procedure to increase biodiversity on population and community level is presented with respect to the impact on genetic variation within restored populations and among natural and restored populations of herbaceous target species.

Chapter Two focuses on the genetic diversity within and the genetic differentiation among populations of the rare and endemic plant species *Cochlearia bavarica*. Amplified Fragment Length Polymorphisms (AFLP) were used to analyze the genetic variation of 32 remnant populations of the species. With respect to conservation management, recommendations are presented to increase the success of future population reintroduction and reinforcement.

In **Chapter Three** the restoration of species-rich grassland communities by the transfer of green hay and threshed plant material was investigated. Species diversity and composition on restoration sites compared to their corresponding source sites was analyzed. Further genetic variation within and among populations on source and restoration sites of the common grassland species *Knautia arvensis* and *Plantago lanceolata* was examined using AFLPs. The study revealed no significant differences among source sites and their corresponding restoration sites, neither in species diversity and composition nor in genetic variation within populations of the two plant species. Only marginal differences in genetic variation among populations on source sites and their corresponding restoration sites could be found. The transfer of local plant material is

highly suited to preserve species composition of species-rich grasslands and the natural genetic pattern of typical grassland plant species on a small geographical scale.

Chapter Four and Five focus on the restoration of species-rich grassland communities by sowing commercially produced regional seed mixtures. Even though this is a common approach in restoration ecology today, there are only few long-term studies investigating if local seed mixtures can actually be applied successfully to restore species-rich grassland communities. Further, it is often questioned whether commercially produced seed material is viable enough to establish vital populations and how the sowing may affect genetic variation of neighboring natural populations. Hence, the outcome of a large-scale grassland restoration project which started about 15 years ago in south-eastern Germany provides more information about the impact of this restoration measure on species diversity and genetic variation of plant species and populations on restored sites.

The success of applying local seed mixtures to restore species-rich grasslands was analyzed within **Chapter Four**. Local seed mixtures can be used successfully to restore species-rich grassland communities in practice: of all species that were present in the local seed mixtures 62 % were contained in the current and on average covered up two thirds of the total vegetation cover. Moreover, restoration can be improved by using specific seed densities and species that are ecologically more suitable to restored sites.

In **Chapter Five** deal with the impact of the restoration management on genetic variation within and among restored and natural populations of typical grassland species. Genetic variation of three common plant species (*K. arvensis*, *Silene vulgaris* and *P. lanceolata*) was analyzed with AFLPs. The study revealed that using commercially produced seed mixtures in restoration caused no decease in genetic diversity within restored populations of common grassland species but did not match exactly the local genetic pattern of the study species. However, commercially produced seed material reflects the genetic potential of an entire seed transfer zone and provides seeds for the reestablishment of genetically viable populations.

Finally, in **Chapter Six** the results of the four main chapters are reviewed in the context of nature conservation. Benefits and disadvantages of species (re)introductions are

discussed. Recommendations are given to enhance the success of species reintroduction in restoration projects.

CHAPTER ONE: GENERAL INTRODUCTION

BIODIVERSITY – A THREATENED TREASURE

Biodiversity is the variety of life on Earth (Gaston 2000). Whether desserts or tropical rainforests, the deep sea, coral reefs or rivers, valleys or mountain ranges - ecosystems all over the planet provide habitats for millions of distinct species. Especially the distribution of plant species depends on biotic and abiotic conditions within a habitat since they are adapted to their local environment (Joshi, Schmid et al. 2001). Therefore, the diversity of species is spaced heterogeneously over our planet and there occur species poor areas like the Arctic or Antarctica as well as hotspots like the Mediterranean ecosystems (Gaston 2000).

However, biodiversity cannot only be described by species richness. It ranges from molecules to ecosystems and The Convention on Biological Diversity (CBD) characterized three different biotic levels as integral parts of biological diversity (CBD 1992 a): genetic diversity, species diversity and ecosystem diversity. Genetic diversity comprises all different genes contained in all living species of microorganisms, fungi, animals and of course plants. Species diversity includes all species with their differences within and among varying species. Different habitats, biological communities, ecological processes and the variation within individual ecosystems contribute to ecosystem diversity. This diversity is the product of evolutionary processes over thousands of years and provides the raw material for evolution.

Generally, all living creatures gain from biodiversity as it is the foundation of intact ecosystems which provide so called ecosystem services like clean and fresh water, fiber or fuel, the prevention of soil erosion, protection from floods and storms, nutrient cycling, oxygen from photosynthesis, the supply of resources, living space or immaterial benefits like cultural and aesthetic values (Tallis and Kareiva 2005).

Even though biodiversity is crucial for human life, there is a rapid loss of species richness in nearly all ecosystems over the world. Of course, extinction is a natural process but nowadays the actual extinction rate is about 1.000 times higher than it would be naturally expected (Pimm, Jenkins et al. 2014).

The international Union for Conservation of Nature (IUCN) maintain a *Red List of Threatened Species* of the world (IUCN 2022). According to the Red List version 2021-3, the IUCN currently evaluated the vulnerability of over 142.500 species. More than

40.000 of these species (more than 28 %) are listed as “critically endangered”, “endangered” or “vulnerable”. This includes 34 % of the listed amphibians, 23 % of the listed mammals, 18 % of the listed reptiles and 13 % of the listed birds. Further, 58 % of the listed mosses, 38 % of the listed ferns and allies and 40 % of the listed gymnosperms but also flowering plants are threatened.

THE LOSS IN BIOLOGICAL DIVERSITY AND ITS CONSEQUENCES

In the past centuries, humans have altered the world’s ecosystems in an adverse way for instance by transforming prairies, forests or wetlands into agricultural and urban systems, by changing global biochemical cycles or by increasing the concentration of atmospheric CO_2. Further, human activities like over-exploitation (over-hunting, over-fishing, over-collecting), pollution or the introduction of invasive alien species reveal major threats to biodiversity (Chapin Iii, Zavaleta et al. 2000).

In particular plant species suffer from the destruction of their natural habitats as they are incapable of moving away from habitat degradation or changing environmental conditions. Modern land use practices, increased urbanization and landscape fragmentation (Sala, Chapin et al. 2000, Fahrig 2003) can diminish habitat quality or may provoke habitat destruction. Due to the depletion of adequate habitats plant populations may become smaller or in the worst case populations may get extinct. Further, the increased distance between the remaining populations hamper the exchange of pollen or seed between remnant populations (Honnay, Coart et al. 2006). Gene flow decreases and self-pollination or mating events between related individuals may become more frequent, resulting in inbreeding and decreased genetic variation. Inbreeding depression may cause the accumulation of deleterious alleles and decrease the fitness of individuals (Keller and Waller 2002). On the other hand large geographical distances and restricted gene flow among populations may enhance genetic drift (Vitousek 1994). Consequently, the genetic variation within populations declines while the differentiation among them increases (Aguilar, Quesada et al. 2008). However, genetic diversity within and among populations is a prerequisite for evolutionary further advancedment (McKay, Christian et al. 2005). Hence, genetic impoverishment reduces the ability of plant individuals to adapt to changing environmental conditions (Heywood 1991, Booy, Hendriks et al. 2000).

In summary, the loss of biodiversity may cause a chain reaction or so called extinction vortex: environmental stresses like habitat degradation, smaller population sizes and decreased genetic diversity mostly resulted in decreased fitness and viability of individuals and may push a population towards extinction (Gilpin 1986). Potential damage from stochastic catastrophic events also contribute to an increased extinction probability (Godwin, Lumley et al. 2020).The loss of species can alter processes, functioning and the structure of whole ecosystems. As a consequence, the resilience of ecosystems to environmental changes declines and ecosystem services become more and more degraded and unsustainable (Tallis and Kareiva 2005).

However, the actual observed biodiversity is constantly changing due to dynamic processes and genetic, species and ecosystem variety are not fixed (Pagel 2020). The quantity and quality of an ecosystem service is positively related to the species diversity within the respective ecosystem (Balvanera, Pfisterer et al. 2006). Considering this in the context of restoration, it should be possible to increase the stability of ecosystems and the respective ecosystem service, by restoring certain habitats and increasing species-richness. Therefore, restoration is a key issue in conservation.

FROM POPULATIONS TO HABITATS - SPECIES INTRODUCTION IN RESTORATION

During the past decades, the interest in restoring degraded landscapes back to more species and habitat rich systems has grown among restoration practitioners, conservationists or forest managers (Hobbs and Norton 1996, McKay, Christian et al. 2005, van der Mijnsbrugge, Bischoff et al. 2010).

The restoration of habitats by improving site conditions might principally be a good conservation approach to allow plant populations or communities to recover (Menges 2008). For example, low-input agriculture, decreased fertilizer application, re-introduction of adequate management regimes in abandoned habitats, the removal of nutrients from the soil, rewetting drained habitats or the renaturation of natural-near river courses may improve and stabilize biotopes.

However, increasing plant species-richness by these restoration approaches is often limited due to a lack of viable seeds in the soil or the absence of dispersal vectors (Münzbergova and Herben 2005). Additionally, landscape fragmentation and the low potential for long-distance dispersal of many plant species may hamper seed dispersal

between populations on restoration sites and potential source populations (Clark, Poulsen et al. 2007).

The controlled introduction of plant species is, therefore, an often-required supplementary procedure in restoration and ranges from the reintroduction of plant populations to the creation of whole plant communities (Jones 2003).

Population reintroduction in general is defined as the controlled placing of certain plant material (seeds, seedlings, individuals) into a managed or natural habitat to establish genetically variable populations (Akeroyd and Wyse Jackson 1995). By increasing gene flow among populations the extinction probability of populations should decrease (Vergeer, van den Berg et al. 2005). The restoration technique is often implemented to restore populations of rare or endangered plant species (cf. Chapter Two). Also common plant species or whole plant communities can be established via the introduction of seed-containing local plant material (cf. Chapter Three) or commercially produced regional seed mixtures (cf. Chapter Four and Five). Especially in grassland conservation the restoration of whole plant communities have become increasingly important since species-rich and extensively managed grasslands declined drastically in central Europe during the recent decades (Poschlod 2017).

A more traditional but proven restoration technique on a small geographical scale is the introduction of local plant material from species-rich source sites via transfer of seed-containing chaff, threshed plant material or green hay (non-dried fresh plant material) (Kiehl, Kirmer et al. 2010). The usage of seed-containing plant material has two advantages: First, it is potentially possible to move the species-richness of a whole plant community from a source site to a potential restoration site. Second, at the same time it is possible to establish genetically variable populations that are locally adapted to specific regions (van der Mijnsbrugge, Bischoff et al. 2010). However, the procedure depends on the availability of appropriate sources (in general species rich plant communities) in the vicinity of restoration sites which may be hard to find in highly fragmented and intensively used landscapes.

Therefore, the application of commercially produced seed mixtures is a promising and comparatively simple alternative, when local seed material cannot be harvested on suitable donor sites around the restoration sites. With respect to the concept of seed transfer zones (Prasse, Kunzmann et al. 2010), seed mixtures from 22 zones within

eight producing areas according to similar environmental conditions (Bucharova, Bossdorf et al. 2018) can be purchased from different seed producers in Germany. The innovative concept plans that it is only allowed to collect, mix, reproduce and sow seeds within one seed transfer zone. This ensures, that local adaptations of plant species to specific environmental conditions maintain what is assumed to increase the success of restoration outcome.

GENETIC CONSEQUENCES OF SPECIES (RE)INTRODUCTION

The introduction of target plant species is a state-of-the-art method in conservation practice (Kirmer, Mann et al. 2009, van der Mijnsbrugge, Bischoff et al. 2010). However, population and species (re)introductions have not always been successful. Godefroid, Piazza et al. (2011) found in a comprehensive literature study generally low recruitment as well as low survival, flowering or fruiting rates in restored populations of reintroduction projects.

The outcome of reintroduction projects can be positively influenced by suitable site preparation, working in protected areas, using seedlings instead of seeds and a high number of introduced individuals (Godefroid, Piazza et al. 2011). A consistent long-term monitoring after reintroduction allows examining the establishment of target species on the restoration site. Thus, failure can be recognized early which enables restoration practitioners or project managers to counteract with appropriate measures (Godefroid, Piazza et al. 2011). Additionally, the success of a restoration project can be increased by considering genetic variation and diversity within native species (Walker 2004). The origin of plant material used in restoration, the collection and also the propagation of source seeds are decisive to obtain genetic variation in restored populations and the successful outcome of restoration management (Godefroid, Piazza et al. 2011).

It is generally known, that geographical or ecological differences among habitats may cause the development of ecotypes and local adaptations in plant populations (Leimu and Fischer 2008). Also life-history characteristics (mating system, pollination vector or dispersal unit) can affect genetic differentiation among populations (Reisch and Bernhardt-Römermann 2014). Therefore, seed material used for restoration should match the gene pool of the populations occurring in the surroundings of the restoration

site (McKay, Christian et al. 2005). Otherwise non-local genotypes may be maladapted to the local environment resulting in decreased fitness of plant individuals (van der Mijnsbrugge, Bischoff et al. 2010).

Further, the mixing of foreign and locally adapted genotypes may also reduce genetic variation if locally adapted alleles were replaced by the new genotypes (genetic swamping) and can lead to outbreeding depression (Hufford and Mazer 2003). Therefore, the introgression of maladapted genes or the disruption of co-adapted gene complexes may reduce overall population fitness in subsequent generations (Hufford and Mazer 2003). Both scenarios are problematic especially in the restoration of long-lived perennials because lower performance of individuals becomes apparent only after several years in subsequent generations (McKay, Christian et al. 2005, van der Mijnsbrugge, Bischoff et al. 2010).

Additionally, collecting source seeds from small populations can negatively affect the genetic variation within restored populations because small populations are less attractive to pollinators (Agren 1996, Kunin 1997). Therefore, source populations may suffer from reduced cross pollination, mating with related individuals or even self-fertilization (Van Treuren, Bijlsma et al. 1994). This may result in inbreeding and as a consequence in reduced fitness and decreased genetic variation (Friar, Ladoux et al. 2000). On the other hand, collecting seed material from large populations but only a small number of source individuals may also reduce genetic variation in restored populations due to genetic drift. A frequency shift of gene variants can reduce genetic diversity or local adaptations (Espeland, Emery et al. 2017) and lead to increased homozygosity and random loss or fixation of deleterious alleles (Young, Petersen et al. 2005).

Another important step in restoration, which can affect negatively the genetic variation of restored plant populations, is the cultivation process within a seed-farm because stock individuals can be used several years in multiple reproduction cycles. This could increase the risk of inbreeding depression (Schoen and Brown 2001) and unintended selection due to the adaptation to environmental conditions at the farm sites during cultivation (Espeland, Emery et al. 2017, Nagel, Durka et al. 2019).

In summary, the (re)introduction of native plant species is often a challenge and it is not always easy to consider genetic diversity and differentiation of plant populations.

However, due to the fact that various techniques can be applied on different geographical scales, population or species (re)introduction are an important approach in restoration ecology.

THESIS OUTLINE AND RESEARCH QUESTIONS

In the past centuries, humans have altered the world's ecosystems in an unprecedented way, resulting in large losses of biodiversity. Consequently, many regions in the world risk ecological collapse. The preservation and protection of biodiversity is, therefore, a key issue in conservation all over the world. The reintroduction of native plant species seems to be a suitable tool to counteract the decrease in biological diversity on different geographical scales.

In **Chapter One** the research question of this thesis is placed into the broader context of biodiversity and its ongoing loss. The restoration of plant populations and communities by means of the reintroduction of locally adapted plant material is presented and the impact on genetic variation within restored populations and among natural and restored populations of herbaceous target species is described.

In **Chapter Two** the diversity and differentiation of the rare and endangered plant species *Cochlearia bavarica* was investigated with respect to population reintroduction. Aim of this study was to increase the success of future reintroduction and reinforcement by providing data, to avoid negative effects of inbreeding and outbreeding and to preserve the natural genetic pattern of the species. Amplified Fragment Length Polymorphisms (AFLP) were used to analyze genetic variation.

In **Chapter Three** the outcome of a practical restoration project in south eastern Germany is presented: Species-rich grassland communities were restored by the transfer of green hay and threshed plant material. On source sites and their corresponding restoration sites no significant differences were found, weather in species diversity and composition nor in genetic variation within populations of the study species *Knautia arvensis* and *Plantago lanceolata*. The study revealed only marginal differences in genetic variation among populations on source sites and their corresponding restoration sites. The transfer of local plant material is highly suited to preserve species composition of species-rich grasslands and the natural genetic pattern of typical grassland plant species on a small geographical scale.

Since the implementation of seed transfer zones, mixtures with locally adapted seeds are easily available for natural regions in Germany and the application of commercially produced seed mixtures has become common approach in restoration ecology. However, there are only few long-term studies investigating if local seed mixtures can actually be applied successfully to restore species-rich grassland communities. Further, it is often questioned whether commercially produced seed material is viable enough to establish vital populations and how the sowing affect genetic variation of neighboring natural populations. Therefore, **Chapter Four and Five** focus on the restoration of species-rich grassland communities by sowing commercially produced regional seed mixtures. The outcome of a large-scale grassland restoration project started about 15 years ago in southeastern Germany is presented.

Within **Chapter Four** the success of applying local seed mixtures to restore species-rich grasslands was analyzed. The current vegetation on restored sites contained 62% of all species that were present in the local seed mixtures, which covered up on average two thirds of the total vegetation cover. Therefore, local seed mixtures can be used successfully to restore species-rich grassland communities in practice. Further, restoration can be improved by using specific seed densities and species that are ecologically more suitable to restored sites.

In **Chapter Five** the impact of restoration management on genetic variation within and among restored and natural populations of typical grassland species was investigated using amplified fragment length polymorphisms (AFLPs). The analysis included three common plant species: *Knautia arvensis* and *Silene vulgaris* (both insect-pollinated) and *Plantago lanceolata* (wind-pollinated). Using commercially produced seed mixtures in restoration caused no decease in genetic diversity within restored populations of the study species but did not match exactly the local genetic pattern of the study species. However, commercially produced seed material reflects the genetic potential of an entire seed transfer zone and provides seeds for the reestablishment of genetically viable populations.

Finally, in **Chapter Six** the results of the four main chapters are reviewed in the context of nature conservation. Benefits and disadvantages of species reintroductions are discussed. Recommendations are given to enhance the success of species reintroduction in restoration projects.

CHAPTER TWO

REINTRODUCTION OF THE ENDANGERED AND ENDEMIC PLANT SPECIES *COCHLEARIA BAVARICA* – IMPLICATIONS FROM CONSERVATION GENETICS

FRANZISKA KAULFUß AND CHRISTOPH REISCH

ABSTRACT

Population reintroduction is a common practice in conservation, but often fails, also due to the effects of inbreeding or outbreeding depression. *Cochlearia bavarica* is a strongly endangered plant species endemic to Bavaria in Germany, constantly declining since the late 1980s. Therefore, population reintroduction is intended.

In this study, we analyzed genetic diversity within and genetic differentiation between all 32 remnant populations of the species in Swabia and Upper Bavaria using amplified fragment length polymorphisms. Our aim was to increase reintroduction success by providing data to avoid negative effects of inbreeding and outbreeding and to preserve the natural genetic pattern of the species.

Genetic diversity within populations was low but similar to other rare and endemic species and varied strongly between populations but did not depend on population size. Our analysis revealed a strong geographic pattern of genetic variation. Genetic differentiation was strongest between Swabia and Upper Bavaria and at the population level, whereas differentiation between subpopulations was comparatively low. Isolation by distance and genetic differentiation was stronger among populations from Upper Bavaria than from Swabia.

From the results of our study, we derived recommendations for a successful reintroduction of the species. We suggest using rather genetically variable than large populations as reintroduction sources. Moreover, the exchange of plant material between Swabia and Upper Bavaria should be completely avoided. Within these regions, plant material from genetically similar populations should preferably be used for reintroduction, whereas the exchange among subpopulations seems to be possible without a negative impact on genetic variation due to natural gene flow.

KEYWORDS: conservation, genetic variation, inbreeding, outbreeding, reinforcement, reintroduction

INTRODUCTION

The loss of plant species is a worldwide problem, mainly due to land use changes (Poschlod, Bakker et al. 2005, Maurer, Weyand et al. 2006) such as agricultural intensification (Storkey, Meyer et al. 2012) and abandonment of traditional management methods (Poschlod and WallisDeVries 2002). The associated process of habitat fragmentation intensifies the loss of plant species (Fahrig 2003, Schleunig, Niggemann et al. 2009), since small and isolated remnant populations suffer from a higher extinction probability (Matthies, Brauer et al. 2004). The actual extinction rate is, therefore, 100 to 1000 times higher than it would be naturally expected (Thuiller 2007).

Population reintroduction, comprising reintroduction in the narrow sense, reinforcement and translocation (Akeroyd and Wyse Jackson 1995), is meanwhile a common practice in conservation to alleviate the proceeding loss of plant species. Generally, the aim of population reintroduction is to establish genetically variable populations, to increase gene flow (Akeroyd and Wyse Jackson 1995, Godefroid, Piazza et al. 2011, Betz, Scheuerer et al. 2013) and to minimize the probability of population extinction (Vergeer, van den Berg et al. 2005).

However, population reintroduction is a challenge and often fails (Godefroid, Piazza et al. 2011). One main reason for the lack of success is the origin of the plant material used for reintroduction, especially when reintroduced plants or seeds derive from small populations or only from a few individuals (Godefroid, Piazza et al. 2011). Small populations are less attractive for pollinators (Aizen and Feinsinger 1994, Agren 1996, Kunin 1997), which reduces cross-pollination and increases self-fertilization or mating with related individuals (Van Treuren, Bijlsma et al. 1994). Using plant material from small populations with limited genetic variation may increase indeed the census population size but even reduce effective population size (Robichaux, Friar et al. 1997, Friar, Ladoux et al. 2000). Reintroduced populations may, therefore, suffer from inbreeding depression (Robichaux, Friar et al. 1997, Friar, Ladoux et al. 2000, Frankham, Ballou et al. 2002).

Similar results can be evoked when reintroduced populations are founded with only a few individuals. Genetic variation of the reintroduced population may be reduced due to this founder-effect (Vergeer, van den Berg et al. 2005). Furthermore, genetic drift

may cause the random loss of alleles, increasing homozygosity and the fixation of deleterious alleles (Ellstrand and Elam 1993, Young, Boyle et al. 1996). Both, inbreeding and genetic drift, result in decreased genetic diversity and fitness (Charlesworth and Charlesworth 1987, Booy, Hendriks et al. 2000, Young, Petersen et al. 2005, Ouborg, Vergeer et al. 2006) and populations may thus lose their ability to adapt to changing environmental conditions (Heywood 1991, Booy, Hendriks et al. 2000, Reed, Lowe et al. 2003).

Moreover, the success of population reintroduction may be limited due to the adaptation of populations to the environmental conditions of their habitat. It has been demonstrated previously, that ecological differences among habitats result in different local adaptions or ecotype development (Joshi, Schmid et al. 2001, McKay, Christian et al. 2005, Becker, Colling et al. 2006, Leimu and Fischer 2008, Reisch and Poschlod 2009). Mixing different genotypes adapted to specific habitat conditions can result in the erosion of co-adapted gene complexes (Frankham, Ballou et al. 2002). Local adaptations get lost and outbreeding depression may result in decreased fitness and performance of the populations (Fischer and Matthies 1998, Keller, Kollmann et al. 2000, Montalvo and Ellstrand 2000, Montalvo and Ellstrand 2001, Krauss, Zawko et al. 2005, Bischoff, Cremieux et al. 2006, Mijnsbrugge, Bischoff et al. 2010), which may consequently decrease reintroduction success.

Cochlearia bavarica Vogt is a rare, endemic and endangered plant species comprising a limited number of small and isolated populations (Fischer, Hock et al. 2003). The species occurs in only two regions of Bavaria and the number and size of populations constantly declined since the late 1980s due to changes in land use, habitat loss and fragmentation (Fischer, Hock et al. 2003). *Cochlearia bavarica* has, therefore, been included in the “German National Strategy on Biodiversity” and in two large conservation projects (“Wildpflanzenschutz Deutschland” and “Löffelkraut & Co”). Within these projects it is intended to maintain and develop populations by protecting and restoring natural habitats of *C. bavarica*. Furthermore, it is purposed to augment small populations by population reinforcement and to reduce the loss of populations by population reintroduction.

The genus *Cochlearia* and its species already have been in the focus of many plant systematic and conservation studies (Paschke, Abs et al. 2002a, Koch, Dobeš et al.

2003, Cires, Samain et al. 2011, Brandrud 2014, Olsen 2015). In this study, we analyzed the genetic diversity and differentiation among populations of *C. bavarica*. Our aim was to increase the success of future population reintroduction and reinforcement, by providing data to avoid negative effects of inbreeding and outbreeding and to preserve the natural genetic pattern of the species. In this context the following questions were addressed:

(1) How large is genetic diversity within populations and genetic differentiation among populations of *C. bavarica*?

(2) Which populations may serve as potential sources for population reinforcement of small populations facing extinction or population reintroduction?

(3) Is it possible to draw general conclusions for the reintroduction of *C. bavarica*?

METHODS

Species description

Cochlearia bavarica Vogt is endemic to Bavaria with a narrow distribution in Swabia and Upper Bavaria (Abs 1999). The species is more frequent in Swabia than in Upper Bavaria and originated from hybridisation of *Cochlearia pyrenaica* DC. and *Cochlearia officinalis* L. (Koch, Hurka et al. 1996) and is a habitat specialist of calcareous springs with continuous water supply, small rivers or drainage ditches and occurs in open calcareous fens, woodland clearings and shaded woodland springs (Abs 1999). The species is considered as highly endangered and is legally protected by law (Fischer, Hock et al. 2003).

C. bavarica is a perennial, monocarpic herbaceous plant species (Paschke, Bernasconi et al. 2003) with a sporophytic self-incompatibility system (Fischer, Hock et al. 2003). Plants flower from May to June, the ellipsoid fruits are 5-8 mm long and contain two to six brown or reddish-brown seeds (Vogt 1985). The species is pollinated by flies, bumblebees, other bees, or small moths (Paschke, Abs et al. (2002b), Paschke, Bernasconi et al. (2003)). Vegetative reproduction plays no major role since daughter rosettes are only found in the immediate vicinity of parent plants (Paschke, Abs et al. 2002b).

Table 2.1: Number, geographic location and names of the analyzed populations in Swabia and Upper Bavaria. Subpopulations are displayed indented. Also specified are population label, number of analyzed individuals (n) and the population size (PS). Furthermore, genetic variation measures as Nei's Gene diversity (GD), Shannon's Information Index (SI) and percentage of polymorphic bands (PB). Standard errors are given for mean values.

no.	region	population (-subpopulation)	label	n	PS	GD	SI	PB
1	Swabia	Klessen	KL	15	600	0.1198	0.1769	32.83
2	Swabia	Ollarzried-Daßberg	OL1	15	300	0.1255	0.1842	32.32
3	Swabia	Ollarzried-Boschach	OL2	15	1500	0.1280	0.1879	33.84
4	Swabia	Ollarzried-Mitte	OL3	15	50	0.1239	0.1824	32.32
	Swabia	Ollarzried-Höhe	OL	15	4300	0.1275	0.1883	33.84
5	Swabia	-Höhe 1	OL4	15	1800	0.0968	0.1423	25.25
6	Swabia	-Höhe 2	OL5	15	2000	0.1219	0.1795	31.82
7	Swabia	-Höhe 3	OL6	15	500	0.1213	0.1774	31.31
8	Swabia	Seebach	SE	6	6	0.0996	0.1421	22.73
9	Swabia	Grub-Eheim	GE	15	7500	0.0971	0.1416	24.75
10	Swabia	Hatzleberg	HA	15	200	0.1139	0.1676	30.81
11	Swabia	Liebenthann	LT	15	500	0.1022	0.1521	28.28
12	Swabia	Immenthal	MM	9	15	0.1015	0.1490	26.77
13	Swabia	Katzbrui-Mariengrotte	KB1	15	95000	0.1199	0.1759	31.31
14	Swabia	Katzbrui-Mühle	KB2	15	7500	0.0704	0.1053	20.71
15	Swabia	Mindeltal-Schönlings	MT1	15	6000	0.1141	0.1660	29.29
16	Swabia	Mindeltal-Reichartsried	MT2	15	1500	0.1087	0.1589	27.78
17	Swabia	Mindeltal-Mayers	MT3	15	5500	0.1115	0.1656	30.30
18	Swabia	Algers	AL	15	7000	0.0950	0.1410	26.77
19	Swabia	Gfäll	GF	15	100	0.1108	0.1616	28.28
20	Swabia	Gillenmoos	GM	14	3000	0.1140	0.1655	28.79
21	Swabia	Kemnath 1	KE1	15	8500	0.1004	0.1465	25.76
22	Swabia	Kemnath 2	KE2	15	-	0.0931	0.1375	25.25
23	Swabia	Gennachquelle	GN	14	15	0.1261	0.1856	32.83
24	Swabia	Aufkirch	AU	6	7	0.0860	0.1243	20.71
25	Swabia	Kaltental 1	KA1	15	-	0.1074	0.1567	27.27
26	Swabia	Kaltental 2	KA2	15	15000	0.0822	0.1212	22.73
	Mean all populations of Swabia					0.1074	0.1577	28.18
	Standard error					± 0.0031	± 0.0046	± 0.81
	Upper Bavaria	Glonnquellen	GL	15	2100	0.1057	0.1572	29.8
27	Upper Bavaria	-Glonn 1	GL1	15	500	0.1101	0.1625	29.29
28	Upper Bavaria	-Glonn 2	GL2	15	1400	0.0934	0.139	26.26
29	Upper Bavaria	-Glonn 3	GL3	15	200	0.0942	0.1397	26.26
30	Upper Bavaria	Kupferbachtal 1	KU1	15	500	0.0992	0.1456	26.26
31	Upper Bavaria	Kupferbachtal 2	KU2	15	1000	0.0969	0.1423	26.77
32	Upper Bavaria	Kupferbachtal 3	KU3	15	8000	0.1149	0.1691	30.81
33	Upper Bavaria	Vagen	VA	15	600	0.1038	0.1541	28.79
34	Upper Bavaria	Lungham	LU	15	2000	0.1182	0.174	31.31
35	Upper Bavaria	Thalham	TH	15	1000	0.1093	0.161	30.81
36	Upper Bavaria	Laubensee	LA	15	50	0.0746	0.1133	23.23
	Mean all populations of Upper Bavaria					0.1028	0.1521	28.47
	Standard error					± 0.0048	± 0.0067	± 1.0011

Study design and sampled populations

In this study we analyzed all 32 actually existing populations of *Cochlearia bavarica* (Table 2.1). 24 populations are located in Swabia, and eight populations in Upper Bavaria (Figure 2.1).

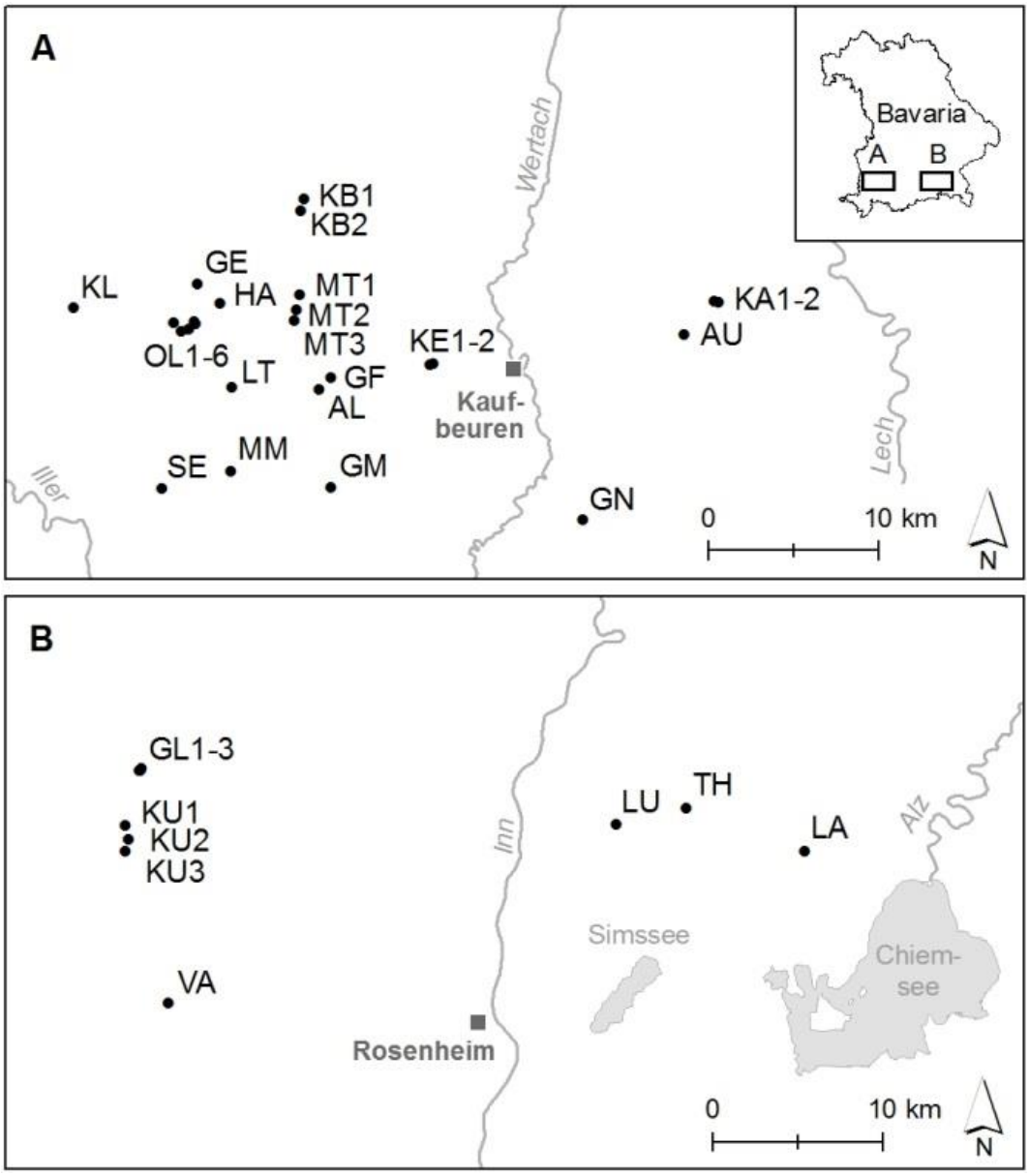


Figure 2.1: Geographic position of the analyzed populations of *C. bavarica* in Swabia (A) and Upper Bavaria (B).

Within the two regions populations of *Cochlearia bavarica* are strongly isolated. However, single populations often consist of several subpopulations with a distance of less

than 200 m in between. Genetic variation was therefore exemplarily analyzed within and among each three subpopulations in one population from Swabia and one population from Upper Bavaria. For molecular analysis, rosette leaves were collected *in situ* from fifteen individuals per population or subpopulation. In total, fresh leaf material of 517 individuals was sampled and dried in teabags over silica gel. Population size was obtained from the monitoring regularly conducted in the conservation projects and ranged from 6 up to 102500 individuals (Table 2.1).

Molecular analysis

Genetic variation was assessed using genome-wide genotyping with AFLPs, amplified fragment length polymorphisms (Vos, Hogers et al. 1995). DNA was isolated from silica gel dried plant material applying the cetyltrimethylammonium bromide method by Rogers and Bendich (1994) in an adaption by Reisch (2007). Concentration of genomic DNA was measured with a spectrophotometer and every sample was diluted with water to a concentration of 7.8 ng/ µL. The AFLP procedure was conducted in accordance with the protocol from Beckman Coulter as described before (Bylebyl, Poschlod et al. 2008, Reisch 2008).

Double strand DNA adapters were produced by adding equal volumes of both single strands of *Eco*RI and *Mse*I adaptors (Biomers) in a 0.2 ml reaction vessel, heating for five minutes at 95 °C with a final 10-minute step at 25 °C.

Digestion of 6.4 µL of genomic DNA (7.8 ng/ µL) and ligation of DNA adaptors were performed by adding 3.6 µL of a core mix consisting of 2.5 U *Eco*RI (Thermo scientific), 2.5 U *Mse*I (Thermo scientific), 0.1 µM *Eco*RI and 1 µM *Mse*I adapter pair, 0.5 U T4 DNA Ligase with its corresponding buffer (Thermo scientific), 0.05 M NaCl and 0.5 µg BSA (BioLabs/NBA) and a following incubation for 2 hours at 37 °C and a subsequent enzyme denaturation step at 70 °C for 15 minutes. The products were diluted 10 fold with 1:10 TE buffer (20 mM Tris-HCl, pH 8.0; 0.1 mM EDTA, pH 8.0).

In the preselective amplification a reaction volume of 5 µL containing the diluted DNA restriction-ligation product, preselective *Eco*RI and *Mse*I primers (Biomers) with a single selective nucleotide (*Mse*I-C and *Eco*RI-A) and an AFLP core mix consisting of 1x Buffer S, 0.2 mM dNTPs and 1.25 U Taq-Polymerase (PeqLab) were amplified under the chosen parameters: 2 minutes at 94 °C; 30 cycles of 20 seconds denaturation at

94 °C followed by 30 seconds annealing at 56 °C and 2 minutes elongation at 72 °C; finally 2 minutes at 72 °C ended the elongation period; 30 minutes at 60 °C and a cool down to 4 °C completed the PCR run. After this the products were diluted 20 fold with 1:10 TE buffer for DNA.

For selective amplification primers with three selective nucleotides were used. *Eco*RI primers were labeled with three different fluorescent dyes for fragment detection (Beckman dye D2, D3 and D4). After an extensive primer screening with eight randomly selected individuals six primer combinations were chosen for further analysis: *Mse*I-CTC/*Eco*RI-AGC and *Mse*I-CAC/*Eco*RI-AAC (D2), *Mse*I-CAA/*Eco*RI-AAG and *Mse*I-CAG/*Eco*RI-AAG (D3), *Mse*I-CTG/*Eco*RI-ACT and *Mse*I-CTA/*Eco*RI-ACA (D4).

Selective amplifications were performed in a reaction volume of 5 µL containing an AFLP Core Mix (1x Buffer S, 0.2 mM dNTPs, 1.25 U Taq-Polymerase (PeqLab), 0.05 µM selective *Eco*RI (Biomers), 0.25 µM *Mse*I (Biomers) primers and 0.75 µL diluted preselective amplification product. The PCR run started with 2 minutes at 94 °C; than 10 cycles of 20 seconds denaturation at 94 °C, 30 seconds annealing at 66 °C (temperature was reduced every subsequent step by 1 °C) and 2 minutes' elongation at 72 °C; than additional 25 cycles of 20 seconds denaturation at 94 °C, 30 seconds annealing at 56 °C and 2 minutes' elongation at 72 °C, completed by a following 30 min step at 60 °C and a cool down to 4 °C.

Selective PCR products were diluted with 5 µL (D2) and with 20 µL (D4) 1xTE buffer for DNA.

Then 5 µL amplified selective PCR product (of each D2, D3 and D4) were added to a stop solution, consisting of 2 µL sodium acetate (3 M, pH 5.2), 2 µL Na_2EDTA (100 mM, pH 8) and 1 µL glycogen (Roche). Participation of DNA took place by adding 60 µL of ice cold ethanol (96 %; -20 °C), an immediate shaking and subsequent centrifugation for 20 minutes at 14000 g at 4 °C. The pelleted DNA was washed once by adding 200 µL of ice cold ethanol (70 %; -20 °C) and again centrifugation for 20 minutes at 14000 g at 4 °C. Afterwards the pelleted DNA was vacuum dried in a vacuum concentrator (Eppendorf) and dissolved in a mixture of 24.8 µL Sample Loading Solution (SLS, Beckman Coulter) and 0.2 µL DNA Size Standard 400 (Beckman Coulter).

According to fragment size, the fluorescence labeled selective PCR products were separated by capillary gel electrophoresis on an automated sequencer (GenomeLab GeXP, Beckmann Coulter) and results were examined with DNA Size Standard 400 using the GeXP software (Beckman Coulter). For further investigations, results were exported as synthetic gel files (.crv) and the fragment pattern of every single individual was analyzed using the software Bionumerics 4.6 (Applied Maths, Kortrijk, Belgium): Each strong and clearly defined fragment was taken into account as either present or absent.

Samples with no clear banding pattern were repeated. Only three samples of *C. bavarica* had to be excluded from the analysis, due to amplification problems.

For quality control of the AFLP procedure, 10 % of all analyzed samples were replicated twice and a genotyping error rate was calculated, according to Bonin, Bellemain et al. (2004), which was 3.2 %.

Statistical analysis

Employing the software Bionumerics 4.6 a binary (0/1) matrix was created for statistical analysis. If present, fragments of a given length were detected as 1 and in the case of absence as 0. Using the matrix, genetic diversity within each population and subpopulation was calculated as the percentage of polymorphic bands (PB), as Shannon's Information Index $SI=\Sigma(p_i)\ln(p_i)$, and Nei's Gene Diversity $H=1-\Sigma(p_i)^2$, where p_i represents the allele frequency, by using the software PopGene 32 (Yeh, Yang et al. 1997). A Man-Whitney-U-test was used to test for significant differences in genetic diversity between regions applying the software IBM Statistics 22 for Windows (IBM Corp). Spearman's rank correlation coefficient was calculated to test the impact of population size on genetic diversity.

Hierarchical analysis of molecular variance, AMOVA (Excoffier, Smouse et al. 1992), were conducted with the software GenAlEx 6.41 (Peakall and Smouse 2006). Thus genetic differentiation within and among subpopulations, populations and between regions was investigated in two- and three-level AMOVAs.

Correlation between genetic distances (Φ_{PT} values calculated in the AMOVA) and geographic distances among populations was tested in a Mantel test with 999 permutations (Mantel 1967).

Genetic distances among populations were calculated as Nei's distance (Ds) following Lynch and Milligan (1994) with non-uniform prior distribution of allele frequencies in the program AFLPsurv (Vekemans 2002). Based on these Ds distances a consensus Neighbor-Net graph was calculated applying the software SplitsTree 4.14.4 (Huson and Bryant 2006). Additionally, distance matrices generated by bootstrapping (1000 bootstrap replicates were performed) were written in AFLPsurv, too. The files were used as input for the NEIGHBOR and CONSENSE procedures from the PHYLIP software package version 3.695 (Felsenstein 1993) to obtain bootstrap support values. Bootstrap values higher than 70 % were plotted in the Neighbor-Net graph.

Genetic relatedness of individuals was analyzed in the software MVSP version 3.12f (Kovach 2007) using principal coordinate analysis (PCoA) based on inter-individual Bray-Curtis similarities.

Moreover, a Bayesian cluster analysis was calculated with the program Structure version 2.3.4 (Pritchard, Stephens et al. 2000, Pritchard, Wen et al. 2007) to infer population structure in the data set and assign individuals into groups. It is assumed that the data set consist of an unknown number of K groups. Every single group is characterized by a set of allele frequencies at each locus and samples from the data set are assigned randomly to groups. The number of groups was calculated using 10000 Markov Chain Monte Carlo (MCMC) simulations with a burn-in-period of 100000 iterations. Analysis for the predefined value of K were run 20 times per K = 1-40 (Falush, Stephens et al. 2003, Falush, Stephens et al. 2007). The program Structure Harvester (Earl and Vonholdt 2012) was used to summarize results. Group assignment was an ad hoc quantity procedure calculating ΔK (Evanno, Regnaut et al. 2005). The best estimate of K for the data set was defined according to the model which gave the consistent results for multiple runs and the highest probability of the data.

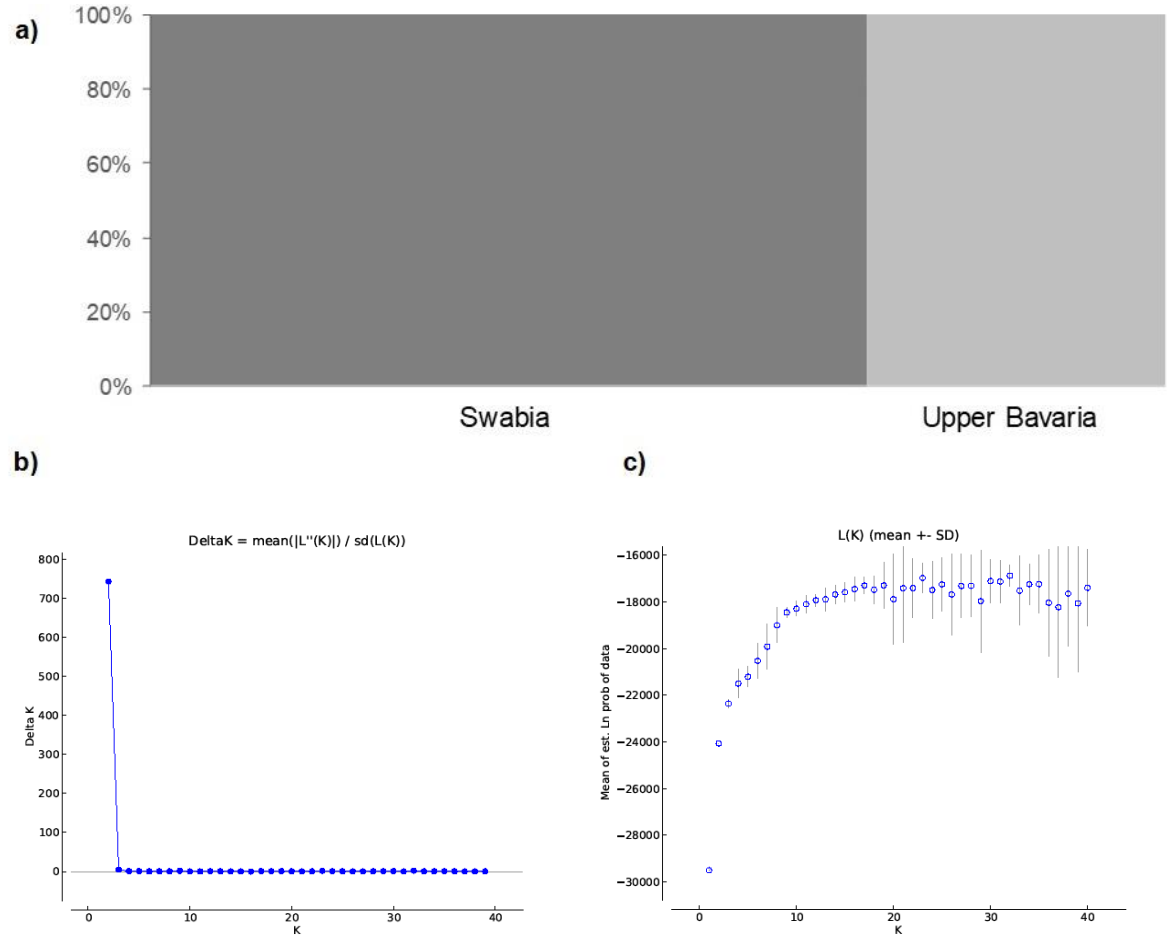


Figure 2.2: Results of the Bayesian Cluster Analysis. Populations were assigned to two groups according to the geographic regions Swabia and Upper Bavaria (**2a**). Results of 20 runs for 1-40 possible groups to infer population structure with Bayesian clustering in STRUCTURE are shown in graph **2b**. Delta K are shown for each of the tested groups K = 1-40. Graph **2c** shows Ln P(D) variance for each of the tested groups.

RESULTS

AFLP banding and genetic diversity

AFLP analysis resulted in 198 fragments. No identical genotypes were detected. Furthermore, there were 4 bands private to the populations from Swabia and eleven bands were found only in populations from Upper Bavaria. 75.76 % of the fragments were polymorphic.

In populations from Swabia, Nei's Gene Diversity (GD) ranged from 0.07 to 0.13 (mean 0.11), Shannon's Information Index (SI) from 0.11 to 0.19 (mean 0.16) and the percentage of polymorphic bands (PB) from 20.71 to 33.34 (mean 28.18). The highest

level of diversity was found in population Ollarzried-Boschach, the lowest in population Katzbrui-Mühle (Table 2.1).

Similar results were found in populations from Upper Bavaria (Table 2.1). GD ranged from 0.08 to 0.12 (mean 0.10) and SI from 0.11 to 0.17 (mean 0.15). The percentage of polymorphic bands varied between 23.23 and 31.31 (mean 28.47). The highest level of diversity was found in population Lungham, the lowest in population Laubensee.

Populations from Swabia and Upper Bavaria did not differ significantly in genetic diversity and the estimated population size was not correlated with genetic diversity (Spearman correlation coefficient: $r_{GD} = -0.22$, $p_{GD} = 0.91$).

Genetic differentiation

In the Bayesian cluster analysis individuals were assigned to two groups ($\Delta K = 743.8$) reflecting the regions Swabia and Upper Bavaria. For K = 2 outputs of all 20 iterations were identical (Figure 2.2 a-c).

In the Neighbor-Net analysis the studied populations were also assigned to these regions (Figure 2.3). Within Swabia, populations formed three groups: one comprised populations from the locations Hatzleberg (HA), Immenthal (MM), Grub-Eheim (GE), Katzbrui (KB), Klessen (KL) and Ollarzried (OL); the second consisted of populations from Liebenthann (LT), Algers (AL) and Gfäll (GF). Populations from the locations Gennachquelle (GN), Gillenmoos (GM), Kemnath (KE), Kaltenthal (KA), Aufkirch (AU), Seebach (SE) and Mindeltal (MT) formed the third group. In Upper Bavaria, the populations Lungham (LU), Thalham (TH) and Laubensee (LA) were clearly separated from a second group, which comprised the populations from Glonnquellen (GL), Kupferbachtal (KU) and Vagen (VA).

The PCoA results were similar to the results from the Bayesian cluster analysis and the Neighbor-Net analysis and also revealed a strong separation of individuals from Swabia and Upper Bavaria (Figure 2.4). At the subpopulation level individuals from different subpopulations were mostly admixed in the two studied populations from Swabia (Figure 2.5) and Upper Bavaria (Figure 2.6). Only subpopulation Ollarzried Höhe 3 exhibited a slightly stronger level of differentiation.

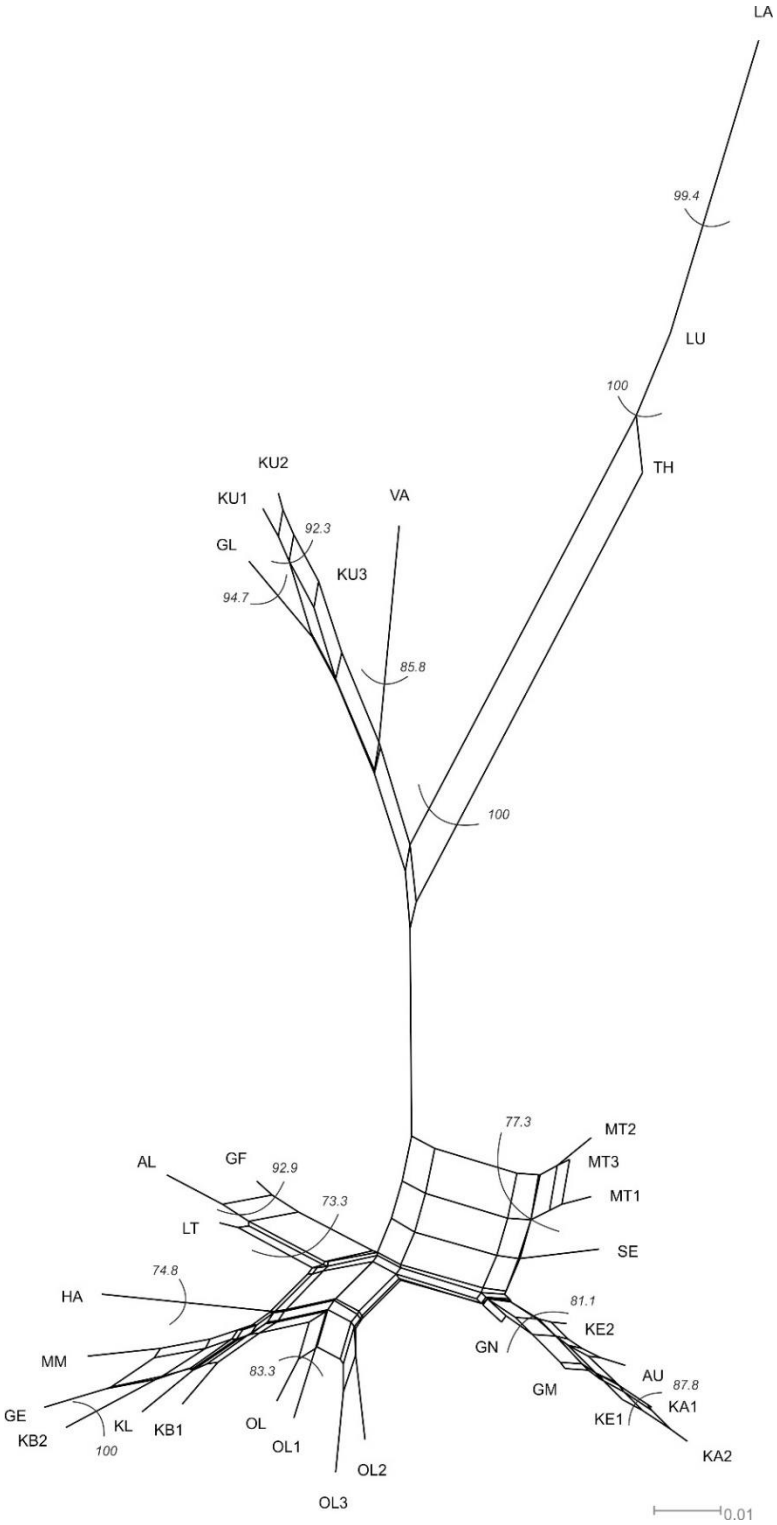


Figure 2.3: Consensus Neighbor-Net of all *C. bavarica* populations based on the AFLP data. Populations from Swabia and Upper Bavaria were clearly separated. Bootstrap values > 70 % are given in italics. The Fit value is 93.47.

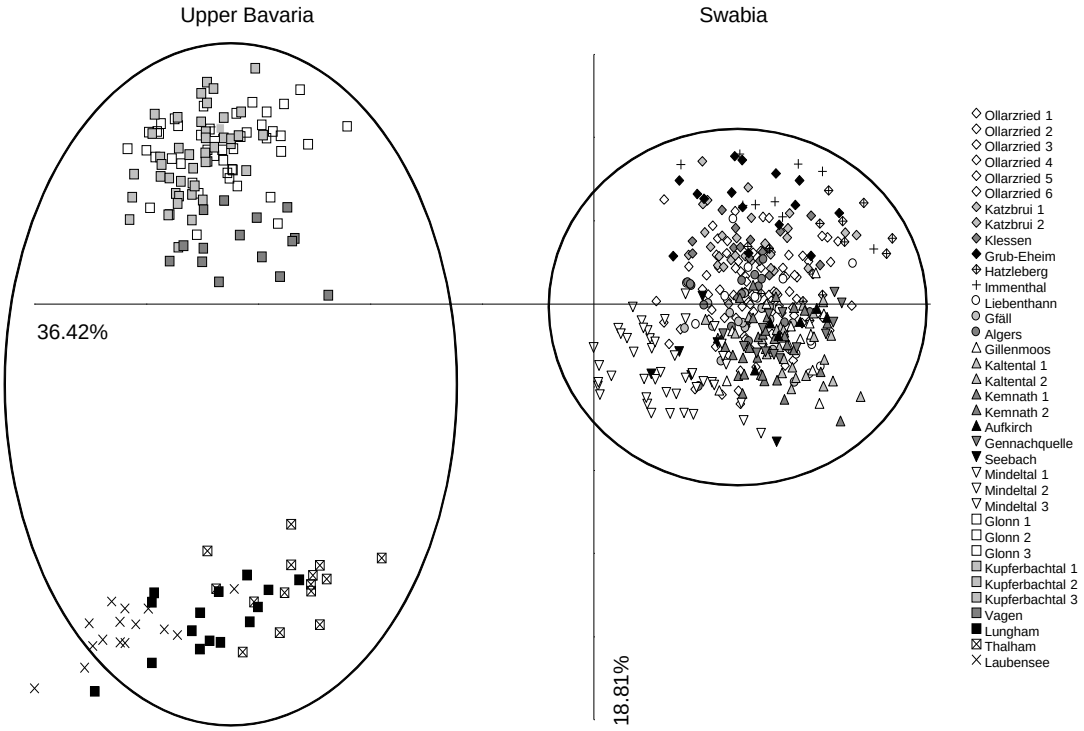


Figure 2.4: Principal coordinates analysis (PCoA) of all sampled individuals of *C. bavarica* from Swabia and Upper Bavaria based on AFLP data. Axis 1 explains 36.34 % of variance; axis 2 explains 18.81 % of variance. Populations from Swabia and Upper Bavaria were clearly separated and formed two groups.

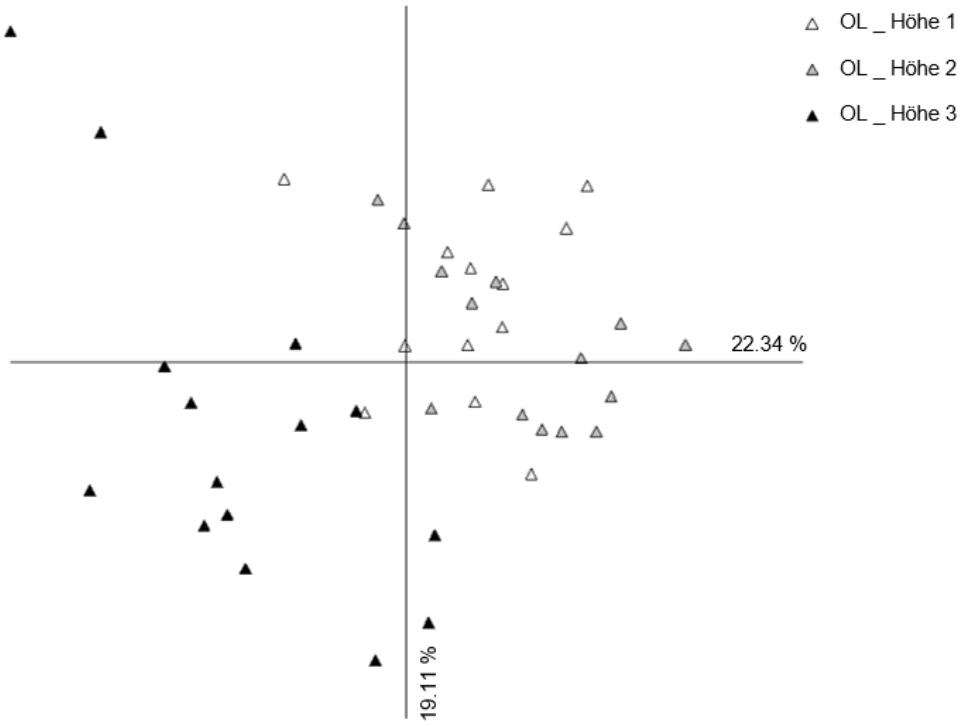


Figure 2.5: Principal coordinates analysis (PCoA) of sampled individuals of *C. bavarica* from Swabia based on AFLP data. Axis 1 explains 22.34 % of variance; axis 2 explains 19.11 % of variance. No geographical pattern could be observed.

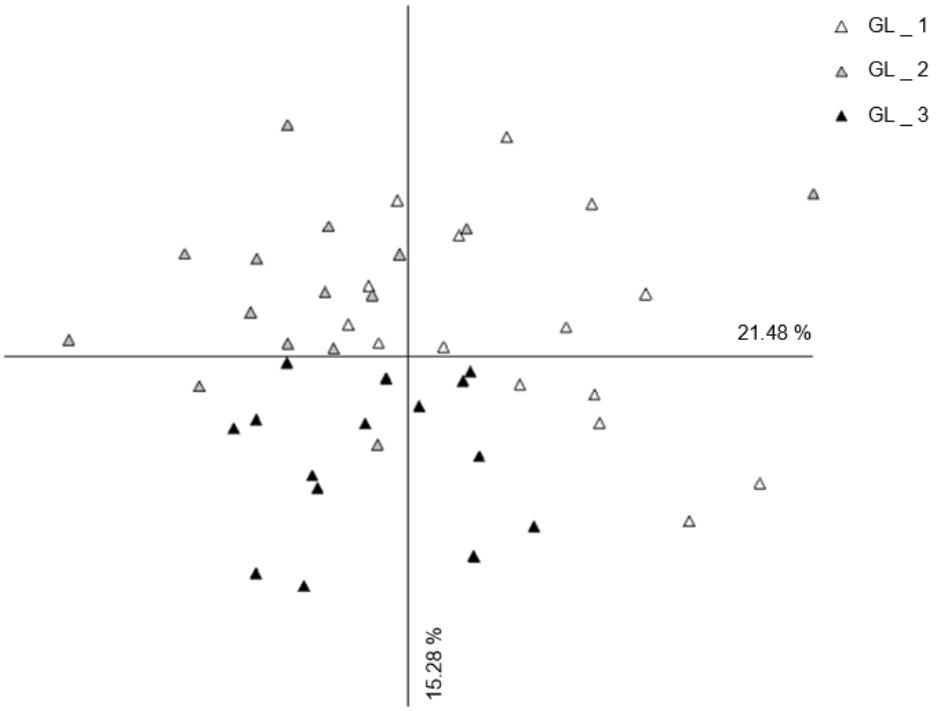


Figure 2.6: Principal coordinates analysis (PCoA) of sampled individuals of *C. bavarica* from Upper Bavaria based on AFLP data. Axis 1 explains 21.48 % of variance; axis 2 explains 15.28 % of variance. No population grouping could be detected.

In the three level analysis of molecular variance (AMOVA) we also observed a very strong genetic differentiation between the two study regions Swabia and Upper Bavaria with a Φ_{PT} value of 0.62 (Table 2.2). Within these regions genetic differentiation among populations was also strong but weaker among populations from Swabia (Φ_{PT} = 0.38) than among populations from Upper Bavaria (Φ_{PT} = 0.51). Further analysis revealed only a low level of genetic differentiation among subpopulations in Swabia (Φ_{PT} = 0.13) and Upper Bavaria (Φ_{PT} = 0.12).

A Mantel test including all populations revealed significant correlation between pair-wise genetic distances and geographic distances (r = 0.80, p = 0.001). Additional Mantel tests have been implemented for each distribution area (Figure 2.7). In Swabia we found only a weak but significant correlation of genetic distance with spatial distance (r = 0.18, p = 0.02). In contrast, this correlation was very strong for populations in Upper Bavaria (r = 0.92, p = 0.001).

Table 2.2: Molecular variance within and among populations of *Cochlearia bavarica* calculated in different analysis of molecular variance (AMOVA) based on 198 AFLP fragments. Levels of significance are based on 999 iteration steps and are indicated by three asterisks ($p < 0.001$). Df indicates degree of freedom, SS the sum of squares, MS the mean squares, % the proportion of genetic variability.

	df	SS	MS	%	ΦPT	
Molecular variation between regions						
Among Regions	1	1515.19	1515.19	34.02	0.62	***
Among Populations	30	3011.66	100.39	27.49		
Within Populations	422	3813.43	9.04	38.49		
Molecular variation among populations within regions						
Swabia						
Among Populations	23	1949.35	84.75	37.57	0.38	***
Within Populations	310	2807.03	9.055	62.43		
Upper Bavaria						
Among Populations	7	1062.31	151.76	51.44	0.51	***
Within Populations	112	1006.4	8.99	48.56		
Molecular variation among subpopulations within populations						
Swabia - Ollarzried-Höhe						
Among Subpopulations	2	60.71	30.36	12.56	0.13	***
Within Subpopulations	42	404.13	9.62	87.44		
Upper Bavaria - Gollquellen						
Among Subpopulations	2	52.89	26.44	12.1	0.12	***
Within Subpopulations	42	362.4	8.63	87.9		

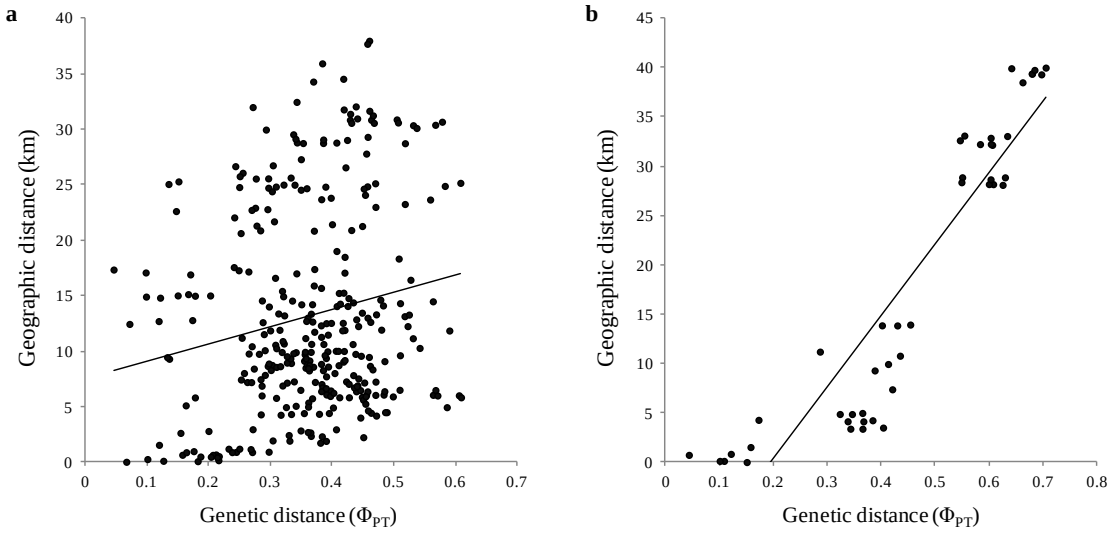


Figure 2.7: Correlation of genetic distance (Φ_{PT}) and geographic distance (km) between populations and subpopulations (Mantel test) for the populations in Swabia (a, $r = 0.18$, $p = 0.02$) and the populations in Upper Bavaria (b, $r = 0.92$, $p = 0.001$) of *C. bavarica.*

DISCUSSION

Genetic diversity

In our study genetic diversity within the analyzed populations of *C. bavarica* was low but within the range observed for species with similar traits (Hamrick and Godt 1996, Nybom and Bartish 2000, Nybom 2004). Nei's Gene diversity of *C. bavarica* was on average 0.10 and, therefore, even slightly lower than previously reported for other rare species (0.12) in a literature survey based on more than 150 plant species (Reisch and Bernhardt-Römermann 2014).

Isolation of predominantly small populations is the most important reason for reduced levels of genetic diversity in populations of rare plant species. Indeed, populations of *C. bavarica* are strongly isolated (Fischer, Hock et al. 2003), with large geographic distances in between. Moreover, many populations are surrounded by dense forests, which enhances isolation since the forests represent effective barriers for pollinators (Paschke, Abs et al. 2002b). Although the species is considered as self-incompatible Fischer et al. (2003) found a certain degree of self-compatibility. With increasing distance between populations gene flow decreases, which means that self-pollination and mating events between related individuals may become more frequent and decrease the level of genetic diversity.

In the last three decades many populations of *C. bavarica* disappeared due to habitat degradation and nutrient enrichment, which resulted in a proceeding fragmentation. This process of habitat fragmentation is a general threat to biodiversity, reducing species richness within small and isolated habitat patches (Fahrig 2003). However, fragmentation also affects genetic diversity since population size decreases and gene flow among small and isolated remnant populations is strongly reduced (Vitousek 1994). The exchange of pollen and seeds between populations is restricted (Honnay, Coart et al. 2006) and consequently genetic diversity within populations is declining. This process of genetic erosion (Oostermeijer 1996, Young, Boyle et al. 1996, Luijten, Dierick et al. 2000) reduces in the long-term the adaptability to changing environmental conditions (Heywood 1991, Booy, Hendriks et al. 2000) and may even cause extinction (Frankham 2005).

Even though the level of fragmentation and isolation is stronger in Upper Bavaria than in Swabia we observed in our study no significant differences in genetic diversity between populations from the two study regions. This is most likely due to the fact that although populations are more frequent in Swabia than in Upper Bavaria the populations are nevertheless strongly isolated. Differences in frequency seem to be too small to result in different levels of genetic diversity.

The positive relationship between population size and genetic diversity has been reported in numerous studies (Hamrick and Godt 1990, Frankham 1996, Godt, Johnson et al. 1996, Fischer and Matthies 1998, Leimu, Mutikainen et al. 2006). However, we observed no significant positive correlation between these two parameters. Previous investigations revealed higher levels of allozyme variation (Paschke, Abs et al. 2002b) in larger than in smaller populations of *C. bavarica*. However, this study was based on data collected 15 years ago, and the populations of *C. bavarica* further declined since then. This may be the reason why our results differ from the previous study on allozyme variation. Indeed, many investigations revealed no correlation between population size and genetic variation mainly due to lag effects or long term survival under highly fragmented conditions (Honnay and Jacquemyn 2007, Kuss, Pluess et al. 2008).

Genetic differentiation

With a Φ_{PT} of 0.62 our study revealed a high level of genetic differentiation between populations of *C. bavarica*. The level of differentiation is much higher than previously reported (Reisch and Bernhardt-Römermann 2014) for other rare species (Φ_{PT} of 0.34), and reflects the strong fragmentation and isolation of *C. bavarica*. Generally, genetic differentiation between populations depends on the interplay of gene flow and drift (Slatkin 1987). Under highly fragmented and isolated conditions gene flow decreases while genetic differentiation due to drift increases (Vitousek 1994). In the case of *C. bavarica*, this process may be enhanced by potential self-pollination further increasing genetic differentiation (Reisch and Bernhardt-Römermann 2014).

However, the level of genetic differentiation varied in our study strongly between different spatial scales. Considering the whole distribution range, we found a strong differentiation between the two regions Swabia and Upper Bavaria. This observation is supported by previous studies revealing a number of alleles being characteristic for

either Swabian or Upper Bavarian populations (Koch, Huthmann et al. 1998, Paschke, Abs et al. 2002b). Within both regions we observed a significant correlation of genetic and geographic distances between populations in the Mantel test. However, the correlation was weaker in Swabia than in Upper Bavaria. In Swabia geographically adjacent populations were not necessarily genetically more similar to each other than geographically more distant populations as shown for the population Seebach and the populations from Mindeltal or the populations Immenthal, Katzbrui, Klessen and Grub-Eheim. In contrast, we observed a clear pattern of isolation by distance in Upper Bavaria. Furthermore, genetic differentiation between populations was lower in Swabia than in Upper Bavaria. This corresponds to the results of the Mantel test and can be ascribed to the fact that populations are and may also have previously been more frequent in Swabia than in Upper Bavaria. Historical gene flow may, therefore, have been stronger among the more frequent populations in Swabia, and more limited among the populations from Upper Bavaria. Referring to the genetic structure within populations we observed only limited differentiation between subpopulations, which were less than 200 m distant to each other. The analysis of molecular variance revealed only low levels of genetic differentiation and the cluster analysis indicated the admixture of individuals. Obviously, pollination seems to be hardly limited at this distance, which is supported by previous studies providing evidence that gene flow by pollen is normally restricted to the nearest vicinity of plant populations to distances of less than one kilometer (Kwak, Velterop et al. 1998, Aavik, Holderegger et al. 2014).

Conclusions with respect to conservation

The aim of this study was to increase the success of future population reintroduction and reinforcement, by providing data to avoid negative effects of inbreeding and outbreeding and to preserve the natural genetic pattern of the species. However, it should be kept in mind that every reintroduction project with its species is unique (Guerrant and Kaye 2007) and that generalizations are, therefore, limited. Nevertheless, it is possible to draw conclusions for a successful reintroduction of *C. bavarica* from our study.

It has been demonstrated that reintroduction success can be enhanced by using plant material from large and stable source populations (Godefroid, Piazza et al. 2011). In

the case of *C. bavarica*, large populations were not necessarily genetically most variable. Since bottlenecks, inbreeding and drift can be avoided best by taking plant material from populations with a high level of genetic diversity we suggest, therefore, to use rather highly variable than large source populations for the reintroduction or the reinforcement of *C. bavarica* such as the population Ollarzried-Boschach in Swabia or the population Lungham in Upper Bavaria. Within these populations plant material should be collected where possible from 50 up to 200 individuals of different age and size classes (Lauterbach 2013) all over the population to sample genetic diversity representatively (Brown and Briggs 1991). Moreover, reintroduction success can be improved by acting at a large scale (Frankham, Ballou et al. 2002). In previous studies 500 up to 5000 individuals have proven as a suitable number of individuals for successful reintroduction (Given 1994, Pavlik 1996, Reed 2005). We strongly recommend, therefore, using a large number of individuals for the planned reintroduction of the species.

Although mixing plant material from multiple source populations has been successfully used for reintroduction (Guerrant and Kaye 2007, Godefroid, Piazza et al. 2011, Maschinski, Wright et al. 2013), because using large numbers of unrelated individuals contributes to a large and diverse gene pool (Vergeer, van den Berg et al. 2005), this approach should be handled with care due the risk of outbreeding depression, which reduces fitness and performance (Fischer and Matthies 1998, Keller, Kollmann et al. 2000, Montalvo and Ellstrand 2000, Montalvo and Ellstrand 2001, Krauss, Zawko et al. 2005, Bischoff, Cremieux et al. 2006, Mijnsbrugge, Bischoff et al. 2010). Furthermore, mixing material from different source populations should be avoided if the spatial genetic pattern of a species should be preserved (Gordon 1994). *C. bavarica* exhibited a very distinct geographic pattern of genetic variation and we would, therefore, strongly advise against using multiple source populations for reintroductions and population reinforcement. Instead, we suggest a graduated procedure for the reintroduction of the species, considering the observed pattern of genetic variation. Since our study revealed a very strong level of genetic differentiation between Swabia and Upper Bavaria the exchange of plant material between these two study regions should be completely avoided. Within these regions we detected different patterns of genetic variation. Whereas we found a clear pattern of isolation by distance in Upper Bavaria, the situa-

tion was more idiosyncratic in Swabia. Most likely due to historic gene flow, geographically adjacent populations were not necessarily genetically similar to each other. We suggest, therefore, different approaches for the two regions. In Upper Bavaria for reintroduction plant material should preferably be used from closely located and, therefore, genetically most similar populations to avoid outbreeding. In Swabia two different approaches are conceivable: if conservationists decide to preserve the current pattern of genetic variation plant material for reintroduction should be taken from the genetically most similar population, if they decide that the present pattern should not be kept since it resulted from former gene flow anyway plant material should be used from the most variable source population. At the subpopulation level we detected only a low level of differentiation with a high degree of admixture between subpopulations due to gene flow. The transfer of plant material between subpopulations should, therefore, be possible without changing the natural genetic pattern of the species and without the risk of outbreeding.

It has already been demonstrated that a specific management of the reintroduction sites increases the reintroduction success. Moreover, a reliable and continuous monitoring allows the evaluation of population reintroduction success (Godefroid, Piazza et al. 2011). Therefore, we strongly recommend a continuous long-term monitoring of the reintroduced *C. bavarica* individuals and a thorough management of the reintroduction sites.

CHAPTER THREE

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 SOUTH-EASTERN GERMANY

FRANZISKA KAULFUß AND CHRISTOPH REISCH

ABSTRACT

Restoration of species-rich grasslands is a key issue of conservation. The transfer of seed-containing local plant material is a proven technique to restore species-rich grassland, since it potentially allows to establish genetically variable and locally adapted populations. In our study we tested how the transfer of local plant material affected the species diversity and composition of restored grasslands and the genetic variation of the typical grassland plant species *Knautia arvensis* and *Plantago lanceolata*.

For our study we selected fifteen study sites in south-eastern Germany. We analyzed species diversity and composition and used molecular markers to investigate genetic variation within and among populations of the study species from grasslands that served as source sites for restoration and grasslands which were restored by transfer of green hay and threshed local plant material.

The results revealed no significant differences in species diversity and composition between grasslands at source and restoration sites. Levels of genetic variation within populations of the study species *Knautia arvensis* and *Plantago lanceolata* were comparable at source and restoration sites and genetic variation among populations at source and their corresponding restoration sites were only marginal different.

Our study suggests that the transfer of local plant material is a restoration approach highly suited to preserve the composition of species-rich grasslands and the natural genetic pattern of typical grassland plant species.

KEYWORDS: Genetic variation, hay, local plant material, molecular marker, restoration, species-rich grassland, threshed plant material, transfer

INTRODUCTION

Species-rich and extensively managed grasslands declined drastically in central Europe during the recent decades (Poschlod 2017). Land use intensification and abandonment caused an ongoing loss of species diversity since the mid-twentieth century (Hejcman, Hejcmanová et al. 2013). On the one hand, higher productivity and mowing frequencies due to increased fertilizer application decreased species richness of grasslands (Jacquemyn, Brys et al. 2003, Zechmeister, Schmitzberger et al. 2003, Socher, Prati et al. 2012). Moreover, atmospheric nitrogen deposition caused a general loss of species richness and shifts in species composition of European grasslands (Wesche, Krause et al. 2012, Diekmann, Jandt et al. 2014). On the other hand the dominance of grasses (Zulka, Abensberg-Traun et al. 2014) and litter accumulation (Jensen and Gutekunst 2003, Ruprecht and Szabó 2012, Piqueray, Ferroni et al. 2015) due to land use abandonment reduced species diversity in grasslands. Consequently, nearly three-quarter of all grassland plant communities are highly endangered today (Rennwald 2000). The restoration of species-rich grasslands is, therefore, a key issue of conservation.

Principally, species-rich grasslands may be restored by improving habitat conditions, e.g. via the reestablishment of traditional management regimes, rewetting or the removal of nutrients from the soil (Bakker 1989, Pfadenhauer and Grootjans 1999). Increasing species richness by these restoration approaches is, however, often limited due to the lack of viable seeds in the soil or the surrounding habitats (Bakker, Poschlod et al. 1996, Bossuyt and Honnay 2008). After decades of intensive grassland management soil seed banks are usually depleted (Bakker, Poschlod et al. 1996, Bissels, Donath et al. 2005) and the immigration of plants from surrounding grasslands is often complicated by landscape fragmentation and the lack of dispersal vectors (Hölzel, Buisson et al. 2012). Creating species-rich grasslands requires, therefore, the introduction of seed material from other sources than the restoration site.

The problem of seed limitation in grassland restoration can be solved in different ways. One possibility is using commercially produced seed mixtures for restoration, which has become a common and comparatively simple approach in recent years (Jongepierová, Mitchley et al. 2007, Török, Deák et al. 2010, Walker, Hermann et al.

2015), since seed mixtures are easily available from a number of different seed producers. Another possibility is the restoration of species-rich grassland by the introduction of local plant material from source sites via transfer of seed-containing chaff, threshed plant material or green hay (non-dried fresh plant material) (Kiehl, Kirmer et al. 2010). These approaches are more traditional methods which have been applied for centuries and represent proven techniques to create new grasslands (Kiehl and Wagner 2006, Coiffait-Gombault, Buisson et al. 2011, Albert, Mudrák et al. 2019).

The transfer of local plant material allows one to move the species richness of a whole plant community from a source site to a potential restoration site and at the same time to establish genetically variable populations that are locally adapted to specific regions (van der Mijnsbrugge, Bischoff et al. 2010).

The use of local seed material is generally recommended in restoration (van der Mijnsbrugge, Bischoff et al. 2010), since plant populations are adapted to local environmental conditions (McKay, Christian et al. 2005). Mixing genetically differing genotypes from geographically different regions may cause a loss of locally adapted genotypes and result in outbreeding depression (Hufford and Mazer 2003). Co-adapted gene complexes can be destroyed and local adaptations get lost which decreases fitness and performance of plant populations (Keller, Kollmann et al. 2000, Montalvo and Ellstrand 2001, Frankham, Ballou et al. 2002). Seed material used for restoration should match the gene pool of the populations occurring in the vicinity of the restoration site (McKay, Christian et al. 2005) and the transfer of locally harvested plant material is, therefore, considered as the “gold standard” to preserve patterns of genetic variation (Dittberner, Becker et al. 2019).

Worldwide, seed production and seed transfer zones have been defined for the commercial production of local seed mixtures used in ecological restoration to avoid the negative effects of mixing local and non-local genotypes (Miller, Bartow et al. 2011, Krauss, Sinclair et al. 2013). In the recent years, genetic differentiation among populations from different seed transfer zones (Bucharova, Michalski et al. 2017, Listl, Poschlod et al. 2017) and the impact of sowing local seeds on the genetic variation of grassland species have been studied intensively (Aavik, Edwards et al. 2012, Reiker, Schulz et al. 2015, Kaulfuß and Reisch 2019). The impact of transferring local plant material on patterns of genetic variation has, however, been hardly analyzed (Dittberner, Becker et al. 2019, Van Rossum, Hardy et al. 2020).

Generally, the restoration process has a strong impact on genetic variation (Mijangos, Pacioni et al. 2015). Previous studies comparing source populations and restored populations of different species often revealed decreased levels of genetic variation in restored populations (Aavik, Edwards et al. 2012, Vandepitte, Gristina et al. 2012), although this was not always the case (Dittberner, Becker et al. 2019, Kaulfuß and Reisch 2019). The observed loss of genetic variation within populations may be caused by bottlenecks occurring during seed harvesting and seed production or by founder effects during recolonization or due to the origin of seeds (Mijangos, Pacioni et al. 2015). Such bottlenecks or founder effects may also occur during grassland restoration by the transfer of seed-containing local plant material. In particular the collection of the material at the source site and the establishment of plants from the seeds at the restoration site are critical steps (Kiehl, Kirmer et al. 2010), potentially limiting the size of the restored populations and consequently also the genetic variation within these populations. Furthermore, the potentially reduced number of transferred individuals and the geographic distance between the selected locations may cause genetic drift increasing variation among populations from source and restoration sites (Kaulfuß and Reisch 2019).

In this study we investigated the impact of grassland restoration by the transfer of green hay and threshed plant material in south eastern Germany on species diversity and composition of the restored grasslands and the genetic variation of the typical central European grassland species *Knautia arvensis* and *Plantago lanceolata*. More specifically we asked the following questions:

(1) Are grasslands at the source and restored sites comparable in species composition and diversity?

(2) Is the level of genetic variation within populations of the study species differing between source populations and restored populations?

(3) How high is genetic variation among source populations and restored populations of the study species?

(4) Is the transfer of green hay and threshed plant material an effective tool in conservation to restore species-rich and genetically diverse grasslands?

METHODS

Study area and sites

For our study, we selected grasslands at 15 study sites in south eastern Germany near Passau (Figure 3.1; Table 3.1). At four of these sites (S1-S4) local plant material was gathered from species-rich grasslands between 2005 and 2014. The plant material from these source sites was then used to establish grasslands on former arable fields at eleven restoration sites (R1.1-R4.1). The variation in the numbers of restoration sites to source sites is due to the fact that our study was part of a practical restoration project by the landscape conservation association of Passau. Seed-containing plant material was obtained by mowing the grasslands at the source sites in June and by threshing the grasslands with an automatic harvester in August. Green hay and threshed plant material were then transferred from S1 to R1.1-R1.2, from S2 to R2.1-R2.3, from S3 to R3.1-R3.5 and from S4 to R4.1.

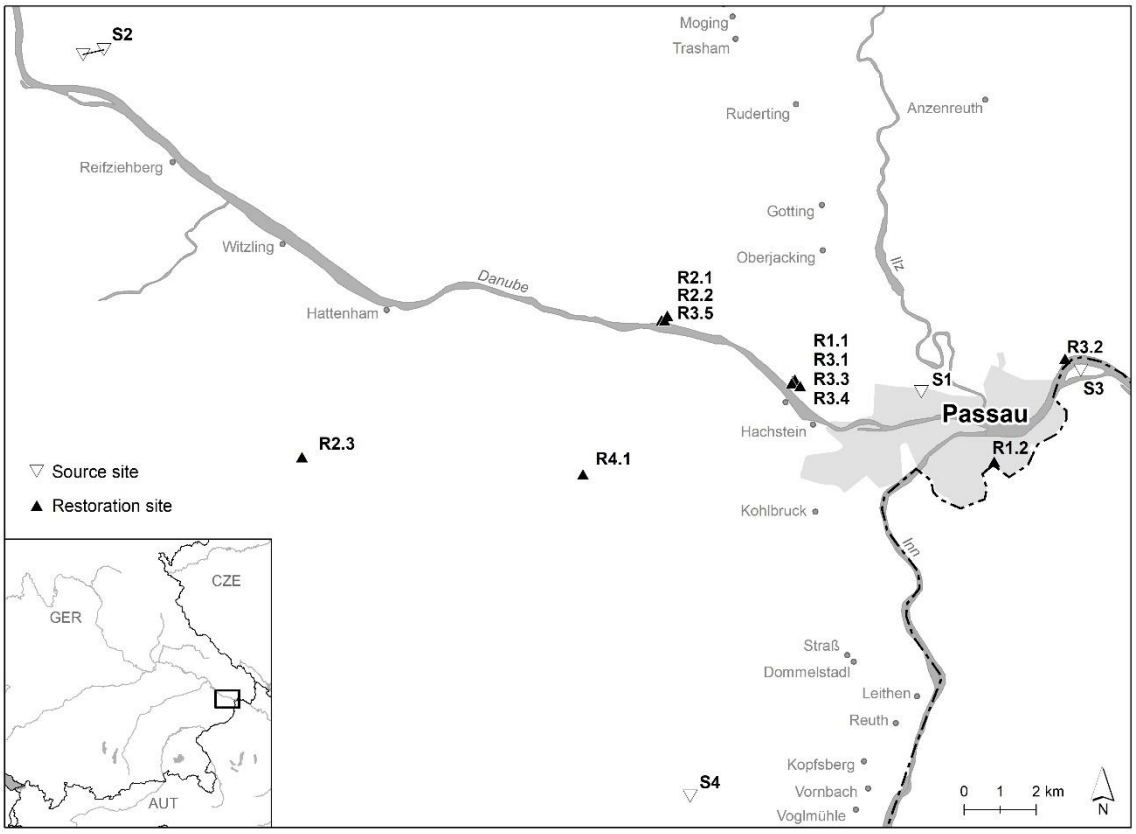


Figure 3.1: Geographic position of the study sites near Passau in south eastern Germany. Source sites are marked with upside down, white triangles. Restoration sites are indicated by black triangles. Restoration sites (R1.1-R4.1) are labeled so that the first number correspond to the source location (S1-4) and the second number corresponding to the replicate (R1.2 corresponds to the 2nd replicate of restoration site sourced from S1).

At the restoration sites topsoil was removed to reduce soil fertility and the number of seeds from the previous vegetation in the soil seed bank (Rasran, Vogt et al. 2007) before the local plant material was spread.

Species diversity and composition of grasslands

At each study site species diversity and composition of the selected grasslands were assessed. Therefore, all species occurring in the grasslands were identified and registered while walking across the study sites in the vegetation period 2016 (Heinz et al. 2012) .

Table 3.1: Study sites with number (No.), site type (S: source or R: restoration site), geographic position (Lat. and Lon.), area of study site (m²), species diversity (SD) and the year of restoration.

No.	Type	Lat.	Lon.	Area [m²]	SD	Year
S1	S	48.583152	13.452477	8000	61	-
S2	S	48.672036	13.146660	12400	86	-
S3	S	48.587754	13.512742	34600	82	-
S4	S	48.483265	13.362789	19500	55	-
Mean source sites					**71**	
R1.1	R	48.586844	13.404991	16000	59	2011
R1.2	R	48.565453	13.479381	11000	52	2008
R2.1	R	48.602194	13.356277	8000	75	2009
R2.2	R	48.603368	13.357393	8000	73	2009
R2.3	R	48.569620	13.218924	10200	71	2012
R3.1	R	48.586531	13.404060	6000	51	2008
R3.2	R	48.590790	13.506841	2400	77	2005
R3.3	R	48.586004	13.403802	3300	56	2012
R3.4	R	48.585256	13.406871	8100	58	2010
R3.5	R	48.602306	13.355209	4000	51	2008
R4.1	R	48.564128	13.324489	2500	38	2014
Mean restoration sites					**60**	

Study species and sampling of plant material

For genetic analysis we selected the two widespread and outcrossing grassland species *Knautia arvensis* (Coult) and *Plantago lanceolata* (L.). *K. arvensis* is a hemicryptophytic, perennial plant species, belonging to the Caprifoliaceae family (Oberdorfer 2001). Its distribution ranges from North-West Africa to Asia and Europe. The plant occurs in fertile meadows, semi-arid grasslands, waysides, forest edges and extensively used fields (Rothmaler 2005). The species forms a basal rosette and paired stem leaves are spear shaped. Growth height is between 25 and 100 cm. The species may be diploid (2n=20) or tetraploid (2n=40), as previously reported (Kaulfuß and Reisch 2019). In the study presented here all individuals were tetraploid, as detected by Flow Cytometry (Dolezel, Greilhuber et al. 2007). *K. arvensis* flowers between July and September and is insect pollinated by bees, bumblebees, butterflies, syrphid-flies and wasps (Oberdorfer 2001). *P. lanceolata* is a hemicryptophytic, perennial plant species, belonging to the Plantaginaceae family (Oberdorfer 2001). The species is widespread all over Europe from the Iberian peninsula to central Asia and occurs in fertile meadows, fields and on loamy or sandy soils (Rothmaler 2005). The species is a perennial rosette-forming herb with lanceolate spreading leaves. Flower stems are 10 to 40 cm high, leafless, hairy and have ovoid inflorescences with many small flowers (Oberdorfer 2001). *Plantago lanceolata* is diploid (2n=12) (Oberdorfer 2001). Consequently, in our study all individuals exhibited the same diploid ploidy level, as revealed by Flow Cytometry (Dolezel, Greilhuber et al. 2007). *P. lanceolata* is mainly wind-pollinated, but insect pollination by short-proboscic bees, flies, beetles and syrphid-flies is also possible (Oberdorfer 2001).

In early summer of 2016, young rosette leaves of the study species were sampled *in situ* from 16 individuals per population in grasslands at source sites and restored sites and dried in teabags over silica gel for further investigation. The number of sampled populations differed slightly between the study species since *K. arvensis* did not occur at all study sites. In total, we sampled plant material of *K. arvensis* from populations at three source sites and nine corresponding populations at the restoration sites. *P. lanceolata* occurred at all study sites and we collected, therefore, plant material from populations at four source sites and eleven corresponding populations at the restoration sites (Table 3.1). At all study sites the population size of *K. arvensis* and *P. lanceolata* was determined by counting the number of individuals in 10 randomly placed one-

square-meter-grids (Reisch, Schmid et al. 2018). The mean number of individuals per square meter was then multiplied with the area of the grassland to calculate population size (Table 3.2).

Molecular analysis

For DNA isolation, the cetyltrimethylammonium bromide (CTAB) protocol by Rogers and Bendich (1994) with adaptions by Reisch (2007) was applied. For every sample, first the concentration of genomic DNA was quantified with a microvolume spectrometer (NanoDrop One, Thermo Scientific) and afterwards a dilution with a standardized concentration of 7.8 ng/ µL was prepared. Genome-wide genotyping with amplified fragment length polymorphisms (Vos, Hogers et al. 1995) was used to assess genetic variation. AFLPs were performed, following the Beckman Coulter protocol as described before (Bylebyl, Poschlod et al. 2008). Primers for selective PCR were chosen, according to Kaulfuß & Reisch (2019). The primer combinations for *K. arvensis* were *Mse*I-CAG/*Eco*RI-ACC (D2), *Mse*I-CTT/*Eco*RI-AGG (D3), *Mse*I-CTT/*Eco*RI-ACT (D4). Primer combinations for *P. lanceolata* were *Mse*I-CTG/*Eco*RI-AGC (D2), *Mse*I-CAA/*Eco*RI-AGG (D3), *Mse*I-CAG/*Eco*RI-ACA (D4). *Eco*RI primers were labeled with fluorescent dyes for fragment detection (Beckman dye D2, D3 and D4). DNA fragments were separated by size with capillary gel electrophoresis performed on an automated sequencer (GeXP, Beckmann Coulter). The results were exported as .crv files. AFLP fragment patterns were evaluated using the software Bionumerics 4.6 (Applied Maths, Kortrijk, Belgium). Each strong and clearly defined DNA fragment was classified as present (1) or absent (0) to create a binary (0/1) matrix, which was the basis for further statistical analysis. We repeated about 10% of the samples and calculated a genotyping error rate (Bonin, Bellemain et al. 2004), which was 4.3 % for *K. arvensis* and 5.6 % for *P. lanceolata*.

Statistical analysis

Based upon the species occurrence list, species diversity was calculated for each site as number of occurring species. We used a one-way ANOVA to test whether species diversity differed significantly between source and restoration sites and Spearman's

rank correlation coefficients to check whether species diversity depended on the year of restoration. All tests were done in IBM Statistics 24 for Windows, IBM Corporation.

Furthermore, we estimated the degree of floristic (dis)similarity in vegetation composition between the source and restoration sites. We performed a nonmetric multidimensional scaling (NMDS) with presence-absence data based on Sorensen similarity index using PC-ORD version 7 software (McCune and Mefford, 2016). The NMDS ordination was performed with 50 runs of real data and 50 randomized (by row) runs with a stability criterion of 0.00001 and a maximum of 200 iterations. Standard stepdown procedures were used to find the appropriate number of axes sufficient to reduce stress, which measures how well the distance ordination space corresponds to the dissimilarity in species composition. A multi-response permutation procedure (MRPP) implemented in PCORD version 7 software (McCune and Mefford 2016) was used to test for differences between the two groups.

Genetic variation within the populations of *K. arvensis* and *P. lanceolata* was calculated as Nei's Gene Diversity (*H*) with the program AFLPsurv (Vekemans 2002). Population size and genetic variation within populations from source and restoration sites were compared using one-way ANOVAs. Spearman's rank correlation coefficients were computed to test for correlation between Nei's Gene Diversity and age of the grasslands as well as population size of *K. arvensis* and *P. lanceolata* at the study sites. All tests were done in IBM Statistics 24 for Windows, IBM Corporation.

The program Structure version 2.3.4 (Pritchard, Stephens et al. 2000, Pritchard, Wen et al. 2007) was used to perform Bayesian cluster analysis. This method enables to examine population structure in the data set and assign individuals into groups without prior definition of populations. The presumably number of groups was computed using 10000 Markov Chain Monte Carlo (MCMC) simulations and a burn-in-period of 100000 iterations. Analysis for the predefined value of K were run 20 times per K = 1-16 for *K. arvensis* and 20 times per K = 1-18 for *P. lanceolata* (Falush, Stephens et al. 2003, Falush, Stephens et al. 2007). Results were summarized with the program Structure Harvester (Earl and Vonholdt 2012). Group assignment was an ad hoc quantity procedure calculating ΔK (Evanno, Regnaut et al. 2005).

The software GenAlEx 6 (Peakall and Smouse 2006) was employed to analyze patterns of genetic similarities between individuals. Therefore, a principal coordinate analysis (PCoA) based on a squared Euclidean distance matrix was calculated. Furthermore, the program was used to compute analysis of molecular variance, AMOVAs (Excoffier, Smouse et al. 1992) to investigate genetic differentiation between populations on source and restored sites.

RESULTS

Species diversity and composition

In total, we observed 165 plant species in the grasslands at all study sites (*Table S 3.1*). They contained many protected (*Centaurium umbellatum*, *Dianthus armeria*, *Dianthus carthusianorum*, *Dianthus deltoides*, *Primula elatior*) and red list species (*Agrostema githago*, RL 1; *Astragalus cicer*, RL 3; *Linum perenne*, RL 1). On average we identified 63 plant species per grassland. At source sites, the number of plant species varied between 55 and 86 with a mean of 71 species (Table 3.1), while the number of plant species at restoration sites ranged from 38 to 77 with a mean of 60 species (Table 3.1). However, species diversity did not differ significantly between source and restoration sites (one-way ANOVA, p=0.178) and did not depend on the year of restoration (Spearman correlation, r=-0.194, p=0.568). NMDS revealed differences in species composition between grasslands at source and restoration sites (Figure 3.2), but MRPP indicated that these differences were not statistically significant (A=-0.008, T=0.47, p=0.63). Grasslands were more similar to each other at source sites than at restoration sites. However, the plots originating from the same source site were not grouped together.

Genetic variation within populations

For *K. arvensis,* AFLP analysis resulted in 127 fragments. 78.57 % of the fragments were polymorphic. In populations at source sites, Nei's Gene Diversity (H_{Ka}) varied between 0.27 and 0.30 with a mean of 0.29. In populations at restoration sites, H_{Ka} ranged from 0.22 to 0.30 with a mean of 0.28 (Table 3.2). Nei's Gene Diversity was not significantly different between populations at source and restoration sites (one-way

ANOVA, p=0.835). Population size (PS) of *K. arvensis* (PS_{Ka}) differed significantly between source and restoration sites (one-way ANOVA, p=0.000), but we observed no significant correlation between H_{Ka} and population size (PS) or year (Y) of restoration (Spearman correlation, r_{Ka_PS}=-0.222, p_{Ka_PS}=0.489; r_{Ka_Y}=0.202, p_{Ka_Y}=0.603).

For *P. lanceolata*, 122 fragments could be detected and 90.35 % of the fragments were polymorphic. In populations at source sites Nei's Gene Diversity (H_{Pl}) ranged from 0.31 to 0.32 with a mean of 0.32 (Table 3.2). In populations at restoration sites H_{Pl} varied between 0.28 to 0.37 with a mean of 0.32. We detected no significant differences between populations at source and restoration sites (one-way ANOVA, p=0.830). Population size (PS) of *P. lanceolata* (PS_{Pl}) differed not significantly between source and restoration sites (one-way ANOVA, p=0.346) and we observed also no significant correlation between Nei's Gene Diversity and population size (PS) or year (Y) of restoration (Spearman correlation, r_{Pl_PS}=-0.033, p_{Pl_PS}=0.910; r_{Pl_Y}=0.012, p_{Pl_Y}=0.973).

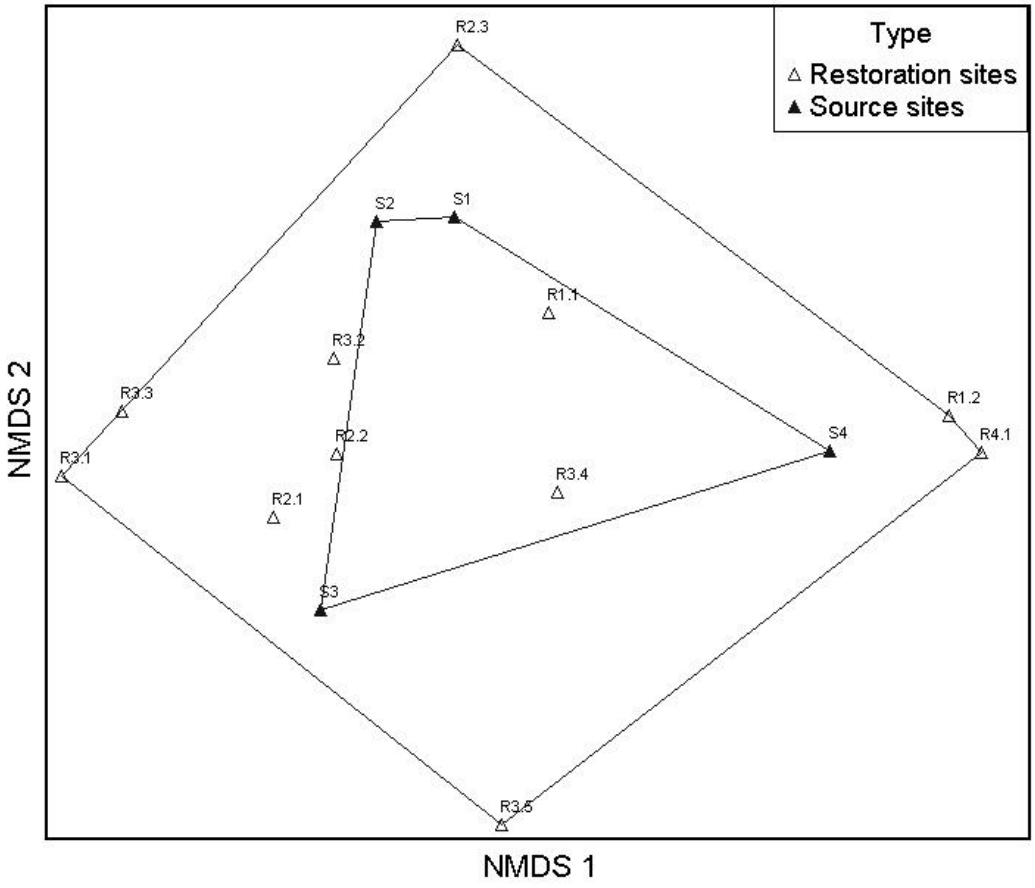


Figure 3.2: Nonmetric Multidimensional Scaling (NMDS) of the study sites based on Sorensen similarity index. Grasslands at source sites (upside down, white triangles) were more similar in their species composition to each other than grasslands at restoration sites (black triangles). S = source site, R = restored site; numbers indicate transfers belonging together (i.e. S1 and R1.1).

Table 3.2: Genetic variation within populations of *K. arvensis* and *P. lanceolata* measured as Nei's Gene Diversity (H_{Ka} and H_{Pl}) at source sites and the corresponding restoration sites, with number (No.), site type (S: source or R: restoration site), population size (PS_{Ka} and PS_{Pl}), and number of analyzed individuals (N_{Ka} and N_{Pl}).

No.	Type	PS_{KA}	PS_{Pl}	N_{Ka}	H_{Ka}	N_{Pl}	H_{Pl}
S1	S	16800	51200	16	0.29	16	0.31
S2	S	28520	16120	16	0.30	16	0.32
S3	S	31140	83040	15	0.27	15	0.32
S4	S	-	79950	-	-	16	0.32
Mean source sites		**25487**	**57578**	**15.6**	**0.29**	**15.7**	**0.32**
R1.1	R	3200	81600	15	0.22	15	0.35
R1.2	R	-	92400	-	-	15	0.31
R2.1	R	7200	20000	15	0.30	16	0.30
R2.2	R	8000	29600	16	0.28	16	0.29
R2.3	R	3060	45900	15	0.30	16	0.28
R3.1	R	3600	18600	15	0.28	13	0.34
R3.2	R	1680	16320	16	0.29	15	0.33
R3.3	R	3630	18810	15	0.29	14	0.35
R3.4	R	1620	59130	14	0.30	13	0.37
R3.5	R	2400	26400	14	0.29	16	0.29
R4.1	R	-	12250	-	-	16	0.30
Mean restoration sites		**3821**	**38274**	**15.0**	**0.28**	**15.0**	**0.32**
***p* (one-way ANOVA)**		**0.000**	0.346		0.835		0.830

Genetic variation among populations

For *K. arvensis*, the principal coordinate analysis (Fig. 3.3a) revealed one group comprising all individuals without any separation of individuals according to population, site type or geographic position of the investigated populations. For the Bayesian cluster analysis, the Evanno's delta K approach indicated that the populations formed 3 genetic clusters (*Fig. S 3.1a*). However, the assignment plots produced for K = 3 showed no distinct grouping by population, site type, or geographic position. Based on the high L(K) values at K = 1-3, it is likely that K=3 is over clustering these data, and only one genetic cluster is present. In the AMOVAs (*Table S 3.2*) molecular variance among all populations was generally low (Φ_{PT}=0.04). Source and restoration sites differed only weakly from each other (Φ_{PT}=0.04). Molecular variance among populations at source sites was, however, slightly higher (Φ_{PT}=0.06) than among populations at restoration sites (Φ_{PT}=0.03). Comparing genetic variance between source and restoration sites for each transfer separately revealed Φ_{PT} values between 0.004 and 0.07 (*Table S 3.2*).

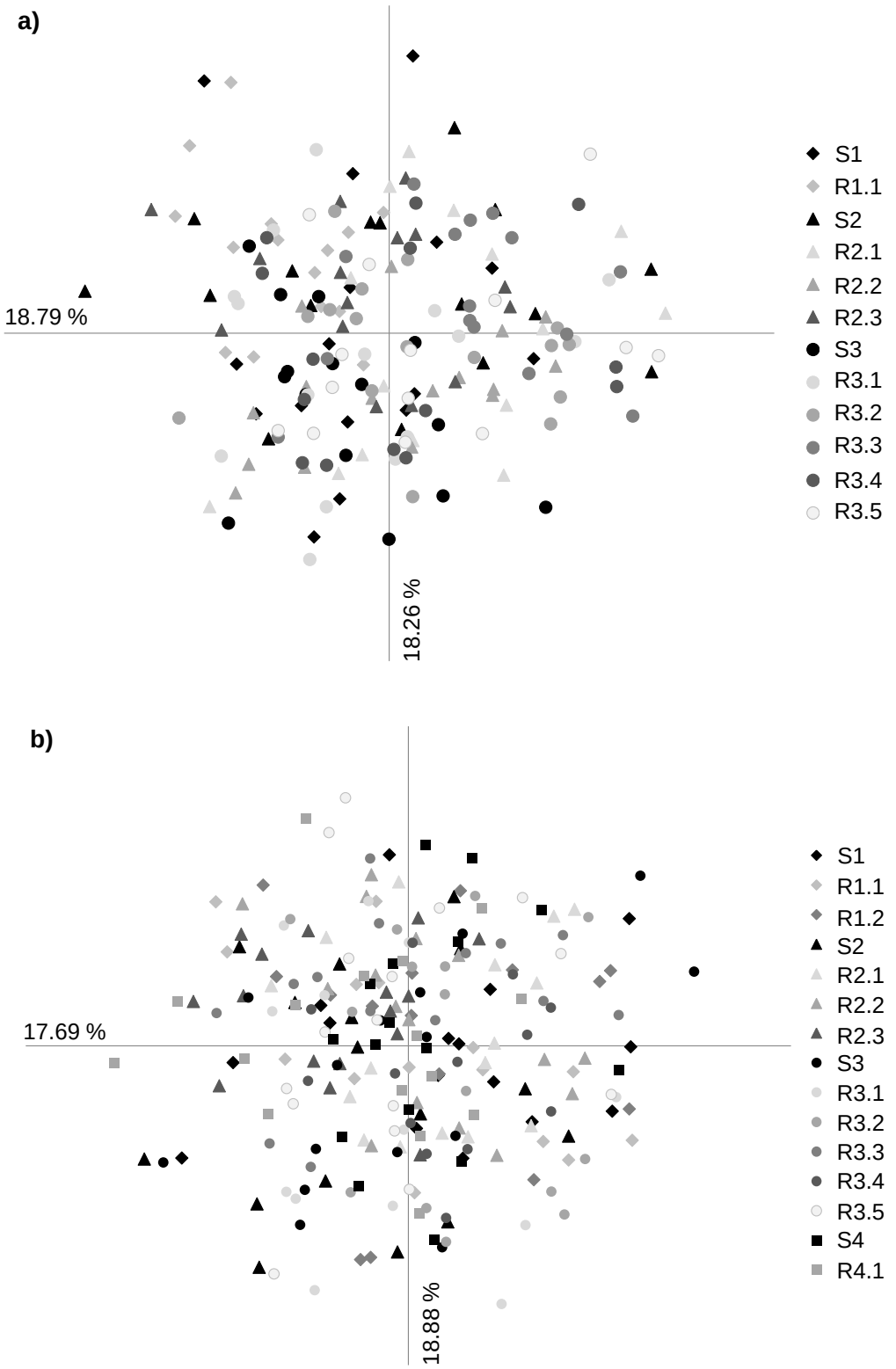


Figure 3.3: Principal coordinate analysis (PCoA) for a) *K. arvensis* and b) P lanceolata. For both species, all investigated populations were admixed and no grouping of individuals according to population, site type or geographic position could be detected. Population labels follow table 3.1.

Six of nine transfers resulted in nonsignificant differentiation or a molecular variance below a Φ_{PT} of 0.04.

Principal coordinate analysis (Figure 3.3b) also revealed one group without any separation of individuals according to population, site type or geographic position of the investigated populations for *P. lanceolata*. For the Bayesian cluster analysis, the Evanno's delta K approach indicated that the populations formed 2 genetic clusters (*Figure S 3.1b*). However, given that there are no observable clustering patterns with the assignment plot, the fact that Evanno's delta K is biased toward K = 2, and that the L(K) also has a high value at K = 1, it is likely that all individuals also cluster into one group.

The AMOVAs (*Table S 3.3*) revealed a very low level of molecular variance among all populations (Φ_{PT}=0.02) as well as between source and restoration sites, among populations from source sites or among populations from restoration sites (all Φ_{PT}=0.02). Comparing genetic variance between source and restoration sites for each transfer separately resulted again in very low levels of molecular variance varying from 0.002 to 0.05 (*Table S 3.3*). Eight of eleven transfers resulted in nonsignificant differentiation or a molecular variance below a Φ_{PT} of 0.03.

DISCUSSION

Impact of restoration on species diversity and composition

In our study we observed no significant differences in species diversity and composition between grasslands at source and restoration sites, which supports the conclusion of previous studies that the transfer of plant species via seed-containing chaff, threshed plant material or green hay (Kiehl, Kirmer et al. 2010) is generally a promising approach to restore species-rich grasslands (Kiehl and Wagner 2006, Coiffait-Gombault, Buisson et al. 2011, Albert, Mudrák et al. 2019). The establishment of a large proportion of species occurring at a source site in restored grasslands requires, however, much effort and the repeated transfer of plant material (Kiehl and Wagner 2006). Species diversity may, hence, for practical reasons be lower at restored than at source sites (Kiehl and Pfadenhauer 2007), a trend we also observed since mean species diversity was slightly lower in grasslands at restored sites than at source sites, although the difference was not significant.

Successful grassland restoration with local plant material depends, in particular, on harvesting time and soil preparation (Schmiede, Otte et al. 2012, Bischoff, Hoboy et al. 2018). Depending on species phenology the composition of seeds within the local plant material used for restoration is strongly affected by harvesting time. Multiple transfers of plant material collected at different times are, therefore, suggested to achieve high transfer rates and similar species composition (Kiehl and Wagner 2006). The grasslands we studied here were restored over a longer period of time and the harvesting process varied for practical reasons seasonally and between years, which may have contributed to the observed differences in species diversity and composition among grasslands from source and respective restoration sites.

Impact of life history traits and restoration on genetic variation

In our study we observed clear differences in genetic variation between the study species. *K. arvensis* and *P. lanceolata* exactly reflected the pattern of genetic variation which has previously been reported for other wind pollinated and more frequently distributed plant species compared to insect pollinated and less frequently distributed species. Generally, the former exhibit higher levels of variation within populations but lower variation among populations, whereas the latter show lower levels of variation within but stronger variation among populations (Reisch and Bernhardt-Römermann 2014). Moreover, populations of *P. lanceolata* were much larger than populations of *K. arvensis*, which may also contribute to the higher level of genetic variation within populations of *P. lanceolata* compared to *K. arvensis* (Leimu, Mutikainen et al. 2006). Hence, the results of our study corroborate the strong impact of life history traits and population size on the genetic variation of plant species, which has been reported in previous studies.

Besides life history traits and population size, genetic variation within and among plant populations may, however, also be influenced by restoration, in particular by bottlenecks caused during the harvesting process (Mijangos, Pacioni et al. 2015). First, the number of seeds harvested for restoration may be limited for practical reasons. It is for instance generally recommended not to harvest the whole source site but only two thirds of the area. Secondly, the number of transferred seeds and their origin may be affected by plant phenology, since not all individuals fruit at the same time. This means

that seed harvesting often comprises not all individuals at the source site but only a subset. Consequently, only a part of the available gene pool is transferred, which may cause a bottleneck and hence reduced levels of genetic variation within restored populations.

Genetic analysis revealed similar levels of genetic variation within populations of *P. lanceolata* from source and restoration sites, which was to be expected due to similar population size. Interestingly we also observed no significant differences in genetic variation between populations of *K. arvensis* despite significantly smaller population size at restoration sites than at source sites. This may be explained in two ways. First, the lack of differences may be a statistical bias due to the low number of *K. arvensis* populations at source sites in our study, which may obscure potential differences in genetic variation. Genetic variation was slightly but not significantly lower in populations from restoration sites, which may support this assumption. Second, high immigration rates, or more specifically gene flow, from nearby grassland sites may have caused a fast recovery of genetic variation within recently founded populations (Tremetsberger, Stuessy et al. 2003). Previous studies of different species often revealed decreased levels of genetic variation in restored compared to source populations (Aavik, Edwards et al. 2012, Vandepitte, Gristina et al. 2012), although this must not be necessarily the case (Kaulfuß and Reisch 2019). Genetic variation within populations restored by transfer of local plant material has hardly been analyzed, but Dittberner et al. (2019) reported, similar to our results, also no loss of genetic variation in floodplain meadow populations of *Arabis sagittata* and *A. nemorensis* restored by hay transfer. This supports our perception that the transfer of local, seed-containing plant material is a restoration approach, which is suitable to restore genetically variable populations.

Restoration by transfer of local plant material may not only reduce genetic variation within populations, but also increase genetic differentiation among populations. Seed harvesting may comprise for seasonal or practical reasons not all individuals at the source site, which means that not the full gene pool available is transferred but only a subset. Moreover, seedling establishment at the restoration site may represent a filter selecting specific genotypes. Both could result in increased levels of genetic differentiation among populations from source and restoration sites. In our study, genetic differentiation among populations of both *K. arvensis* and *P. lanceolata* from source and

restoration sites, was much lower than reported for other common, outcrossing plant species (Reisch and Bernhardt-Römermann 2014). Even more important is, however, that genetic differentiation among source sites and restoration sites was lower (*K. arvensis*) or equivalent (*P. lanceolata*) to genetic differentiation among populations from source sites. These results support the assumption that – at least for the investigated populations in our study – grassland restoration by transfer of green hay and threshed plant material caused neither a decrease in genetic variation within nor an increasing divergence among populations at source and restored sites. This again supports our assessment that the transfer of local plant material seems to be an approach, which allows to restore genetically comparable grassland populations. Our study underpins, therefore, the perception that the transfer of local plant material is indeed the restoration approach most suitable to preserve the natural genetic pattern of plant species.

CHAPTER FOUR

GRASSLAND RESTORATION BY LOCAL SEED MIXTURES: NEW EVIDENCE FROM A PRACTICAL 15-YEAR RESTORATION STUDY

FRANZISKA KAULFUß AND CHRISTOPH REISCH

ABSTRACT

Aim: Local seed-mixtures are frequently used to restore species-rich grasslands. However, it has hardly been tested whether local seed mixtures can actually be applied successfully in grassland restoration practice at larger scales and long-term. To close this gap, we report the results of a large-scale restoration study where grasslands have been restored about 15 years ago using different local seed mixtures.

Location: Bavaria, SE Germany.

Methods: To evaluate the efficacy of the local seed mixtures, we compared species composition of seed-mixtures and present vegetation. Then we tested whether restoration success depends on site characteristics such as size and shape (rectangle or stripe) of the grassland, restoration procedures like topsoil removal, seed-density, and land use or species habitat preferences for light, water and nutrients and its life span (annual, perennial).

Results: On average, the present vegetation contained 62.4% of all species that were present in the local seed mixtures. The species from the local seed mixtures made on average 69.1% of total cover in the established vegetation, whereby species composition of local seed mixture and vegetation significantly differed from each other. The probability of a sown species to establish increased with seed density up to 300 seeds/m². Furthermore, habitat preferences significantly affected species establishment chances with species requiring full illumination, dry and nutrient-poor soil being more successful during restoration, reflecting the high proportion of sites with topsoil removal prior to seeding in our study. Annual species had significantly lower establishment chances as compared to their perennial counterparts.

Conclusions: Our study provides another piece of evidence that local seed-mixtures can successfully be applied in large-scale grassland restoration projects. We provide several practical recommendations how such practices can be further improved by using specific seed densities and creating new local seed mixtures using species ecologically more suitable to the restored sites.

KEYWORDS: Species-rich grassland, local seed mixture, restoration, species trait, vegetation composition, species diversity

INTRODUCTION

Grasslands have a tremendous ecological significance since they represent a large part of all terrestrial habitats. For example, in Europe, grasslands cover about 1.8 million ha (Carlier, De Vliegher et al. 2005), which corresponds to about 40% of the land surface. These grasslands provide key ecosystem services including carbon sequestration, protection from erosion and harboring many plant and animal taxa (Dengler, Janišová et al. 2014).

Currently, grasslands are threatened by modern land use practices (Sala, Chapin et al. 2000). Increased fertilization and mowing frequencies have caused ongoing loss of species (Socher, Prati et al. 2012, Klaus, Hölzel et al. 2013) and this process is enhanced by the increased deposition of atmospheric nitrogen since the middle of the 20th century (Wesche, Krause et al. 2012, Diekmann, Jandt et al. 2014). The restoration of species-rich grasslands is therefore on the nature conservation agenda worldwide.

Modern grassland restoration practices are largely based on the application of local seed-mixtures (Jongepierová, Mitchley et al. 2007, Kiehl, Kirmer et al. 2010, Török, Deák et al. 2010, Walker, Hermann et al. 2015). The main idea of this approach is that seed mixtures used in restoration should be produced from natural seed material originating in the regions, where the restoration practices take place. A main advantage of using local seed material for restoration is that autochthonous plant populations are considerably better adapted to local environmental conditions than non-local populations (McKay, Christian et al. 2005, van der Mijnsbrugge, Bischoff et al. 2010). Furthermore, the local seed mixtures are recommended for use to avoid potential outbreeding effects (Hufford and Mazer 2003) and to increase restoration success (Sackville Hamilton 2001). In Germany, for example, the regions for the local seed mixture production have been mainly defined based upon region-specific geomorphologic and climatic parameters (Prasse, Kunzmann et al. 2010) also taking into account genetic variability of plant species (Durka, Michalski et al. 2017, Listl, Poschlod et al. 2017, Listl, Poschlod et al. 2018). These principles have been also implemented in the seed production; currently, producers located in different parts of the country offer a range of ‘restoration-ready’ local seed mixtures.

Despite their strong relevance for grassland restoration, the success of the local seed mixtures in practice has hardly been verified. Previous research has demonstrated that topsoil removal before sowing (Rasran, Vogt et al. 2007) or disturbance (Freitag, Klaus et al. 2021), and the use of high-diversity seed mixtures (Kirmer, Baasch et al. 2012) can increase the restoration success. Moreover, sowing density and post-restoration land use can also have an impact on grassland restoration (Kiehl, Kirmer et al. 2010). Furthermore, the restoration success may depend on site characteristics such as the size and shape (elongated or regular) of the restored grassland, which in turn affects rates of deposition of fertilizer and herbicides from nearby arable fields (Duncan, Dorrough et al. 2008). Finally, species characteristics such as life history, or habitat preferences, e.g. in terms of light or water requirement for successful establishment, may also have an impact on restoration success.

Yet, restoration recommendations are mainly based on experimental studies (Kiehl, Kirmer et al. 2010, Freitag, Klaus et al. 2021) under more or less controlled conditions that can be very different from real-world restoration projects. Here, the restoration process is usually less standardized due to the involvement of different actors with different requirements for the restoration goals (e.g. conservation agencies, farmers, local stakeholders). Furthermore, local abiotic and biotic conditions in specific restoration projects can strongly deviate from the published studies because of their local nature (Prach, Jongepierová et al. 2013). Therefore, data from real-world restorations are much needed to evaluate the success of grassland restoration under real life conditions. In our study, we attempted to close this gap by analyzing the success of local seed mixtures in a large-scale grassland restoration study conducted at 35 sites in south eastern Germany where about 15 years ago grassland has been restored using different local seed mixtures. First, we estimated whether the species composition of restored grasslands was similar to the species composition of the sown local seed mixtures. Then, we evaluated the effects of site characteristics, restoration procedures and species characteristics on establishment success of individual species present in the local seed mixtures. More specifically, we asked the following questions: (i) Are local seed mixtures a tool which can be used successfully to restore species-rich grassland in practice? (ii) Is the restoration success of local seed mixtures at the community and species level depending on site characteristics, restoration procedures, species habitat preferences and plant life span? Based on the results of our analysis, we finally

make practical recommendations for the further application of local seed mixtures in grassland restoration.

METHODS

Study design

For our investigation we selected 35 study sites at 11 different geographic locations in south eastern Germany (Figure 4.1, Table 4.1). Between 2003 and 2006, the grasslands were restored on ex-arable fields by application of local seed mixtures within the framework of land consolidation projects. Based upon information from the land consolidation agency (Amt für Ländliche Entwicklung Oberpfalz) and the seed producers we identified the local seed mixtures used for restoration at each site, their species composition, and the total weight of the seed mixture (in kg) applied for restoration (*Table S 4.1*). Using the total weight of the applied seed mixture, the relative proportion of each species in a mixture (in %) and average seed weight (http://data.kew.org/sid/sidsearch.html), we calculated the seed density (SD) for each species and mixture as number of sown seeds/m².

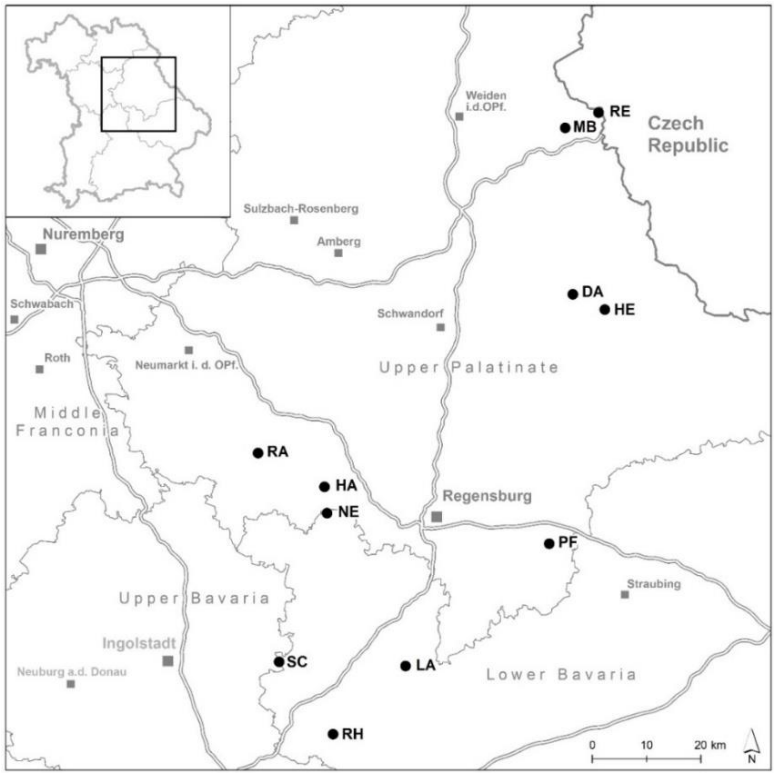


Figure 4.1: Geographic position of the study locations in southeast Germany.

Table 4.1: Selected study sites with name of the study site (ST), year of the grassland restoration (YE), size in ha (SI) and shape (SH; R: regular, S: elongated) of the study site, pre-restoration soil preparation (TR; +: topsoil removal, -: no topsoil removal) and post-restoration land use (LU; NO: none, MW: mowing, MU: mulching) type. For each site the density of the sown seeds (SD) in number of seeds/m² is given. For each site the number of species in the applied seed mixture SR_{mix}, the total number of species in the established vegetation SR_{tot} and the proportion of sown species in established vegetation SR_{pro} in % are given. We also determined the cumulative abundance of sown species in the established vegetation (AB_{mix}), the cumulative abundance of all species in the established vegetation (AB_{tot}) and the relative abundance of sown species in the established vegetation (AB_{rel}).

ST	YE	SI	SH	TR	LU	SD	SR_{Mi}	SR_{tot}	SR_{pro}	AB_{mix}	AB_{tot}	AB_{rel}
DA01	2003	0.27	R	+	NO	970	14.2	21.8	65.5	625.2	808.2	77.0
HA01	2005	0.11	R	+	NO	184	11.8	18.2	64.8	516.8	657.4	78.4
HA02	2005	0.14	R	+	NO	76	12.2	18.2	67.8	609.8	644.4	94.5
HA03	2005	0.07	S	+	NO	443	3.4	14.6	23.2	176.0	610.4	29.9
HA04	2005	0.13	S	+	MW	477	7.8	16.8	46.3	393.6	810.0	48.3
HA05	2005	0.17	R	+	NO	147	9.6	16.8	57.0	531.4	649.0	81.8
HA06	2005	0.32	R	+	NO	341	12.0	18.8	64.7	620.6	806.0	77.4
HA07	2005	0.08	R	+	MW	120	13.0	23.2	56.2	557.2	858.2	64.9
HA08	2005	0.20	S	+	MW	301	5.4	12.8	42.3	357.2	590.2	60.6
HE01	2005	0.11	S	+	NO	139	12.8	17.0	75.7	559.4	648.4	86.3
HE02	2005	0.63	R	+	MW	19	8.6	17.2	49.8	349.8	668.6	52.0
HE03	2005	0.08	R	+	NO	299	2.4	10.2	23.6	148.0	582.4	22.7
LA01	2006	2.60	R	+	MW	166	18.4	23.0	81.7	453.4	523.6	88.0
MB01	2006	1.00	R	-	MW	705	8.0	17.0	46.8	242.4	533.6	44.3
NE01	2005	0.21	S	+	MU	487	6.8	13.6	49.4	261.2	614.2	42.2
NE02	2005	0.37	R	+	MU	56	14.8	18.0	82.5	618.6	652.4	95.0
PF01	2004	0.42	R	+	MW	43	19.0	23.4	81.2	550.4	691.6	79.5
PF02	2004	2.08	R	+	MW	44	16.6	20.6	80.6	576.8	662.8	87.2
PF03	2004	1.14	R	+	MW	45	17.8	27.0	66.0	672.8	777.4	86.5
PF04	2004	0.40	R	+	MW	49	17.0	20.2	84.2	636.2	697.2	91.5
RA01	2006	0.23	R	-	MW	143	26.0	29.6	87.9	715.4	848.0	84.5
RE01	2005	0.10	S	+	NO	90	12.6	23.0	55.0	546.0	892.2	61.1
RE02	2005	0.08	R	+	NO	272	15.2	22.4	68.1	503.6	682.2	73.7
RE03	2005	0.14	S	+	NO	147	5.4	19.4	28.3	344.2	758.8	45.7
RE04	2005	0.11	S	+	NO	129	17.0	26.6	63.8	595.6	905.6	66.1
RH01	2005	0.07	R	+	NO	24	14.2	17.0	84.1	487.2	564.2	86.1
RH02	2005	0.07	R	+	NO	64	16.4	25.6	64.9	522.4	729.2	70.9
RH03	2005	0.10	R	+	NO	13	18.8	23.6	79.5	631.2	780.4	80.4
RH04	2005	0.06	R	+	NO	40	19.2	25.4	74.9	660.0	845.4	77.9
SC01	2006	0.20	R	-	MW	173	12.0	20.6	58.0	487.6	758.2	63.9
SC02	2006	0.08	R	-	MW	392	12.4	22.8	54.4	278.2	693.8	40.2
SC03	2006	0.17	R	-	MW	180	16.2	23.6	69.6	629.8	813.2	78.3
SC04	2006	0.30	S	-	MW	115	5.6	11.6	48.4	376.4	625.2	60.0
SC05	2006	0.44	R	-	MW	79	14.2	19.2	74.1	484.2	646.2	74.8
SC06	2006	0.20	S	-	MW	167	7.4	11.8	62.5	376.8	572.8	66.0
Mean							**12.7**	**19.7**	**62.4**	**488.4**	**702.9**	**69.1**

For each study site, the year of the restoration, the size and shape (elongated or regular) of the site as well as the potential application of pre-restoration soil preparation (topsoil removal or none) and/or post restoration land use (mowing, mulching or none) was recorded (Table 4.1).

The species composition of the restored grasslands was surveyed at each study site (*Table S 4.2*). For this purpose, vegetation surveys were conducted in five randomly distributed study plots with a size of 2x2 m. All species occurring in the plots were identified and their cover was estimated using a decimal scale (Londo 1972).

Data analysis

Based on data on seed mixture composition and the vegetation surveys we calculated for each plot 1) relative proportion of sown species that were in the seed mixture (SR_{pro}) and 2) their cumulative abundance (AB_{rel}) in the established vegetation, as two simple indices (Table 4.1).

To estimate the degree of floristic similarity between seed mixture composition and present vegetation of the restored grasslands, we performed a nonmetric multidimensional scaling (NMDS) with species composition of the established vegetation and the corresponding seed mixtures (species presence/absence matrices) based on Bray-Curtis similarity index using PC-ORD version 7 software (McCune and Mefford 2016).

The NMDS was calculated with 50 runs of real data and 50 randomized (by row) runs with a stability criterion of 0.00001 and a maximum of 200 iterations. We used standard stepdown procedures to find the appropriate number of axes sufficient to reduce stress. Correlations between seed mixture composition and ordination scores for the established species and sites were quantified using Spearman's rank correlation as suggested previously (McCune, Grace et al. 2002). Visual inspection of the ordination diagrams revealed differences between composition of applied seed mixtures and established vegetation. Therefore, a multi-response permutation procedure (MRPP) implemented in the PC-ORD software was used to test whether this difference was statistically significant.

To estimate the efficacy of seed mixtures in grassland restoration, we calculated a generalized linear mixed-effects model (GLMM, family ‘Binomial’) to analyze whether

the establishment of species from the seed mixtures is affected by site characteristics, restoration procedures, density of sown seeds and species characteristics (Table 4.2). The response variable in the model was relative abundance (a value from 0 to 1, where 1 corresponds to abundance of 100%) of a sown species in the established vegetation (*Table S 4.1*). Pre-restoration soil preparation (topsoil removal or not), post-restoration land use (no land use, mowing, mulching), shape of restored grasslands (elongated or regular) and species characteristics were used as fixed factors. Species characteristics included species-specific density of sown seeds (both linear and non-linear terms), habitat preferences expressed as Ellenberg indicator values (EIV) for light (L), soil moisture (F) and nutrients (N) and four functional traits (life span, specific leaf area (SLA), plant height and seed size) (*Table S 4.1*). The EIV are proxies for habitat requirements of adult plants and, except for the F value (the highest value is 12), range from 1 to 9 with highest numbers indicating high requirements for the corresponding environmental factor (e.g. N value of 9 indicates a species occurring on soils with high nutrient contents). The EIV for light was included into the model as interaction term with post-restoration land use to infer possible positive effects of land use (particularly mowing) on establishment of sown seeds with different requirements for light. In the same vein, we considered topsoil removal to influence considerably the soil properties in the restored sites. To account for such effects on species establishment from the sown seeds, the EIV for soil moisture and nutrients were included into the model as interaction terms with the pre-restoration soil preparation. Data on life span were extracted from the LEDA database (Kleyer, Bekker et al. 2008). The model included sites and plant family as random effects to account for site- and family-specific variation in restoration success, respectively. The seed density values in the models were log-transformed to improve the normality of the residuals. Collinearity was not a problem in all models, as the explanatory variables were only weakly correlated with each other. Model assumptions were met in all cases. All statistical analysis were conducted in the R statistical environment (R-Core-Team 2020). The GLMM was fitted with the help of the lme4 package (Bates, Maechler et al. 2015).

RESULTS

In terms of species richness, the seed mixture success varied between 23.2 (site HE_03) and 87.9% (site RA_01) with an average of 62.4 % (Figure 4.2A). The average relative abundance of sown species in the established vegetation was 69.1 % (Figure 4.2B), ranging from 22.7 % (site HE_03) to 95% (site NE_02).

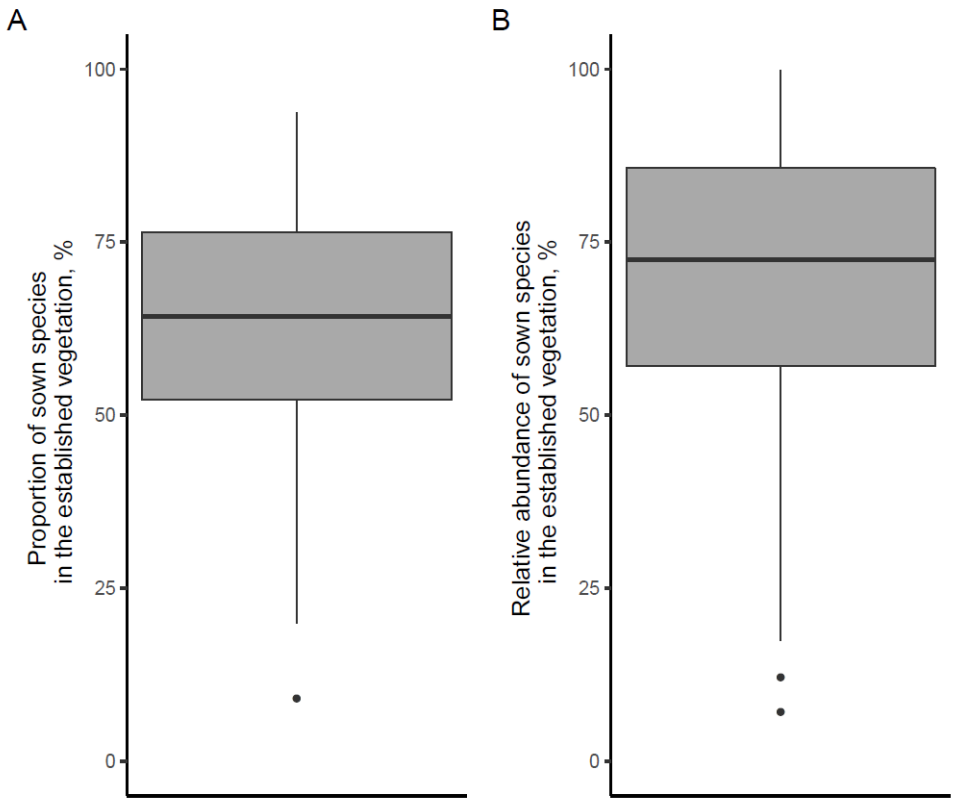


Figure 4.2: Restoration success of the applied seed mixtures in the study area expressed as A) proportion of sown species the established vegetation in % and B) relative abundance of sown species in the established vegetation.

The NMDS (2-dimensional, final stress = 16.5) ordination accounted for 75.5% of total variance in seed mixture and established vegetation composition (when correlating the original distance matrix with distances in ordination space) of which 45.4% and 30,1% could be attributed to axis 1 and 2, respectively.

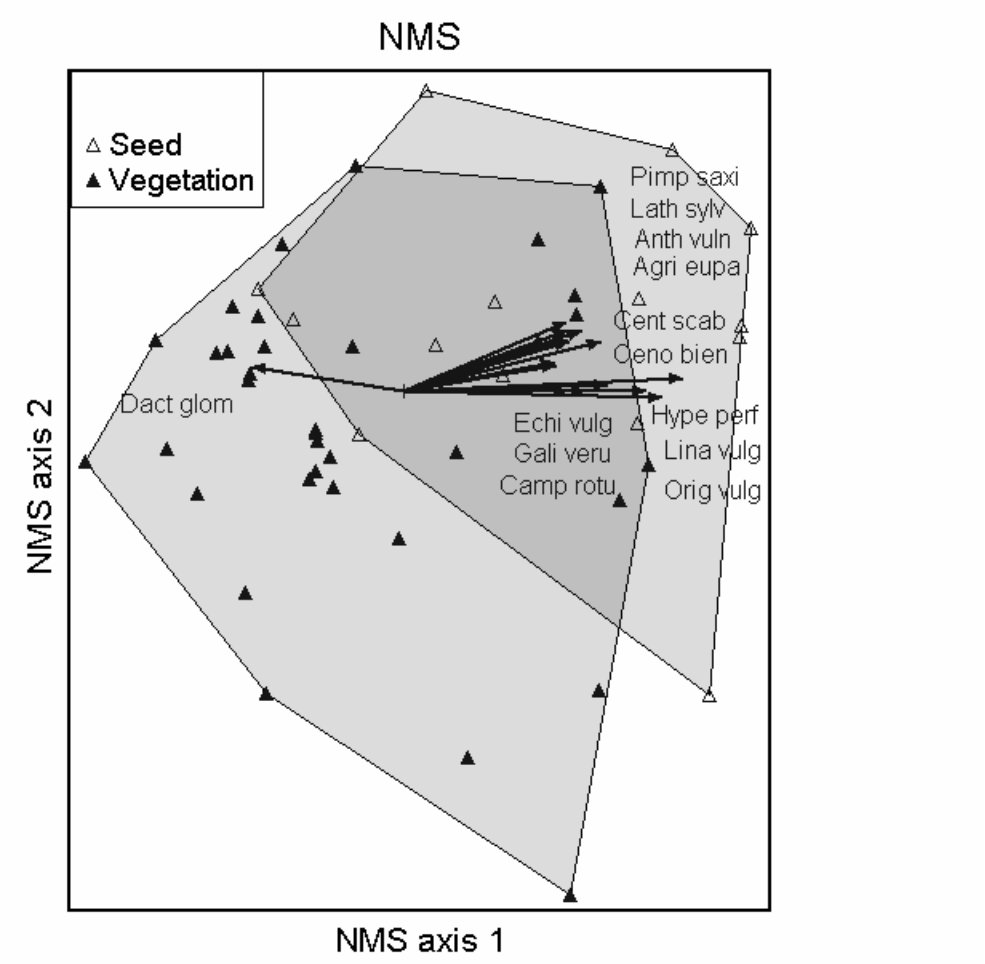


Figure 4.3: Results of the NMDS (3-dimensional, final stress = 16.1) ordination (76% of total variance in seed mixture and established vegetation composition). When correlating the original distance matrix with distances in ordination space, 33%, 26%, and 17% could be attributed to axis 1, 2 and 3, respectively.

The ordination diagram revealed clear differences in species composition between applied seed mixtures and established vegetation (Figure 4.3); the results from the MRPP ($A=0.05$, $T=-9$, $p<0.0001$) indicated that these differences were statistically significant. Grass *Dactylis glomerata* was one of the most frequent species in established vegetation, whereas frequency of many herbs, such as *Campanula rotundifolia*, *Galium verum*, *Hypericum perforatum* and *Origanum vulgare*, was much higher in the seed mixtures as in the established vegetation.

The GLMM revealed several significant effects of the restoration procedure and species traits on the abundance of species from the local seed mixtures in the established vegetation (Table 4.2, Figure 4.4). To begin with, the number of sown seeds positively

affected the success of a species. However, this effect was detected only for the interval from 1 to ca. 300 seeds/m²; seed densities higher than 300 seeds/m² had a small, yet significant negative effect on the restoration success (Figure 4.4A). Second, the species occurring in open, fully illuminated habitats (i.e. with a lower EIV for light) had significantly higher success rates; none of the management types had a significant impact on this relationship (Figure 4.4B). Third, species with low requirements for water (i.e. with lower EIV for soil moisture) also displayed significantly higher success rates (Figure 4.4C); this relationship was significantly weaker at the sites with pre-restoration soil preparation by topsoil removal. Fourth, species occurring on nutrient-poor soils (i.e. with lower EIV N values) had significantly higher probability to establish in the restored grasslands at sites with topsoil removal; this effect was not detected at sites without pre-restoration soil preparation (Figure 4.4D). Finally, life span was found to have a significant impact on the success rates with perennial species having higher proportions in the establish vegetation as compared to annuals (Figure 4.5).

Table 4.2: Results GLMM with the relative abundance of sown species in the established vegetation (AB_{rel}) as response variable. Pre-restoration soil preparation (topsoil removal or not), post-restoration land use (no land use, mowing, mulching) and species characteristics were used as fixed factors. Species characteristics included species-specific density of sown seeds (both linear and non-linear terms), habitat preferences expressed as Ellenberg indicator values (EIV) for light (L), soil moisture (F) and nutrients (N). Bold entries are significant ($p<0.05$) results.

Predictor	Estimate	Standard error ±	p-value
Intercept	0.63	0.35	0.405
Shape (elongated)	0.93	0.35	0.845
log(Seed density)	1.51	0.08	**<0.001**
log(Seed density)^2	0.95	0.01	**<0.001**
Life span (perennial)	7.33	1.66	**<0.001**
Preprocessing (intact soil):EIV Soil moisture	0.86	0.05	**0.010**
Preprocessing (topsoil removal):EIV Soil moisture	0.92	0.03	**0.003**
Preprocessing (intact soil):EIV Soil nutrients	1.04	0.04	0.391
Preprocessing (topsoil removal):EIV Soil nutrients	0.88	0.02	**<0.001**
Management (no management):EIV Light	0.71	0.03	**<0.001**
Management (mowing):EIV Light	0.76	0.04	**<0.001**
Management (mulching):EIV Light	0.82	0.07	**0.028**

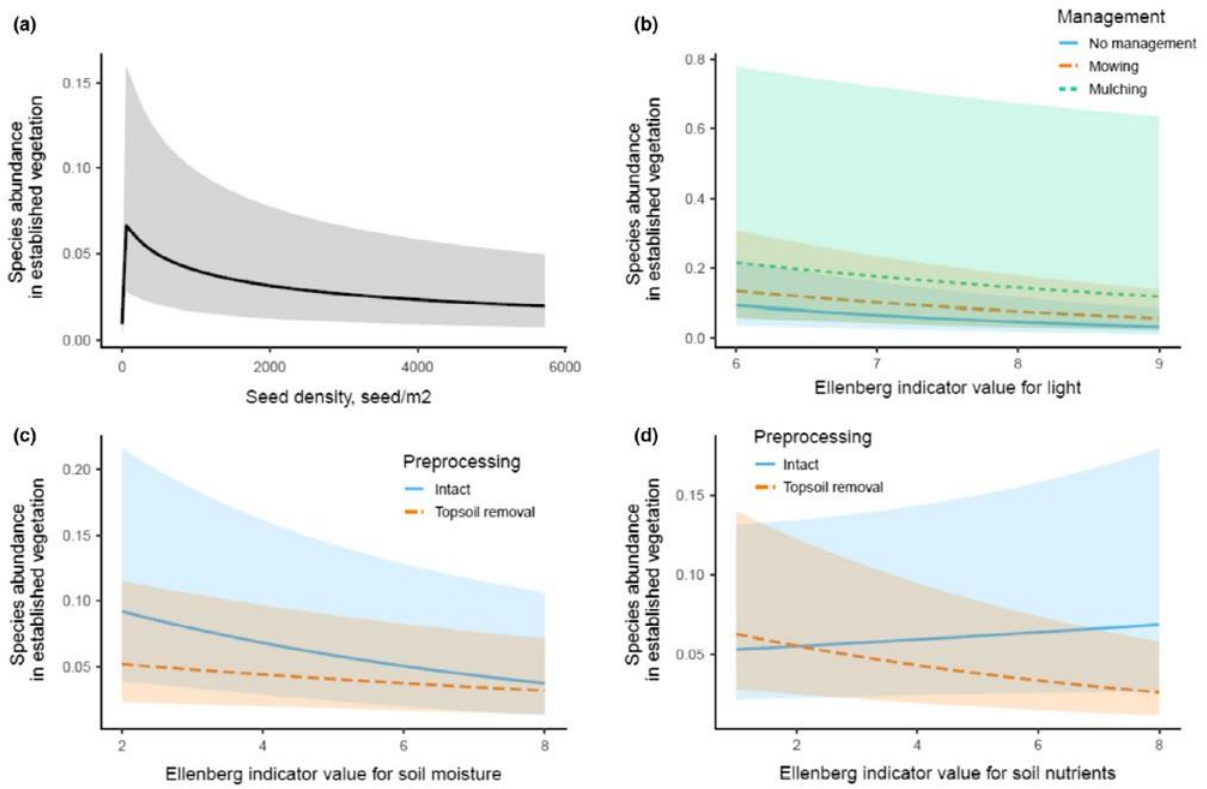


Figure 4.4: Relationship between seed density, species habitat preferences (EIV for light soil moisture and soil nutrients) and restoration success at the species level. Shaded areas indicate 95% confidence intervals.

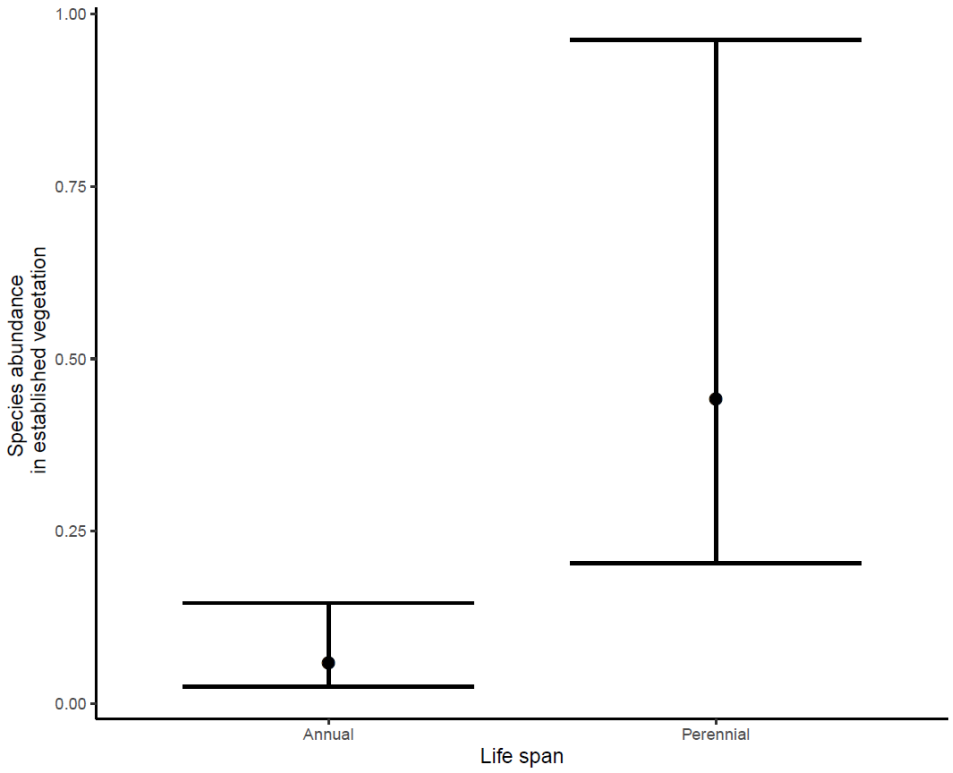


Figure 4.5: Restoration success of annual and perennial species from the seed mixtures.

DISCUSSION

In our study, about two thirds of all species present in the applied seed mixtures were found at the study sites with relatively high abundance (on average 69.4%) about 20 years after the restoration measures had been completed.

The observed high proportion of sown species in the established vegetation was largely in line with former studies; the most comprehensive review on grassland restoration by seed-mixtures reported establishment rates of 32% to 96% (Kiehl, Kirmer et al. 2010). Similarly, Kirmer et al. (2012) observed an establishment rate of 67% for high diversity seed mixtures in ecological restoration of surface mined-land to grasslands. Moreover, the relative abundance of the sown species in the plots was about 70%, which means that on average more than two third of the plots was covered by sown species. Only at seven of 35 study sites the relative abundance of spontaneous species was larger than the relative abundance of sown species. Our study supported, therefore, the observation that the application of local seed mixtures is in general a well-suited approach to restore species-rich grassland in practice.

Despite the comparatively high success rates, the multivariate analysis revealed considerable differences in the species composition of seed mixtures and restored vegetation. The most parsimonious explanation is that many herbaceous species, which established in the first few years after the local seed mixture application, disappeared in the later years due to competition with competitive grasses. The GLMM results suggest that uncompetitive annual species were most affected by this process. Consequently, light demanding herbs included in the seed mixtures, such as *Campanula rotundifolia*, *Galium verum, Hypericum perforatum* or *Origanum vulgare*, declined over time, whereas competitive grasses like *Arrhenatherum elatius*, *Festuca pratensis* or *Dactylis glomerata* increased. This line of argument is supported by research showing that grasses may out-perform herbaceous species in grassland restoration (Pywell, Bullock et al. 2003, Jongepierová, Mitchley et al. 2007, Török, Deák et al. 2010). Moreover, the immigration of species from nearby located grasslands and the loss of species not compatible with the mowing regime (Prach, Jongepierová et al. 2013), may also have contributed to the observed differences between species composition of seed mixtures and present vegetation.

The GLMM revealed a strong impact of seed density on restoration success at the species level. In our investigation at 24 of 35 study sites grassland was restored with less than 200 seeds/m², which is a significantly lower number of seeds than reported in many studies before (Kiehl, Kirmer et al. 2010). It is, therefore, obvious that we observed a strong relationship between restoration success and the number of sown seeds. Our results hence support previous studies showing that increasing seed density boosts the number of established individuals (Sheley and Half 2006). Consequently, Carter & Blair (2012) and Barr et al. (2017) showed that higher seed density results in more successful grassland restoration.

The GLMM also revealed a strong impact of species habitat requirements on restoration success. Previous studies demonstrated that an appropriate balance of species with different ecological requirements is required to ensure a quick restoration of species-rich grassland (Staab, Yannelli et al. 2015). In our study, we also observed that the establishment of species from the seed mixtures depends on their habitat requirements. First, we found that annual species decline over time after the beginning of the restoration. This may be ascribed to the decline of short-living and colorful species like *Papaver rhoeas* or *Centaurea cyanus*, which were added to the seed mixtures to create visually appealing grasslands soon after the application of the seed mixture. These species, which are typical arable field weeds, disappear from the restored grasslands by and by when the vegetation cover gets denser, and the habitat conditions are not suited any more for annual species such as arable weeds – a process which is often observed in the course of vegetation succession (Boscutti, Vianello et al. 2017). Second, and even more interesting, we observed that the success of individual species in grassland restoration depends on light, water and nutrient conditions required for establishment and persistence. Plant species adapted to bright, dry and nutrient-poor habitat conditions had a significantly higher probability to establish and persist in the restored grasslands, than species favoring other ecological conditions, in particular at study sites with topsoil removal. Therefore, our results corroborate the observation that top soil removal before restoration supports the establishment of grassland plant species adapted to relatively nutrient-poor site conditions (Rasran, Vogt et al. 2007). Therefore, species composition of the restored grasslands, even after 20 years, still reflects the habitat conditions at the beginning of the restoration process. The open, dry and nutrient-poor environmental conditions shortly after topsoil removal represent

a strong filter selecting those species, which were able to cope with these conditions, while other species were deleted from the species pool. Therefore, our results illustrate clearly that the interaction of habitat conditions and realized niche requirements has a large impact on the development of the restoration process.

Based upon our results two general recommendations can be made for the successful restoration of species-rich grassland with local seed mixture in the future. First, seeding density should always be high enough to ensure successful restoration – following our results there will likely be no additional benefit from sowing much in excess of 300 seeds per m^2 per species, as establishment success levels off thereafter.

Second, it would be advantageous when seed mixtures would mainly contain species favoring light, dry and nutrient-poor habitat conditions, in particular when topsoil removal has been applied to prepare the restoration sites, because these species exhibit a significantly higher probability to establish and persist in the restored grasslands.

CHAPTER FIVE

RESTORATION OF GRASSLANDS USING COMMERCIALLY PRODUCED SEED MIXTURES: GENETIC VARIATION WITHIN AND AMONG NATURAL AND RESTORED POPULATIONS OF THREE COMMON GRASSLAND SPECIES

FRANZISKA KAULFUß AND CHRISTOPH REISCH

ABSTRACT

The use of local seed material is a common practice in restoration. However, the impact of sowing on genetic variation of natural populations is still unclear.

Aim of this study was, therefore, to test if genetic variation within and among populations restored with local seed material corresponds to the genetic variation of neighboring natural populations. We investigated each ten natural and restored populations of three common forbs (*Knautia arvensis, Silene vulgaris* and *Plantago lanceolata*), situated in five study regions in south-eastern Germany.

Our study revealed significant genetic differentiation between natural and restored populations of the insect-pollinated *K. arvensis* and *S. vulgaris* although differentiation was much stronger for *K. arvensis* since most restored populations contained another ploidy level than natural populations. For the wind-pollinated *P. lanceolata*, genetic differentiation between natural and restored populations was comparable to the genetic differentiation between its natural populations. Genetic diversity within restored populations of each species was equivalent or even higher than within natural populations.

Our study provides evidence that the local genetic structure especially of common insect-pollinated forbs may be affected by the application of regional seed mixtures in restoration. *Regional admixed provenancing* in seed production is an important approach to preserve regional patterns and to provide seeds for the reestablishment of genetically variable populations. The method would however be an even more powerful tool in restoration when ploidy levels would be checked before seed production and seed transfer zones would be smaller.

KEYWORDS: Conservation; genetic diversity; genetic differentiation; genetic variation; inbreeding; outbreeding; seed mixtures; sowing

INTRODUCTION

Ecological restoration of species-rich grasslands often depends on the availability of viable seeds in the soil seed bank of restoration sites or on native target species in the surrounding environment (Bakker, Poschlod et al. 1996). Landscape fragmentation can hamper seed dispersal between restoration sites and potential source populations (Münzbergova and Herben 2005, Hölzel, Buisson et al. 2012) and, therefore, the introduction of target species is a state-of-the-art method in conservation practice and especially sowing of local seed material has become a common tool in restoration ecology (Jongepierova, Mitchley et al. 2007, Török, Deák et al. 2010, Walker, Hermann et al. 2015).

In forestry, guidelines for the use of local seed material have been established for several decades (FoVHgV 2003). Also across the world, the usage of local seeds and the implementation of seed transfer zones gain in importance for restoration purposes, for example in Australia (Krauss, Sinclair et al. 2013), Canada (Ukrainetz, O'Neill et al. 2011), the USA (Miller, Bartow et al. 2011) and Europe (Malaval, Lauga et al. 2010, Jørgensen, Elameen et al. 2016). In Germany, a seed transfer zone concept including a seed transfer zone map and seed zone-specific species lists has been implemented since 2010 (Prasse, Kunzmann et al. 2010). Seed transfer zones were determined on basis of the German system of 89 natural regions (Meynen, Schmithüsen et al. 1953-62), which were grouped together to 22 seed transfer zones within eight producing areas according to similar environmental conditions (Bucharova, Bossdorf et al. 2018). Within a seed transfer zone, source seeds from several large populations have to be collected, mixed thoroughly, reproduced and can be transferred only within this zone. This seed sourcing strategy is called *regional admixture provenancing* and offers great advantages for restoration: The system provides almost unlimited amount of locally adapted seed material for a huge number of species in every part of Germany. Generally, in restoration the use of local seeds or plant material is recommended (Mijnsbrugge, Bischoff et al. 2010) because plants are adapted to their surrounding environmental conditions. Ecological (isolation-by-environment) or geographical (isolation-by-distance) differences among habitats may cause the development of ecotypes and local adaptations (Joshi, Schmid et al. 2001, Bischoff, Cremieux et al. 2006, Leimu and Fischer 2008). That is why blending genotypes originating from genetically

differing seed sources may result in outbreeding depression (Hufford and Mazer 2003). Co-adapted gene complexes can be destroyed and local adaptations get lost which leads to decreased fitness and performance of plant populations (Keller, Kollmann et al. 2000, Montalvo and Ellstrand 2001, Frankham, Ballou et al. 2002). This may be avoided, when seeds reflect the gene pool of the naturally occurring individuals and populations near the restored areas (McKay, Christian et al. 2005).

However, genetic differentiation between populations does not only depend on ecological or geographic distances among populations, but also on life-history characteristics, such as mating system, pollination vector or dispersal unit (Hamrick and Godt 1996, Reisch and Bernhardt-Römermann 2014). For example, an outcrossing wind-pollinated plant species is likely to show lower genetic differentiation over large geographic distances as it is the case for an endemic outcrossing and insect-pollinated plant species. Considering the natural differentiation of plant populations due to abiotic and biotic factors the questions arise, how strong populations restored with local seed mixtures may vary from natural ones and if it is possible to ensure, that the genetic differentiation between natural and restored populations corresponds to the spatial genetic differentiation pattern of naturally occurring populations.

Furthermore, the production of seed material including sampling method of source seeds and propagation within a seed-farm may have major impacts on genetic diversity. This matters for example, when seeds were collected from small populations because these are less attractive to pollinators (Agren 1996, Kunin 1997). Reduced cross pollination increases mating with related individuals or even self-fertilization (Van Treuren, Bijlsma et al. 1994), which may increase inbreeding and lead to reduced fitness and decreased genetic variation (Friar, Ladoux et al. 2000, Frankham, Ballou et al. 2002). Furthermore, collecting seed material from a small number of source individuals in a large source population may cause genetic drift. A frequency shift of gene variants can reduce genetic diversity or local adaptations (Espeland, Emery et al. 2017) and lead to increased homozygosity and random loss or fixation of deleterious alleles (Ellstrand and Elam 1993, Young, Petersen et al. 2005). Seed sampling is followed by cultivation of source seed and their reproduction. Stock individuals can be used several years for the production of local seeds. The multiple reproduction cycles may decrease genetic variation and increase the risk of inbreeding (Schoen and Brown 2001). Additionally, the plants are exposed to different environmental conditions than

in their naturally habitat and unintended selection during the cultivation stage might be inevitable (Espeland, Emery et al. 2017, Nagel, Durka et al. 2019)

To avoid these negative effects caused by cultivation processes, there are procedural rules to follow for seed production. Therefore, commercially produced seed material is expected to exhibit a high genetic variability which is maintained by mixing source seeds of several large source populations. This procedure shall ensure the preservation of genetic variation. Finally, multiplying plant material only for a short period (for example 5 generations) should decrease the risk of unintended selection during the propagation and also the possibility of inbreeding depression and genetic erosion due to multiple reproduction cycles (Prasse, Kunzmann et al. 2010, ErMiV 2011).

Although there are some genetic studies discussing seed origin and genetic differentiation among seed transfer zones (Michalski and Durka 2012, Bucharova, Michalski et al. 2017, Durka, Michalski et al. 2017, Listl, Poschlod et al. 2017, Listl, Poschlod et al. 2017), the impact of sowing local seeds on the genetic variation of forbs has much less frequently been studied. Only few studies directly compared genetic variation of plant populations from natural and restored grasslands (Aavik, Edwards et al. 2012, Reiker, Schulz et al. 2015).

Aim of this study was, therefore, to test if genetic variation within and among populations restored with local seed material corresponds to the variation of neighboring natural populations. We selected three widely distributed, outcrossing forbs (*Knautia arvensis, Silene vulgaris* and *Plantago lanceolata*) and analyzed the genetic diversity and differentiation of natural and restored populations in a comparative approach using amplified fragment length polymorphisms (AFLPs). We asked the following questions

(i) How strong is genetic differentiation among natural and restored populations?

(ii) How large is the genetic diversity of natural and restored populations?

(iii) Are local seed mixtures a promising tool to restore both species and genetic diversity of species-rich grasslands?

METHODS

Study species and design

For our study, we selected three common outcrossing forbs, occurring in natural and restored grasslands: the insect-pollinated *Knautia arvensis* (Coult.) and *Silene vulgaris* (Garcke) and the wind-pollinated *Plantago lanceolata* (L.).

We sampled plant material for molecular analysis in populations of the study species from ten natural (N) and ten restored (R) species-rich grasslands. The populations were located in five study regions across Bavaria in Germany (Figure 5.1; Table 5.1). Study sites were not identical since not all species occurred at all sites simultaneously. However, we always compared ten natural populations and ten restored populations.

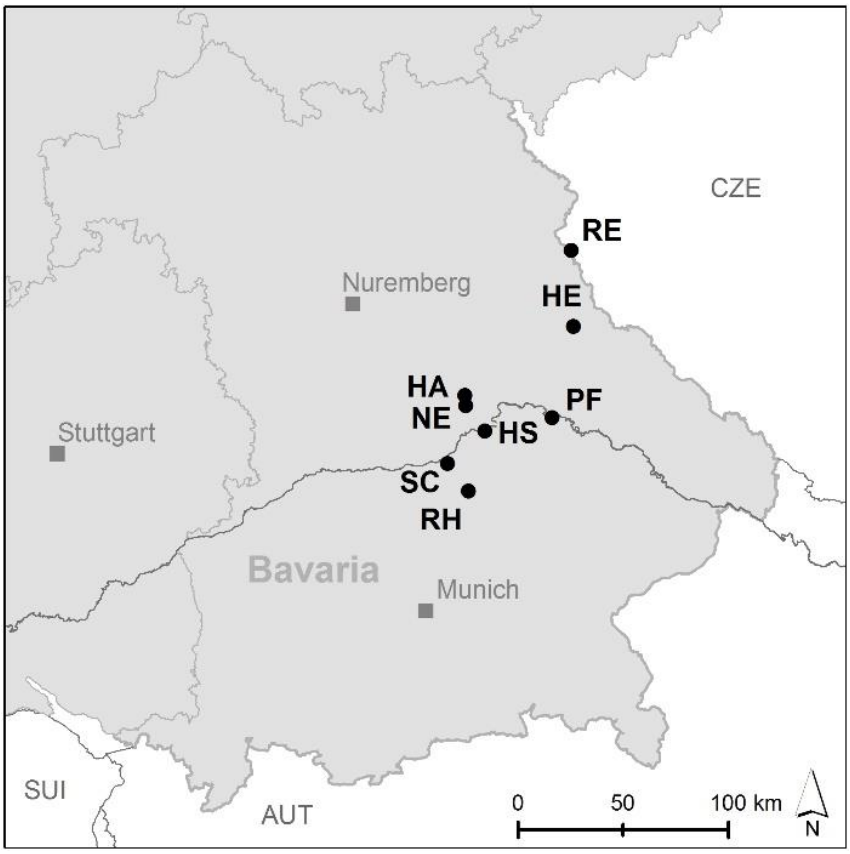


Figure 5.1: Geographic position of the study regions in south eastern Germany. 10 natural and 10 restored populations of the study species *Knautia arvensis*, *Silene vulgaris* and *Plantago lanceolata* were investigated in each of the five study regions: Study regions for *K. arvensis* were HE, NE, PF, RH and SC. Populations of *S. vulgaris* were situated within HA, NE, PF, RH and SC and the investigated populations of *P. lanceolata* were situated within HS, NE, PF, RE and RH. HE = Hetzmannsdorf, NE = Netzstall, PF = Pfatter; RH = Rannertshofen; SC = Schwaig; HA = Haag; HS = Herrnsaal; RE = Reichenau.

Table 5.1: Number, study region, names, categories and geographic location of analyzed populations of a) *Knautia arvensis*, b) *Silene vulgaris* and c) *Plantago lanceolata*. Also specified are the number of analyzed individuals (N) and Nei's Gene diversity (H), including all means with standard errors for natural and restored populations. HE = Hetzmannsdorf, NE = Netzstall, PF = Pfatter; RH = Rannertshofen; SC = Schwaig; HA = Haag; HS = Herrnsaal; RE = Reichenau.

Nr.	Study region	Population	Category	Latitude	Longitude	N	H
a)							
1	HE	HE_N1	natural	4.934.628	1.250.623	16	0.21
2	HE	HE_N2	natural	4.933.422	1.250.729	13	0.20
3	NE	NE_N1	natural	4.902.381	1.186.284	16	0.20
4	NE	NE_N2	natural	4.904.336	1.186.960	16	0.20
5	PF	PF_N1	natural	4.897.689	1.236.611	14	0.19
6	PF	PF_N2	natural	4.896.185	1.246.250	15	0.19
7	RH	RH_N1	natural	4.864.168	1.181.630	15	0.19
8	RH	RH_N2	natural	4.866.278	1.181.008	15	0.17
9	SC	SC_N1	natural	4.877.587	1.171.611	16	0.19
10	SC	SC_N2	natural	4.878.536	1.172.866	15	0.14
	Mean natural populations						**0.19[a]**
	Standard error						**0.01**
11	HE	HE_R1	restored	4.935.013	1.251.966	14	0.19
12	HE	HE_R2	restored	4.936.063	1.253.116	16	0.22
13	NE	NE_R1	restored	4.901.584	1.181.664	15	0.23
14	NE	NE_R2	restored	4.901.077	1.182.420	16	0.22
15	PF	PF_R1	restored	4.896.704	1.237.576	15	0.22
16	PF	PF_R2	restored	4.897.702	1.239.393	15	0.26
17	RH	RH_R1	restored	4.865.359	1.183.969	16	0.21
18	RH	RH_R2	restored	4.865.378	1.183.551	16	0.17
19	SC	SC_R1	restored	4.877.228	1.169.663	16	0.20
20	SC	SC_R2	restored	4.878.370	1.170.407	16	0.21
	Mean restored populations						**0.21[b]**
	Standard error						**0.01**
b)							
1	HA	HA_N1	natural	4.907.680	1.182.332	15	0.38
2	HA	HA_N2	natural	4.908.555	1.181.543	15	0.35
3	NE	NE_N1	natural	4.904.336	1.186.960	15	0.36
4	NE	NE_N2	natural	4.904.375	1.189.968	16	0.36
5	PF	PF_N1	natural	4.897.689	1.236.611	15	0.37
6	PF	PF_N2	natural	4.898.161	1.241.632	15	0.36
7	RH	RH_N1	natural	4.870.202	1.179.640	15	0.31
8	RH	RH_N2	natural	4.866.394	1.181.020	14	0.32
9	SC	SC_N1	natural	4.877.587	1.171.611	14	0.29
10	SC	SC_N2	natural	4.878.536	1.172.866	15	0.36
	Mean natural populations						**0.35[a]**
	Standard error						**0.01**
11	HA	HA_R1	restored	4.906.022	1.182.210	16	0.36
12	HA	HA_R2	restored	4.906.692	1.182.260	16	0.36
13	NE	NE_R1	restored	4.901.584	1.181.664	15	0.34
14	NE	NE_R2	restored	4.901.077	1.182.420	15	0.35
15	PF	PF_R1	restored	4.896.704	1.237.576	16	0.37
16	PF	PF_R2	restored	4.897.702	1.239.393	16	0.35
17	RH	RH_R1	restored	4.865.359	1.183.969	15	0.37
18	RH	RH_R2	restored	4.865.378	1.183.551	15	0.38
19	SC	SC_R1	restored	4.877.228	1.169.663	13	0.35
20	SC	SC_R2	restored	4.878.370	1.170.407	15	0.37
	Mean restored populations						**0.36[a]**
	Standard error						**0.00**

Table 5.1 (continued).

Nr.	Study region	Population	Category	Latitude	Longitude	N	H
c)							
1	HS	HS_N1	natural	4.892.020	1.194.383	15	0.32
2	HS	HS_N2	natural	4.891.838	1.192.749	16	0.30
3	NE	NE_N1	natural	4.901.309	1.181.329	16	0.30
4	NE	NE_N2	natural	4.904.336	1.186.960	16	0.29
5	PF	PF_N1	natural	4.897.689	1.236.611	14	0.34
6	PF	PF_N2	natural	4.898.944	1.241.210	14	0.35
7	RE	RE_N1	natural	4.865.605	1.185.441	16	0.33
8	RE	RE_N2	natural	4.864.168	1.181.630	15	0.32
9	RH	RH_N1	natural	4.968.725	1.251.595	15	0.33
10	RH	RH_N2	natural	4.967.111	1.251.398	15	0.30
	Mean natural populations					**15**	**0.32[a]**
	Standard error						**0.01**
11	HS	HS_R1	restored	4.891.721	1.196.153	15	0.32
12	HS	HS_R2	restored	4.891.367	1.193.815	16	0.31
13	NE	NE_R1	restored	4.901.584	1.181.664	15	0.29
14	NE	NE_R2	restored	4.901.077	1.182.420	15	0.31
15	PF	PF_R1	restored	4.896.704	1.237.576	16	0.34
16	PF	PF_R2	restored	4.897.702	1.239.393	16	0.32
17	RE	RE_R1	restored	4.865.359	1.183.969	14	0.31
18	RE	RE_R2	restored	4.865.400	1.184.102	15	0.28
19	RH	RH_R1	restored	4.968.182	1.251.814	15	0.34
20	RH	RH_R2	restored	4.967.763	1.251.950	14	0.31
	Mean restored populations					**15**	**0.31[a]**
	Standard error						**0.01**

Natural grasslands were historically old, which means that they have been continuously used as grassland since 19 th century and were identified using historical cadastral maps and recent maps from 2005 and 2015. Restored populations were located on former arable land. After topsoil removal ten to fifteen years ago, commercially produced local seed mixtures from a big, German seed farming company have been applied at these sites to restore species-rich grassland.

For molecular analysis, fresh leaf material was collected in situ from sixteen individuals per population. In total, material of 320 individuals was sampled and dried in teabags over silica gel.

Ploidy levels of the study species

As a first step, we applied Flow Cytometry (FCM) to identify potentially occurring different cytotypes, investigating the same plant material that was used for AFLP analysis. For each species, we tested one individual per population. Methodological details are attached in *Appendix S 5.1*.

Molecular analysis

DNA was isolated from 15mg dried plant material applying the cetyltrimethylammonium bromide protocol by Rogers and Bendich (1994) with adaptions by Reisch (2007). All samples were standardized at a concentration of 7.8 ng/ µL. The AFLP method was performed in accordance with the Beckman Coulter protocol as described before (Bylebyl, Poschlod et al. 2008).

Restriction-Ligation was performed in a reaction volume of 10 µL, containing genomic DNA, *Eco*RI (MBI Fermentas) and *Mse*I (MWG Biotech) restriction enzymes and T4 DNA Ligase (MBI Fermentas). The samples were incubated for two hours at 37 °C.

PCRs were performed in a reaction volume of 5 µL. Preselective primers had one selective nucleotide (*Eco*RI-A; *Mse*I-C). For selective amplification, a primer screening was conducted. For each species, 30 combinations were tested and then three combinations per species were selected for further analysis. The fluorescence labeled selective primers had three selective nucleotides (*Table S 5.1*).

The fluorescence labeled products were diluted with 5 µL (D2) and with 20 µL (D4) 1:10 TE buffer for DNA and then according to their size, separated by capillary gel electrophoresis on an automated sequencer (GeXP, Beckmann Coulter). Results were examined using the software Bionumerics 4.6 (Applied Maths, Kortrijk, Belgium). For quality control of the AFLP procedure a genotyping error rate was calculated (Bonin, Bellemain et al. 2004), which was 3.1 % for *K. arvensis*, 2.9 % for *S. vulgaris* and 4.7 % for *P. lanceolata*.

Statistical analysis

For band detection, each strong and clearly defined fragment was taken either into account as present (1) or absent (0). The generated binary (0/1) matrix was used for further statistical analysis.

Bayesian cluster analysis were calculated with Structure, version 2.3.4 (Pritchard, Stephens et al. 2000) to infer population structure in the data set and assign individuals into groups. The potential number of groups was calculated using 10000 Markov Chain Monte Carlo (MCMC) simulations with a burn-in-period of 100000 iterations. Analysis for the predefined value of K were run 20 times per K = 1-22 (Falush, Stephens et al.

2003). Results were summarized by employing the program Structure Harvester (Earl and Vonholdt 2012). Group assignment was an ad hoc quantity procedure calculating ΔK (Evanno, Regnaut et al. 2005). According to the model, which gave the consistent results for multiple runs and the highest probability of the data, the best estimate of K for the data set was determined.

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

Hierarchical analysis of molecular variance, AMOVA (Excoffier, Smouse et al. 1992), were also conducted with the software GenAlEx 6 . Thus genetic differentiation within and among populations was investigated in two- and three-level AMOVAs.

Correlation between genetic distances (Φ_{PT} values calculated in the AMOVA) and geographic distances among populations was tested in a Mantel test with 999 permutations (Mantel 1967).

Gene diversity H was calculated using AFLPsurv (Vekemans 2002). A Wilcoxon-test was used to test for significant differences in genetic diversity between natural and restored populations applying the software IBM Statistics 24 for Windows (IBM Corp).

RESULTS

Ploidy levels of the study species

FCM revealed different ploidy levels for *Knautia arvensis*. All natural populations and the restored populations from Schwaig (SC) were tetraploid. The restored populations of Hetzmannsdorf (HE), Netzstall (NE), Pfatter (PF) and Rannertshofen (RH) were diploid. We detected no different ploidy levels for *Silene vulgaris* and *Plantago lanceolata*.

Genetic differentiation among natural and restored populations

The Bayesian cluster analysis resulted in two groups for all study species. For *K. arvensis* (ΔK = 282.2), the first group included all natural and the restored populations SC_R1 and SC_R2 (tetraploid populations). The remaining restored populations (diploid populations) formed the second group (Figure 5.2a).

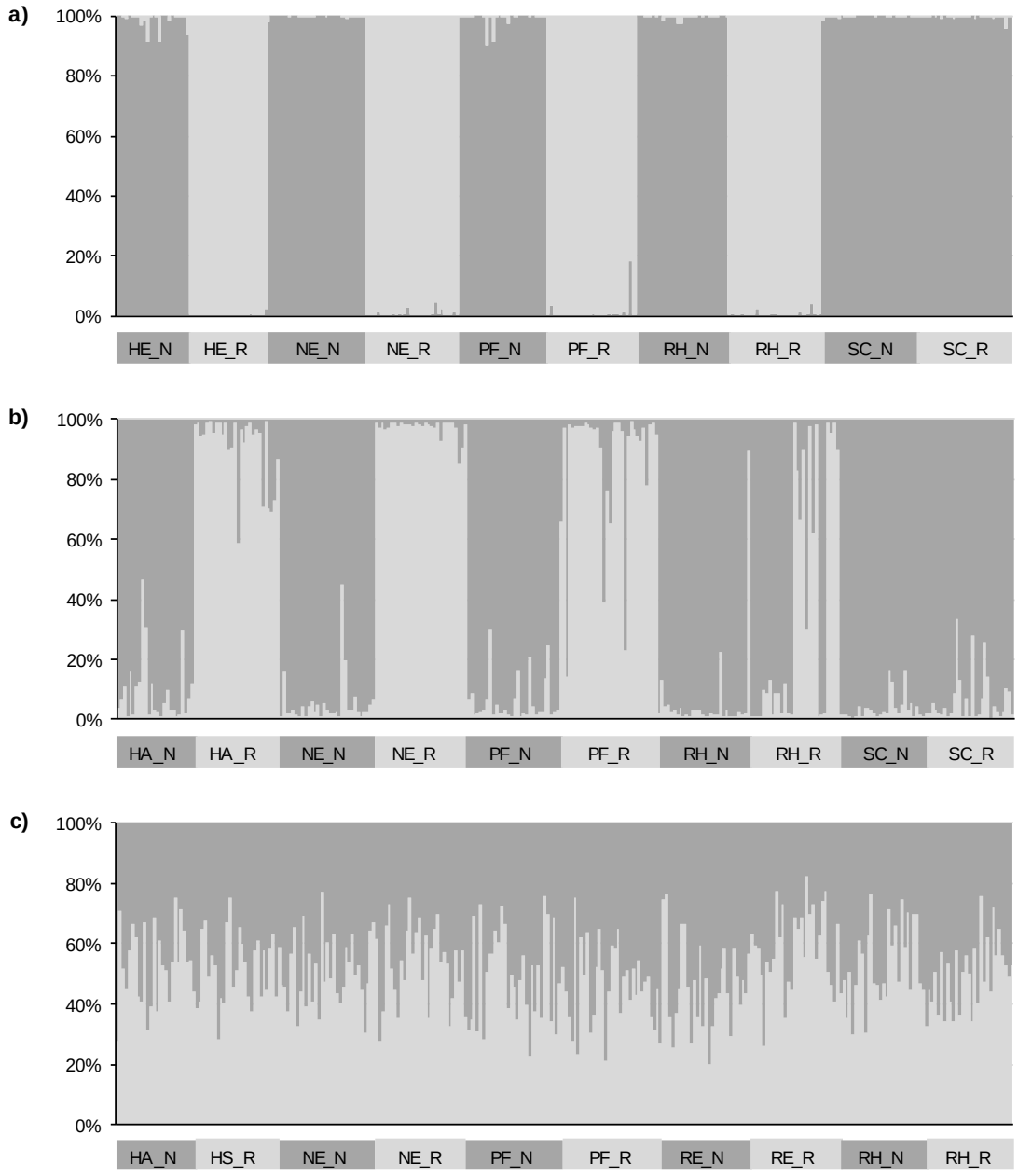


Figure 5.2: Bayesian Cluster Analysis for a) *Knautia arvensis* based on 137 AFLP fragments, b) *Silene vulgaris* based on 121 AFLP fragments and c) *Plantago lanceolata* based on 127 AFLP fragments. Populations of *K. arvensis* (ΔK_{Ka} = 282.2; 2c) and *S. vulgaris* (ΔK = 153; 3c) were assigned to two groups. Individuals of *P. lanceolata* (2 groups, ΔK = 3.4) were admixed.

For *S. vulgaris* (ΔK = 153) all natural populations and most individuals of the restored populations from the regions RH and SC formed the first group. The restored populations belonging to the regions HA, NE and PF built the second group (Figure 5.2b). Although the individuals of *P. lanceolata* (ΔK = 3.4) were assigned in two groups we detected no population grouping according to the grassland type or study region (Figure 5.2c). For every species, K = 2 outputs of the 20 iterations were identical.

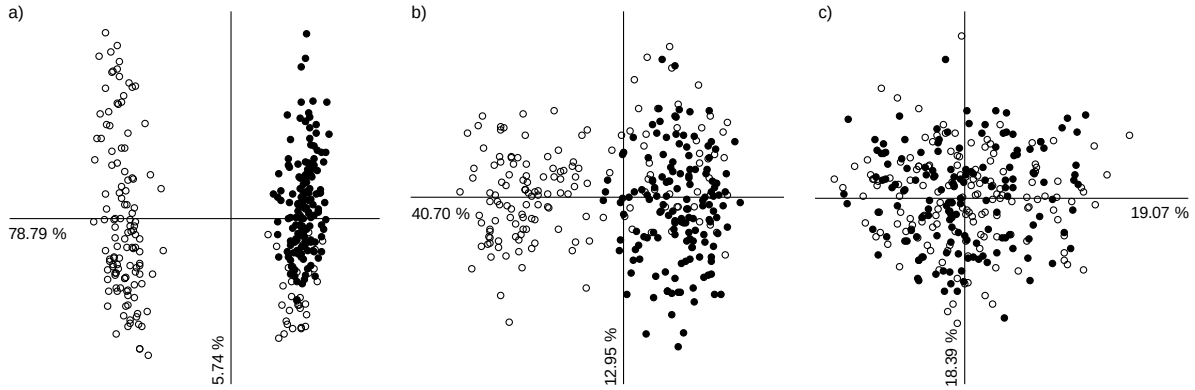


Figure 5.3: Results of the principal coordinates analysis (PCoA) based on AFLP data of the study species. Triangles, light gray: individuals of natural populations. Triangles, dark gray: individuals of restored populations. a) *Knautia arvensis*: Axis 1 explained 78.79 % of variance; axis 2 explained 5.74 % of variance in the data set. Individuals were separated clearly into two groups, consisting of natural individuals (group 1) or natural and restored individuals (group 2). b) *Silene vulgaris*: Axis 1 explained 78.79 % of variance and axis 2 explained 5.74 % of variance in the data set. PCoA resulted in two slightly mixed groups. Group 1: natural individuals. Group 2: natural and restored individuals. c) *Plantago lanceolata*: Individuals of natural and restored populations were admixed. 21.48 % of variance in the data set was explained by axis 1 and 15.28 % by axis 2

The PCoA resulted in a strong separation of natural and restored populations for *K. arvensis*. According to the findings of FCM analysis, diploid restored populations from the study regions HE, NE, PF and RH built the first group. The tetraploid restored populations SC_R1 an SC_R2 were grouped with the tetraploid natural populations (Figure 5.3a). Natural and restored populations of *S. vulgaris* showed stronger admixture and we identified two clusters according to the findings of the Bayesian cluster analysis (Figure 5.3b). The natural and restored populations of *P. lanceolata* were admixed and no groups were distinguishable (Figure 5.3c).

The AMOVA (Table 5.2) revealed low genetic differentiation between the study regions for all study species. However, we observed very strong genetic differentiation for *K. arvensis* (Table 5.2a) among all populations (Φ_{PT} = 0.49). With respect to the results of FCM, the ploidy levels were taken into count for further analysis: the genetic differentiation between tetraploid restored and natural populations was with a Φ_{PT} value of 0.19 comparable low. However, we found lower levels of genetic differentiation among all natural populations (tetraploid populations; Φ_{PT} = 0.13). Additionally, we conducted separate AMOVAs among all diploid and among all tetraploid populations and found a genetic differentiation comparable to the differentiation between the natural populations (for both Φ_{PT} = 0.14). For *S. vulgaris* (Table 5.2b), the AMOVA resulted

in low genetic differentiation among all populations (Φ_{PT} = 0.13) and slightly stronger differentiation between natural and restored populations (Φ_{PT} = 0.16). Moreover, a low genetic differentiation among natural (Φ_{PT} = 0.09) and restored (Φ_{PT} = 0.09) populations was observed. The conducted AMOVAs for *P. lanceolata* (Table 5.2c) between all populations, natural and restored populations as well as among natural and among restored populations revealed low genetic differentiation (for all Φ_{PT} = 0.03).

Table 5.2: Results of the analysis of molecular variance (AMOVA) for a) *Knautia arvensis*, b) *Silene vulgaris* and c) *Plantago lanceolata*. Levels of significance are based on 999 iteration steps and indicated by asterisks (p < 0.001). df indicates degree of freedom, SS the sum of squares, MS the mean squares, % the proportion of genetic variability.

	df	SS	MS	%	ΦPT	
a)						
Molecular variation among all populations						
Among populations	19	2377.25	125.12	48.66	0.49	***
Within populations	285	2307.59	8.10	51.34		
Molecular variation between study regions						
Between study regions	4	430.55	107.64	0.00	0.49	***
Among populations	15	1946.70	129.78	49.65		
Within populations	285	2307.59	8.10	50.35		
Molecular variation between natural and restored populations (tetraploid)						
Between natural and restored populations	1	68.897	68.897	8.83	0.19	***
Among populations	10	231.557	23.156	10.46		
Within populations	171	1.334.355	7.803	80.71		
Molecular variation among natural populations (tetraploid)						
Among populations	9	220.49	24.50	12.74	0.13	***
Within populations	141	1078.60	7.65	87.26		
Molecular variation among all diploid populations						
Among populations	7	210.965	30.138	14.23	0.14	***
Within populations	114	973.240	8.537	85.77		
Molecular variation among all tetraploid populations						
Among populations	11	300.454	27.314	14.09	0.14	***
Within populations	171	1.334.355	7.803	85.91		
b)						
Molecular variation among all populations						
Among populations	19	842.17	44.32	12.94	0.13	***
Within populations	281	3848.93	13.70	87.06		
Molecular variation between study regions						
Between study regions	4	202.52	50.63	0.84	0.13	***
Among populations	15	639.65	42.64	12.21		
Within populations	281	3848.93	13.70	86.95		
Molecular variation between natural and restored populations						
Between natural and restored populations	1	228.16	228.16	7.89	0.16	***
Among populations	18	614.01	34.11	8.30		
Within populations	281	3848.93	13.70	83.81		
Molecular variation among natural populations						
Among populations	9	300.40	33.38	9.26	0.09	***
Within populations	139	1840.54	13.24	90.74		
Molecular variation among restored populations						
Among populations	9	313.61	34.85	8.79	0.09	***
Within populations	142	2008.39	14.14	91.21		

Table 5.2 (continued).

	df	SS	MS	%	ΦPT	
c)						
Molecular variation among all populations						
Among populations	19	286.66	15.09	03.07	0.03	***
Within populations	283	2885.81	10.20	96.93		
Molecular variation between regions						
Between study regions	4	63.98	16.00	0.18	0.03	***
Among populations	15	222.68	14.85	2.92		
Within populations	283	2885.81	10.20	96.90		
Molecular variation between natural and restored populations						
Between natural and restored populations	1	16.17	16.17	0.07	0.03	***
Among populations	18	270.49	15.3	03.3		
Within populations	283	2885.81	10.20	96.90		
Molecular variation among natural populations						
Among populations	9	131.11	14.57	2.59	0.03	***
Within populations	142	1473.77	10.38	97.41		
Molecular variation among restored populations						
Among populations	9	139.38	15.49	3.49	0.04	***
Within populations	141	1412.03	10.01	96.51		

According to the FCM results, three Mantel-tests for *K. arvensis* were conducted, for all populations, for the diploid populations and for all tetraploid populations. We found no correlation between pairwise genetic and geographic distances for the species (r_{Ka} = 0.01, p_{Ka} = 0.62; $r_{Ka\_diploid}$ = 0.04, $p_{Ka\_diploid}$ = 0.34; $r_{Ka\_tetraploid}$ = 0.03, $p_{Ka\_tetraploid}$ = 0.39). The Mantel-tests for *S. vulgaris* and *P. lanceolata* also revealed no correlation between pairwise genetic distances and geographic distances (r_{Sv} = 0.05, p_{Sv} = 0.18; r_{Pl} = -0.05, p_{Pl} = 0.30).

Genetic diversity of natural and restored populations

For *K. arvensis*, 82.48 % of the fragments were polymorphic. In natural populations, Nei's Gene Diversity (H) ranged from 0.14 to 0.21 (mean 0.19; Table 5.1a). In restored populations, H values were significantly higher than in natural populations and ranged from 0.17 to 0.23 (mean 0.21; p = 0.04).

For *S. vulgaris*, 89.26 % of the fragments were polymorphic. H ranged from 0.29 to 0.37 (mean 0.35) for natural populations and from 0.34 to 0.38 (mean 0.36) for restored populations. No significant difference could be detected between natural and restored populations (p = 0.39; Table 5.1b).

For *P. lanceolata* the percentage of polymorphic fragments was 83.46 %. Nei's gene diversity ranged from 0.29 to 0.35 (mean 0.32) for natural populations and from 0.28

and 0.34 (mean 0.31) for restored populations (Table 5.1c). Between natural and restored populations, no significant difference could be detected b (p = 0.80).

DISCUSSION

Genetic differentiation between natural and restored populations

Gene flow, genetic drift and adaptation to local environmental conditions and their interactions strongly affect genetic differentiation (Slatkin 1987). The exchange of pollen, seeds or plant material among populations should reduce genetic differences between populations (Slatkin 1987) and result in comparatively low levels of differentiation especially among populations of widespread and outcrossing forbs. It is, therefore, assumed that local seed mixtures originating from delineated seed transfer zones reflect the spatial genetic structure of common forbs (Hufford and Mazer 2003).

However, our study revealed varying degrees of differentiation between natural and restored populations for the three investigated plant species. The differentiation was stronger between natural and restored populations of the two insect-pollinated species than between natural and restored populations of the wind-pollinated species, which can be attributed to the large-scale dispersal of pollen via wind, reducing the degree of differentiation.

For *Knautia arvensis* the genetic differentiation between natural and restored populations (Φ_{PT} = 0.59) was very strong. This is mainly because different ploidy levels occurred in natural and restored populations. It has already been shown before that *K. arvensis* exhibits various cytotypes which do not interbreed (Kolar, Stech et al. 2009, Durka, Michalski et al. 2017) and therefore, function as effective breeding barriers (Kohler, Mittelsten Scheid et al. 2010). Consequently, the two ploidy levels can be regarded as separate taxonomic units.

Considering only tetraploid populations, genetic differentiation between natural and restored populations was with a Φ_{PT} value of 0.19 slightly higher than the genetic variation between natural populations (Φ_{PT} = 0.13). Genetic differentiation among diploid or tetraploid populations was comparable. This provides evidence that the natural genetic structure of the species seems not to be strongly affected by applying local seed material, when the correct ploidy level is used.

In our study, all natural populations of *K. arvensis* were tetraploid, may be because of the limited population number. However, according to previous investigations, in our study region located in the Danube region both diploid and tetraploid populations of *K. arvensis* may occur (Kolar, Stech et al. 2009, Durka, Michalski et al. 2017). The restoration of grassland with diploid populations closely located to tetraploid natural populations may therefore be acceptable, although not being optimal since the local genetic pattern is clearly affected.

Our study revealed also a significant differentiation between natural and restored populations of *Silene vulgaris* (Φ_{PT} = 0.16), although populations were more strongly admixed than observed for *K. arvensis*. However, genetic differentiation between natural and restored populations was twice as high as among natural populations. Thus, the local seed material did not match the natural spatial genetic pattern of the species exactly. This observation goes in line with findings of a former study by Aavik, Edwards et al. (2012) who also detected significant genetic differentiation between natural and restored populations of the widespread, outcrossing plant species *Lychnis flos-cuculi* L. in grasslands.

For *P. lanceolata* it has been reported in former studies that genetic differentiation between populations may depend on geographic distances between populations and on environmental distances between habitats (Bischoff, Cremieux et al. 2006, Crémieux, Bischoff et al. 2010). In our study genetic differentiation between natural and restored populations was, however, comparable to the genetic differentiation between natural populations and even lower than previously reported for other wind-pollinated species (Reisch and Bernhardt-Römermann (2014). Therefore, the application of local seed material did not distort the natural spatial genetic structure of the species in our study area.

Summing up, our investigation revealed a slight but significant genetic differentiation between natural and restored populations of insect pollinated forbs, which means that commercially produced seed mixtures did not fully reflect the local genetic structure of the species. This means not necessarily that the concept for the production of local seed mixtures failed. Mixing the seed material from several source populations within the seed transfer zone is supposed to guarantee high levels of genetic variation within populations but it is clear that this approach must cause genetic differentiation at the same time.

Thus, commercially produced seed material reflects the genetic potential of the entire seed transfer zone, but matches not exactly the local genetic pattern. Nevertheless, seed material from a commercially produced seed mixture will still be genetically closer to natural populations than seed material from anywhere.

Genetic diversity of natural and restored populations

In the context of using local seeds for restoration, it is often questioned whether commercially produced seed material is variable enough to establish vital populations (Espeland, Emery et al. 2017, Nagel, Durka et al. 2019). For example, the source populations of collected stock seeds maybe had been inbred due to small population size, isolation or fragmentation (Aavik, Edwards et al. 2012). Genetic diversity can also be reduced, when only a few source individuals are sampled, which may cause bottleneck effects and enhance genetic drift (Friar, Ladoux et al. 2000). Furthermore, the seed stock for several reproduction cycles, which may lead to inbreeding and reduced genetic diversity (Schoen and Brown 2001). Studies showed, that genetic diversity of populations can be negatively affected by bottlenecks, isolation or small population size (Ellstrand and Elam 1993) and that fragmentation can have a negative impact on genetic diversity of common plant species as well as on rare ones (Honnay and Jacquemyn 2007).

However, we detected no reduced genetic diversity in the restored populations of our three study species. In contrast to the apprehensions, the observed genetic diversity of the restored populations was equal or even higher compared to the genetic diversity of the investigated natural populations. Our results support the few existing previous studies, where the authors also reported no decreased levels of genetic diversity in restored grassland plant populations (Aavik, Edwards et al. 2012, Reiker, Schulz et al. 2015). Furthermore, the genetic diversity observed in natural and restored populations of *K. arvensis* (mean H = 0.20), *S. vulgaris* (mean H = 0.36) and *P. lanceolata* (mean H = 0.32) was even higher than reported in literature (Reisch and Bernhardt-Römermann 2014).

The level of genetic diversity we observed in restored populations of the three study species support the system used for seed production in Germany. With the applied

regional admixture provenancing (Bucharova, Bossdorf et al. 2018) it seems to be possible to maintain high genetic diversity of common forbs in local seed mixtures. The system is based on 89 natural regions across Germany, defined by Meynen, Schmithüsen et al. (1953-62). These 89 natural regions were summarized to 22 seed transfer zones. For the production of local seed mixtures stock seed from at least five large source populations distributed across a seed transfer zone are collected, mixed thoroughly and then be propagated for up to five generations (Prasse, Kunzmann et al. 2010), which seems to be an suitable time span to avoid decreased genetic diversity.

Conclusions

The use of local seed mixtures is a frequently applied and effective practice in ecological restoration of species-rich grasslands (Zahlheimer 2009, Prasse, Kunzmann et al. 2010, Kiehl, Kirmer et al. 2014). Nevertheless, such a general procedure may raise concerns about the quality of commercially produced seed material. It seems to be questionable whether the natural spatial genetic pattern of common plant species can be maintained while producing local seed material with a sufficient level of genetic diversity.

In our study, the seed material used for restoration reflected the natural genetic structure of the species to a very different degree. In the case of *K. arvensis* restored populations in four of five study regions differed in ploidy level from the corresponding natural populations. In our study area both ploidy levels of *K. arvensis* may occur (Kolar, Stech et al. 2009). Differing ploidy levels between natural and restored populations may therefore be acceptable. Nevertheless, it would be better to use the same ploidy level for restoration to preserve the local genetic pattern of the species. The distribution pattern of the cytotypes needs, therefore, to be investigated more precisely and should be carefully considered when sampling source populations in future. The difference between natural and restored populations was smaller in *S. vulgaris* than in *K. arvensis*. However, genetic differentiation between natural and restored populations was also nearly twice as large as between natural populations of the species, indicating that the natural genetic structure of *S. vulgaris* is affected at the local scale by the

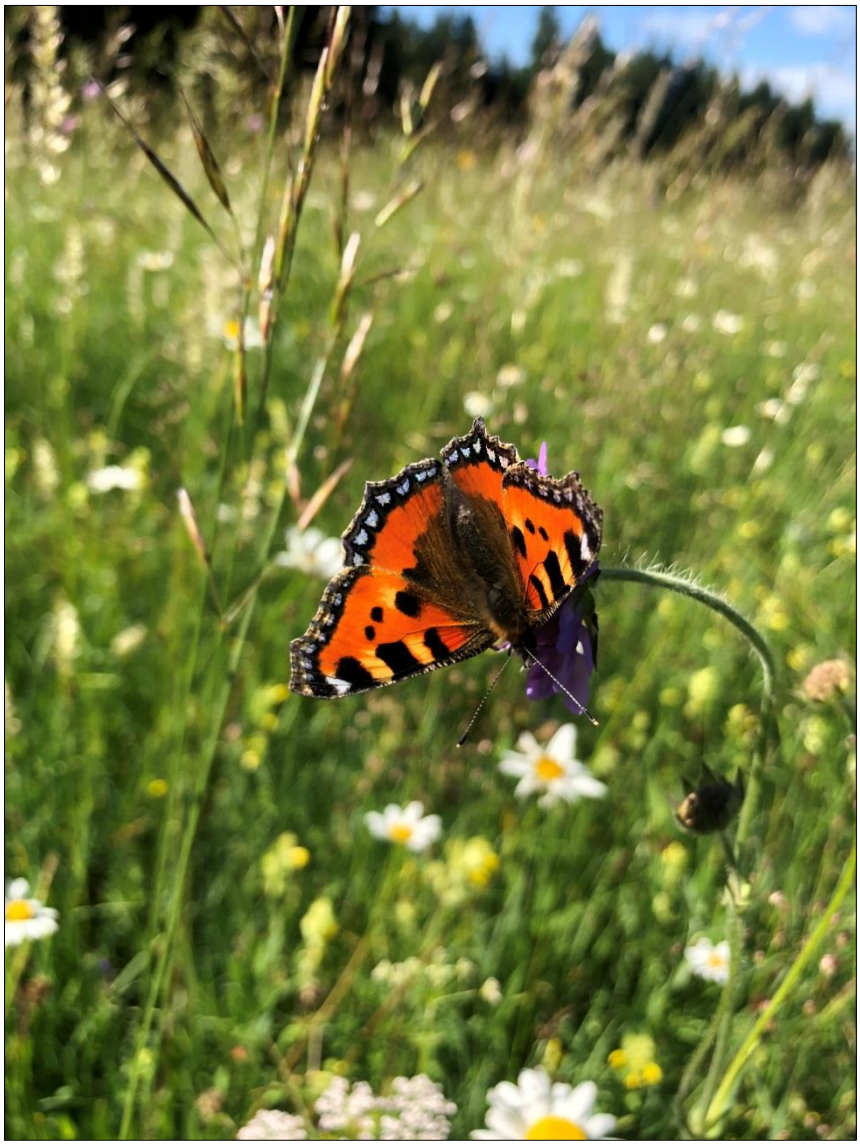
application of commercially produced seed material for restoration. For the wind-pollinated *P. lanceolata* genetic differentiation between natural and restored populations was within the range of the natural populations. The use of local seed material for restoration has therefore no impact on the local genetic structure of this species.

The application of local seed material is a big step forward in restoration practice and with the system of seed production and seed transfer zones an almost unlimited amount of regionally specific seeds for restoration is provided for a wide range of plant species. Our study clearly shows that the local genetic structure especially of insect pollinated plant species may be affected by the use of commercially produced seed material. It is clear that the system of *regional admixed provenancing* is not designed to match exactly the genetic structure of plant populations at a very small local scale, but rather to protect the broader patterns of genetic variation. Furthermore, commercially produced seed material may match the ecological conditions within a seed transfer zone. Finally, using regional seed mixtures for restoration is still better than using seeds from far away. However, there are possibilities to improve the system, for example by including different habitat types in the seed collection process or by minimizing the size of seed transfer zones, although we are aware that the size of zones has to be large enough to allow profit for the seed producers. Further genetic analyses are needed to better understand the patterns of genetic variation in common forbs, which may then contribute to optimize the system of *regional admixed provenancing*.

Whereas natural and restored populations often differed genetically in our study, genetic diversity was comparable within both grassland types. The results presented here clearly support the assumption that highly diverse populations of forbs can be created using commercially produced local seed material. The implemented *regional admixture provenancing* strategy (Bucharova, Bossdorf et al. 2018) seems, therefore, to be an appropriate method to produce genetically diverse local seed material and with further genetic research and some adjustments in sampling and multiplying strategy of source seeds, the procedure will become an even more powerful tool in conservation management.

CHAPTER SIX:

GENERAL DISCUSSION, CONCLUSION & PERSPECTIVES



GENERAL DISCUSSION AND CONCLUSIONS

The rapid loss of biodiversity in nearly all ecosystems over the world is mostly caused by human activities. Especially changes in land use practice due to agricultural intensification (Muller, Dutoit et al. 1998) and landscape fragmentation (Fischer and Stocklin 1997) contribute to the drastically decrease in species richness during the past decades. However, when processes, functioning and the structure of ecosystems are altered their resilience to environmental changes declines (Tallis and Kareiva 2005). As the function and stability of ecosystems is essential since they provide important ecosystem services for human life, the preservation and protection of biodiversity is, therefore, a key issue in conservation and numerous restoration techniques are applied including *in-situ* and *ex-situ* conservation management.

In ecological restoration it is generally recommended to use local plant material for the reintroduction of plant species. The application of local seed material should increase establishment, performance and survival of (re)introduced individuals and, therefore, restoration success. However, in the context it is often questioned whether the applied local plant material is genetically variable enough to establish vital populations (Espeland, Emery et al. 2017, Nagel, Durka et al. 2019) or if the introduction of local plant material can maintain the natural spatial genetic structure of plant species. Further the production of local seed mixtures raises concerns as stock seeds are used for several reproduction cycles which may lead to inbreeding and reduced genetic diversity (Schoen and Brown 2001). Thus, the introduction of plant material requires much effort and research to ensure successful establishment, survival and reproduction of the restored populations (Maxted, Hawkes et al. 2000).

Therefore, investigating the genetic variation of plant species prior to a planned reintroduction measure may enhance restoration success. By detecting genetic distribution patterns of target plant species, genetically variable source populations can be identified to prevent negative consequences on genetic variation due to restoration management. However, unless the importance of genetic variation in restoration is acknowledged, it is hard to consider genetic diversity and differentiation within and among plant populations in practical restoration projects (McKay, Christian et al. 2005). The present thesis examined the impact of different restoration techniques on genetic variation within and among restored and natural populations of rare and common plant

species as well as on species composition of newly created plant communities. Moreover, the outcome of practical restoration projects was investigated to derive recommendations improving restoration management with respect to conservation genetics.

IMPACT OF RESTORATION ON SPECIES RICHNESS AND COMPOSITION

The restoration techniques investigated in this thesis are both known as successful methods to restore species-rich plant communities: By using seed-containing local plant material in restoration it seems to be possible to transfer the species richness of a whole plant community from a source to a suitable restoration site and establish viable and locally adapted plant populations (van der Mijnsbrugge, Bischoff et al. 2010). To avoid a depletion of the potential soil seed bank and to ensure generative rejuvenation in source populations and, thus, to maintain their genetic variation, restoration practitioners recommend to harvest only two thirds of a plant community on source sites (Kiehl and Pfadenhauer 2007). Therefore, harvesting may not comprises all individuals or plant species occurring at the source site but only a subset. Additionally, harvesting time due to plant phenology may function as a filter for the species richness of plant communities on restoration sites (Schmiede, Otte et al. 2012, Bischoff, Hoboy et al. 2018). The repeated transfer of plant material at different times in vegetation period can counteract these limitations and increase restoration success (Kiehl and Wagner 2006). For example, the combined application of green hay and threshed plant material turned out to be an effective restoration method to “copy” species diversity and composition from grasslands at source sites to restoration sites.

Nonetheless, seed-containing plant material only can be transferred when stable and viable source communities are available near restoration sites. Within a fragmented and impoverished landscape, these habitats could be hard to find. Therefore, the availability and usage of commercially produced local seed mixtures is a great opportunity mostly with successful outcome: Former studies observed high proportions of sown plant species in the established vegetation on restoration sites (Kiehl, Kirmer et al. 2010, Kirmer, Baasch et al. 2012) which goes in line with the findings of this thesis.

GENETIC CONSEQUENCES OF USING LOCAL SEED MATERIAL IN RESTORATION

An important point for the successful outcome of restoration projects is the application of genetically viable source plant material to establish vital populations. Sources should be chosen carefully because landscape fragmentation may have a negative impact on genetic diversity of plant populations (Honnay and Jacquemyn 2007). Due to fragmentation, the geographical distances among plant populations increase and hamper gene flow between them. Mating of related individuals or self-pollination may increase, resulting in inbreeding and reduced genetic variation within populations (Ellstrand and Elam 1993) while the differentiation among them increases. Additionally, random genetic drift may also reduce genetic diversity and inbreeding depression may result in the accumulation of deleterious alleles and reduced population fitness (Young, Boyle et al. 1996, Keller and Waller 2002).

Thus, the collection of source material is a critical step at the beginning of a restoration project. Small and isolated populations should be avoided as sources since they could suffer from inbreeding. Additionally, sampling source seeds from only a few plant individuals per population may cause bottlenecks and enhance genetic drift (Friar, Ladoux et al. 2000, Mijangos, Pacioni et al. 2015) in the newly established populations.

Since the harvesting process is affected by plant phenology (Schmiede, Otte et al. 2012, Bischoff, Hoboy et al. 2018) and may not comprises the entire seed set from source populations (Kiehl and Pfadenhauer 2007) only a part of the available gene pool might be collected. If seed-containing plant material is used in restoration this possible lack of genetic variation could be covered by the repeated application of source material or the combination of different harvesting techniques (Kiehl and Wagner 2006).

To maintain high genetic diversity in local seed mixtures on the other hand, multiple source populations are used for the production of the mixtures to cover a wide genetic range of the target plant species. Additionally, source seed material is used only for a short time, to avoid negative effects of inbreeding during propagation (Prasse, Kunzmann et al. 2010). The results of our study showed that these precautions work at least for the investigated study species and ensure the production of genetically variable local seed material for restoration.

Furthermore, plant species develop specific genetic distribution patterns based on different environmental habitat conditions, life history traits and the resulting local adaptations. In this context, it is often questioned if the introduction of local plant material can maintain this natural spatial genetic structure of a target species.

The restoration with seed-containing local plant material normally took place in a small geographic area. For example, the furthest distance between populations on source and restored sites of the investigated study species in this thesis was about 25 km beeline. By implementing this restoration technique, it is expected that it is possible to establish new plant populations that are locally adapted to specific regions and their environmental habitat conditions (van der Mijnsbrugge, Bischoff et al. 2010). Therefore, the natural genetic structure of a target species can be preserved as found in this thesis.

In contrast, the size of a seed transfer zone is much bigger and due to production procedure the seeds in a mixture may be genetically differentiated to occurring natural populations around the restoration area. This large-scale approach raises concerns about a possible adulteration of the natural genetic distribution pattern of target species because the introduction of foreign genotypes can cause serious genetic consequences. By mixing populations from different local environmental conditions, the newly introduced genotypes may replace locally adapted alleles. Thereby, genetic variation get lost due to so-called genetic swamping or may lead to outbreeding depression (Hufford and Mazer 2003). As a result, plant individuals may suffer from decreased fitness and lose their adaptability to changing environmental conditions (Hufford and Mazer 2003, McKay, Christian et al. 2005).

However, genetic differentiation among target species does strongly depend on life history traits like pollination system or dispersal strategy. For example, wind-pollinated plant species show lower levels of genetic differentiation due to large-scale dispersal via pollen, while populations of insect-pollinated plant species develop stronger spatial genetic distribution patterns (Reisch and Bernhardt-Römermann 2014). Therefore, the application of local seed mixtures within a seed transfer zone may affect the genetic differentiation among natural and restored populations of target species in varying degrees depending on their life history traits.

This is confirmed by the findings in this thesis. As expected, the wind-pollinated study species revealed comparatively low levels of differentiation among populations on restored and natural sites. The result goes in line with a former study (Durka, Michalski et al. 2017) and support the system of seed transfer zones for wind-pollinated plant species.

On the other hand, the differentiation between populations on natural and restored sites of the insect-pollinated plant species was clearly stronger. Therefore, commercially produced seed mixtures did not fully reflect the local genetic structure of the plant species. These results goes in line with findings of Listl, Poschlod et al. (2017) who reported a low fit of the geographical differentiation patterns of wild plant populations compared to the setting of local seed transfer zones. Consequently, sampling and propagation of source seed material of insect-pollinated plant species may need to be adjusted. For example, smaller collection areas could help to maintain lower levels of genetic differentiation among natural and sown populations.

However, if plant species develop different levels of ploidy, sampling, propagation and application of commercially produced seed mixtures should be performed very carefully. In the case of *K. arvensis* the different cytotypes function as effective breeding barriers (Kohler, Mittelsten Scheid et al. 2010) since gene flow between diploid and tetraploid individuals is completely inhibited (Kolar, Stech et al. 2009, Durka, Michalski et al. 2017). This illustrates the complexity and limitations of using commercially produced local seed mixtures on a larger geographical scale in restoration. More research on the genetic variation of insect-pollinated plant species is needed. Especially species known to develop various levels of ploidy should be investigated before restoration measures took place to avoid the introduction of mismatched cytotypes.

CONCLUSIONS AND FUTURE PERSPECTIVES

This thesis showed, that the (re)introduction of local plant species is a useful tool in conservation. With respect to the investigated restoration projects, the applied techniques had been mostly successful.

For rare and endangered plant species, inferring the genetic variation within and among populations prior to a planned reintroduction measure can significantly contribute to restoration success. By detecting the spatial genetic pattern of a target species, genetically variable source populations can be identified and the introduction of genetically too differentiated seed material can be avoided.

Analyzing the genetic variation is not only a state-of-the-art-method in conservation for rare and endangered plant species but also for common ones. Since all plant species are adapted to their local environmental conditions, each species develops a spatial genetic distribution pattern. Therefore, knowledge about these patterns is important for restoration management and its success. The thesis showed that the introduction of plant species by applying local seed-containing plant material is a suitable method to maintain the natural spatial genetic distribution pattern of target species.

However, speaking of seed transfer zones, the results of this thesis obtained that the larger the area for the seed collection of a certain species is, the harder it is to match or preserve their natural spatial genetic pattern. This is especially true for the investigated insect-pollinated study species compared to the wind-pollinated one. Further research on the genetic structure of “critical” plant species like *K. arvensis* is needed for a better understanding of their spatial genetic distribution pattern and to implement this knowledge in restoration practice. Improvements for restoration with local seed mixtures could be smaller seed transfer zones or considering environmental differentiation of habitats in propagation. However, for these adjustments seed-farms need more space for propagation and appropriate source populations that could be difficult to find. Further, it could be challenging the producers to maintain the profitability since it is likely that these adjustments will increase production costs.

Although not all genetic consequences of sowing commercially produced local seeds can yet be assessed for every used plant species, the mixtures are highly demanded since the application of local plant material in the wild is mandatory in Germany (§ 40 BNatSchG). However, in some cases the usage of local seed mixtures is not possible.

Due to the high demand in restoration the availability of certain seed mixtures within a seed transfer zone cannot be guaranteed (personal communication). Further practitioners criticize that exceptions for the application of seed material from neighboring seed transfer zones are rarely permitted (Jedicke, Aufderheide et al. 2022). Therefore, the production of local seed mixtures with a wide range of plant species is sometimes not possible because species which are only available in neighboring seed transfer zones may not be allowed in the mixtures (Jedicke, Aufderheide et al. 2022). Thus, finding a practicable solution for these restrictions is vital since the mixtures are urgently needed for restoration, especially in highly fragmented, intensively used and cleared landscapes (personal communication).

On the other hand, the application of seed-containing local plant material is known as the “gold standard” to maintain local adaptations and high genetic variation in restored populations and species richness on restoration sites. However, the usage of seed-containing local plant material in restoration is restricted to the availability of appropriate source sites. In regions with a low number of suitable habitats the demand on seed-containing plant material from these areas could be high. It is obvious that an uncontrolled draw-off may lead to genetic impoverishment of the plant populations growing on these source sites. Therefore, it is necessary to protect them against “over-harvesting”. The same shall be applied to populations of plant species that function as sources for the production of local seed mixtures. Hence, a “donor site database” or equally a “source population database” would be necessary in order to record when and how often harvesting takes place. At the same time regular examinations on the genetic variation of target plant species would be advisable. In Bavaria donor site databases are only locally available and exist not for all regions; seed producers most probably may have databases, but not available for public (personal communication). There are concerns among conservation authorities whether these databases should be publicly available for a wide range of operators including restoration practitioners, companies that produce seed mixtures or perform the transfer of seed-containing plant material (personal communication) to avoid the exploitation of donor populations and sites. However, as long as a donor site database all over Bavaria is in process of development and rules for harvesting are not generally mandatory for everyone it cannot be ruled out that the uncontrolled harvest may be a threat for the preservation of species richness and genetic variation of populations on these sites.

Besides the investigated *in-situ* restoration measures, there are further possibilities to preserve plant species like the conservation of populations *ex-situ* away from their natural habitats. According to the Global Strategy for Plant Conservation, at least 75 % of all threatened species should be conserved *ex-situ* and 20 % of this material should be available for restoration projects and reintroduction measures (CBD 1992 b). For example the cultivation in botanic gardens or storage of seeds in gene banks may contribute to the preservation of species diversity and genetic variation (Lauterbach 2013). However, it could be possible that populations cultivated *ex-situ* become inbred and, therefore, the adaptations to their source environment get lost. Furthermore, the restored *ex-situ* populations may diverge genetically from their sources over time (Schoen and Brown 2001). It has been observed that *ex-situ* populations compared to their corresponding source populations showed lower levels of genetic diversity. Additionally strong genetic differentiation among restored populations and their sources were detected (Lauterbach, Burkart et al. 2012, Brutting, Hensen et al. 2013). On the other hand, gene banks for plant species are an important tool to preserve intraspecific biodiversity and genetic variation *ex-situ* for conservation. By collecting several seed accessions from populations of a plant species within its distribution area, it would be potentially possible to conserve the spatial genetic distribution pattern of a plant species over decades until it is needed for restoration. The advantage of this conservation method is, that the stored seed material represent the genetic variation of *in-situ* populations at the time of sampling including potentially already lost alleles (Greene, Kisha et al. 2014). But gene banks require much effort: seeds must be collected, tested and eventually recollected, seed longevity and quality must be good, genetic variation should be investigated and enough space for storage rooms must be available, too (Listl 2016). Nevertheless, this method provides essential backup if wild populations get extinct due to deterioration of habitat conditions or habitat loss.

However, the conservation of plant populations in their natural habitats should be preferred if possible (Lauterbach 2013). Therefore, genetic conservation areas may be a useful alternative in restoration ecology to preserve local adaptations of plant populations and additionally intraspecific variation of whole ecosystems under *in-situ* conditions (Maxted, Dulloo et al. 2011). At the moment genetic conservation areas are mostly implemented to preserve genetic variation of fodder or forage crops, crop wild relatives or medicinal plant species (Maxted, Scholten et al. 2007). The concept could

also be applied for other threatened plant species or ecosystems, for instance grasslands (Pagel 2020). Especially protected areas are suggested as appropriate locations to establish genetic conservation areas since they are assumed to be more sustainable (Maxted, Dulloo et al. 2011). However, to maintain a broad range of genetic variation of target species, genetic conservation areas needed to be large enough. Since even within protected areas biodiversity declines (Leuschner, Wesche et al. 2013) and generally highly diverse habitats and ecosystems disappear in our landscape, appropriate sites could be hard to find.

The results represented in this thesis showed that more research is needed to avoid negative impacts of restoration management on genetic variation of target plant species. Investigating additional transfers of seed-containing plant material in other regions could help to improve restoration success. In the context of commercially produced seed material, the investigation of other common plant species could help to detect possible general spatial genetic patterns for plant species with the same life-history traits. Additionally, the genetic distribution pattern of species that develop different cytotypes should be better taken into account. Additionally, the successful outcome of restoration projects may be enhanced by considering epigenetic variation. This mechanism contributes to phenotypic plasticity of populations and, therefore, enables the rapid adaptation to changing environmental conditions for plant individuals (Gaspar, Bossdorf et al. 2019). In this context, common garden experiments or genetic analysis with different molecular tools like MSAPs or next-generation sequencing may also contribute to a better understanding of local adaptations (Holderegger and Segelbacher 2016)

In summary, a profound knowledge on genetic variation of plant species, suitable habitat conditions, the reestablishment of gene flow among populations as well as a subsequent long-term monitoring are key factors for sustainable conservation measures (Godefroid, Piazza et al. 2011). Combining this knowledge in practical projects may significantly increase restoration success.

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SUPPLEMENTARY MATERIAL

SUPPLEMENTARY MATERIAL – CHAPTER THREE

Table S 3.1: List of species occurring in the grasslands at the selected study sites.

Study site	S	R1.	R1.	S	R2.	R2.	R2.	S	R3.	R3.	R3.	R3.	R3.	S	R4.
Achillea millefolium	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1
Aegopodium podagraria	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Agrimonia eupatoria	0	1	0	1	0	0	1	0	0	0	0	0	0	1	0
Agrostema githago	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
Agrostis capillaris	1	1	1	1	1	1	1	1	0	0	0	1	1	1	1
Agrostis giganthea	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
Agrostis stolonifera	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Ajuga reptans	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0
Alchemilla vulgaris	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Allium carinatum	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Allium oleraceum	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Alopecurus pratense	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1
Anthoxanthum odoratum	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1
Anthriscus cerefolium	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Aquilegia spec.	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0
Arabis hirsuta	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0
Arenaria serpyllifolia	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Arrhenatherum elatius	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Artemisia vulgaris	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Astragalus cicer	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Astragalus spec.	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Bellis perennis	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Betonica officinalis	1	1	1	1	0	0	1	0	0	1	1	1	0	0	0
Brachypodium sylvaticum	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Briza media	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1
Bromus erectus	0	0	0	1	1	0	1	1	1	1	1	1	0	0	0
Calamagrostis epigejos	1	0	0	0	0	0	0	1	0	1	0	0	0	1	0
Calliergonella cuspidata	0	1	0	0	1	1	0	0	1	0	1	1	1	1	1
Campanula glomerata	0	0	0	0	1	1	0	1	1	1	1	0	1	0	0
Campanula patula	1	1	1	1	1	1	1	0	0	1	0	1	0	1	1
Campanula rapunculus	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0
Campanula rotundifolia	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0
Carec flacca	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Carex hirta	0	0	1	0	0	1	0	1	0	0	0	0	1	0	0
Carex pallescens	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1
Centaurea jacea	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Centaurea nigra	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0
Centaurea scabiosa	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Centaurium umbellatum	0	1	0	1	1	1	0	1	1	1	1	1	1	0	0
Cerastium holosteoides	0	0	1	1	1	1	0	1	1	1	1	1	1	0	1
Cirsium oleraceum	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0
Cirsium vulgare	0	1	0	0	1	1	0	1	0	0	0	0	0	0	0
Cisium arvensis	0	0	0	1	0	0	0	1	0	0	0	0	1	0	0
Clinopodium vulgare	0	1	0	0	1	0	0	0	0	0	0	1	0	0	0
Colchicum autumnale	0	0	0	1	0	0	0	1	1	0	0	0	1	1	0
Convolvulus arvensis	1	1	1	1	1	0	1	0	1	1	0	0	1	1	0
Coronilla varia	0	1	0	1	1	1	1	1	1	1	1	0	1	0	0
Crepis biennis	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1

Cynosurus cristatus	0	1	1	1	0	0	1	1	0	1	0	0	0	1	1
Dactylis glomerata	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Daucus carota	1	1	0	1	1	1	1	1	1	1	1	0	1	1	0
Dianthus carthusianorum	1	0	0	1	1	1	1	0	1	0	1	0	0	0	0
Dianthus deltoides	0	0	1	1	0	1	1	0	0	1	0	1	0	1	0
Diantus armeria	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0
Echium vulgare	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0
Elymus repens	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0
Equisetum arvense	0	1	1	1	0	0	0	1	0	0	1	0	1	1	0
Erigeron annuus	1	1	0	1	1	1	0	1	1	1	1	1	1	1	0
Eruca sativa	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Erysimum cheiranthoides	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Euphorbia cyparissias	0	0	0	1	1	0	0	1	0	0	0	1	0	0	0
Euphorbia spec.	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Festuca arundinaceae	1	0	0	1	1	1	0	1	0	0	0	0	0	1	0
Festuca ovina agg.	0	0	0	0	0	0	0	1	1	1	1	1	0	0	0
Festuca pratensis	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Festuca rubra agg.	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0
Filipendula ulmaria	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Filipendula vulgaris	0	0	0	0	1	1	0	0	0	1	1	0	0	0	0
Galium mollugo agg.	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Galium verum	1	1	0	1	1	1	1	1	1	0	1	0	1	0	0
Genista tinctoria	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0
Geranium dissectum	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
Geranium palustre	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Geranium pratense	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Glechoma hederaceae	0	0	0	0	0	0	0	1	0	1	0	0	0	1	0
Helianthemum nummularium	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Heracleum sphondylium	1	1	1	1	1	0	0	1	0	1	0	0	1	0	0
Hieracium pilosella	1	0	0	1	0	1	1	1	0	1	1	0	0	0	0
Hieracium spec.	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Holcus lanatus	1	1	1	1	1	1	1	1	0	1	0	1	1	1	1
Hypericum perforatum	1	1	1	1	0	0	1	1	0	1	1	1	0	0	0
Hypochoeris radicata	0	1	0	1	1	1	1	1	1	1	0	1	0	1	1
Juncus effusus	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Juncus tenuis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Knautia arvensis	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0
Lathyrus pratensis	1	1	1	1	1	1	0	1	0	1	0	1	1	1	1
Leontodon hispidus	1	0	0	0	1	1	1	1	1	1	1	1	0	1	0
Leucanthemum vulgare	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1
Linaria vulgaris	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Linum cartharticum	1	1	0	1	1	0	0	0	1	0	1	1	1	0	0
Linum perenne	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Lolium perenne	0	0	1	0	0	0	1	0	0	0	0	0	0	0	1
Lotus corniculatus	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Luzula multiflora	1	0	1	0	0	0	1	0	0	0	0	0	0	0	0
Lychnis flos-cuculi	0	0	1	1	1	0	1	0	0	0	0	0	0	1	1
Lychnis viscaria	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Lysimachia nummularia	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0
Lysimachia vulgaris	1	0	0	0	0	0	0	1	0	1	0	0	0	0	0
Malva moschata	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0
Medicago falcata	0	1	0	1	1	1	1	1	1	1	1	1	0	0	0
Medicago lupulina	1	0	0	1	1	1	1	1	1	1	1	1	0	0	0
Medicago sativa	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Melilotus officinalis	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Mentha arvensis	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0
Mentha longifolia	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Molinea caerulea	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ononis spinosa	0	0	0	1	1	1	0	1	0	1	1	1	0	0	0
Origanum vulgare	0	1	0	1	1	1	1	0	1	0	1	0	0	0	0
Orobanche gracilis	0	1	0	1	0	1	1	0	1	0	1	1	0	1	1
Peucedanum oreoselinum	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0

Phleum pratense	0	1	1	0	0	1	1	0	0	0	0	0	1	1	0
Picris hieracioides	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0
Pimpinella major	0	1	1	1	1	1	0	1	1	0	1	1	0	1	0
Pimpinella saxifraga	0	0	0	1	1	1	1	1	1	1	1	0	0	0	0
Plagionium affine	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Plantago lanceolata	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Plantago media	1	0	0	1	1	1	1	1	1	1	1	1	1	1	0
Poa annua	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0
Poa pratensis	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Poa trivialis	0	1	1	1	0	0	1	1	0	1	0	1	1	1	1
Polygala vulgaris	1	0	0	1	0	0	1	0	0	0	1	0	0	0	0
Potentilla anserina	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Potentilla argentea	0	1	0	1	0	0	0	0	0	0	0	1	0	0	0
Potentilla erecta	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Potentilla recta	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0
Potentilla reptans	0	1	0	1	1	1	1	1	0	1	0	1	1	1	0
Primula elatior	1	0	0	1	0	1	0	0	0	1	0	0	1	0	0
Prunella grandiflora	0	0	0	0	0	?	1	0	0	0	0	0	0	0	0
Prunella vulgaris	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1
Ranunculus acris	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Rhinantus minor	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Rhythidiadelphus squarrosus	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0
Rumex acetosa	1	1	1	1	1	1	0	1	0	1	0	1	0	0	0
Rumex crispus	0	1	1	0	0	0	0	0	0	0	0	1	1	1	1
Salvia pratensis	1	1	0	1	1	1	1	1	1	1	1	0	0	0	0
Sanguisorba minor	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0
Sanguisorba officinalis	1	0	1	0	1	1	0	1	1	1	1	1	1	0	0
Scabiosa columbaria	1	0	0	1	1	1	0	1	1	1	1	1	1	0	0
Scleropodium purum	0	1	0	1	0	0	1	0	0	1	0	1	0	0	0
Sedum acre	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Senecio jacobaea	0	0	0	1	1	1	1	0	0	0	1	0	0	0	0
Seseli libanotis	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0
Silene vulgaris	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1
Stellaria graminea	1	0	1	0	0	0	0	0	0	1	0	1	1	0	0
Symphytum officinale	0	0	0	0	1	1	0	1	0	0	0	0	1	0	0
Tanacetum vulgare	0	1	0	1	1	0	1	0	1	1	0	0	0	0	0
Taraxacum officinale	1	0	1	1	0	1	1	1	1	1	1	0	1	1	1
Thuidium tamariscinum	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0
Thymus pulegioides	1	0	0	0	0	0	1	0	1	1	0	0	0	0	0
Tragopogon pratensis	1	1	0	1	1	1	0	1	1	1	1	0	0	0	0
Trifolium campestre	0	1	0	1	0	1	1	0	0	0	0	0	0	0	0
Trifolium dubium	0	0	1	0	0	0	1	1	0	1	0	1	0	1	1
Trifolium medium	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0
Trifolium montanum	0	0	0	0	1	1	0	1	0	1	0	0	0	0	0
Trifolium pratense	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Trifolium repens	0	1	1	1	1	1	1	1	0	1	0	1	0	1	1
Trisetum flavescens	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1
Tussilago farfara	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Verbascum lychnitis	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0
Veronica chamaedris	1	0	1	1	1	1	1	1	0	1	0	1	0	1	1
Vicia cracca	0	0	0	1	1	1	0	1	0	1	0	0	1	1	0
Vicia hirsuta	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0
Vicia sativa	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0
Vicia sepium	0	0	1	0	0	0	0	0	0	1	0	0	1	0	0
Vicia tetrasperma	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0

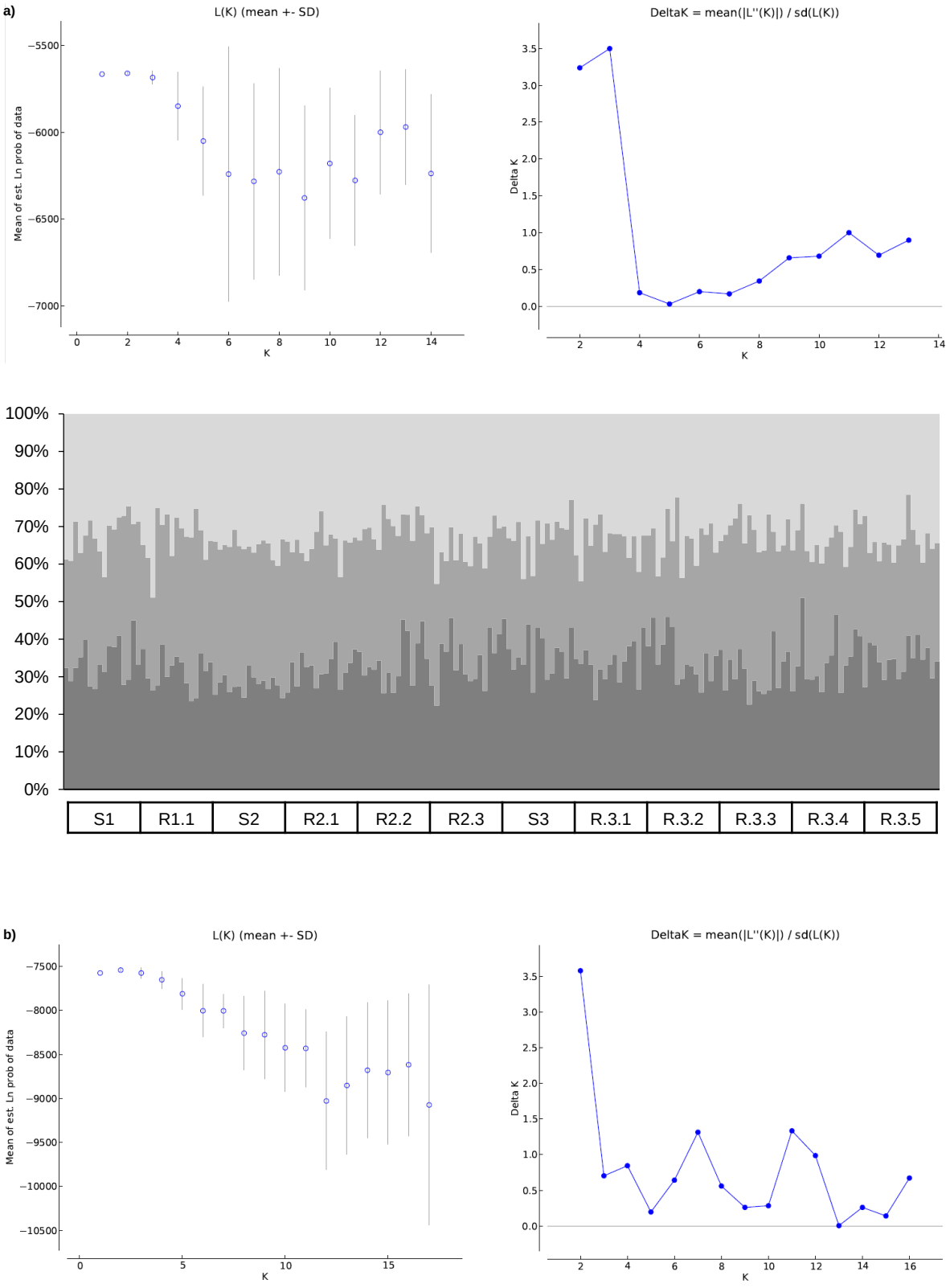
Table S 3.2: Molecular variance within and among populations of *K. arvensis* calculated in different analysis of molecular variance (AMOVA) based on 127 AFLP fragments. Levels of significance (p-values) are based on 999 iteration steps. (Df: degree of freedom; SS: sum of squares, MS: mean squares. %: proportion of genetic variance).

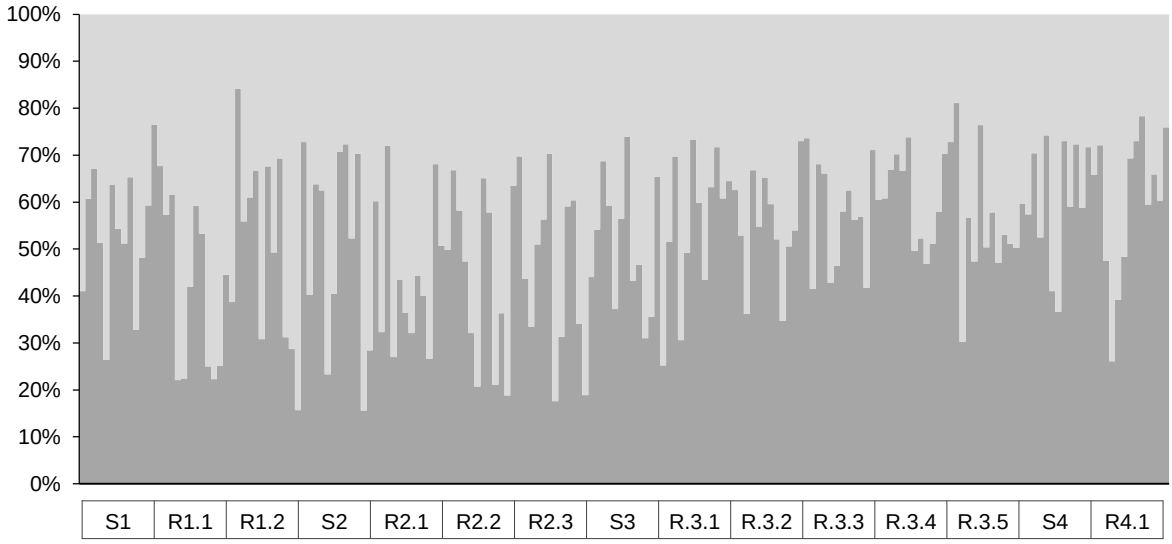
	df	SS	MS	%	ΦPT	p
Molecular variation among all populations						
Among Pops	11	168.25	15.30	4.01	0.040	0.001
Within Pops	170	1592.52	9.37	95.99		
Molecular variation among populations at source and restoration sites						
Among site type	1	18.26	18.26	0.46	0.043	0.001
Among Pops	10	149.99	15.00	3.80		
Within Pops	170	1592.52	9.37	95.74		
Molecular variation among populations at source sites						
Among Pops	2	34.760	17.380	5.55	0.055	0.001
Within Pops	44	398.304	9.052	94.45		
Molecular variation among populations at restoration sites						
Among Pops	8	105.417	13.177	2.69	0.027	0.001
Within Pops	126	1173.768	9.316	97.31		
Molecular variation among single transfers:						
Transfer 1: S1 vs. R1.1						
Among Pops	1	18.24	18.24	6.85	0.069	0.003
Within Pops	29	247.30	8.53	93.15		
Transfer 2: S1 vs. R1.1						
Among Pops	1	12.08	12.08	1.29	0.013	0.137
Within Pops	29	291.40	10.05	98.71		
Transfer 2: S2 vs. R2.2						
Among Pops	1	15.78	15.78	4.08	0.041	0.003
Within Pops	30	281.56	9.39	95.92		
Transfer 2: S2 vs. R2.3						
Among Pops	1	10.67	10.67	0.45	0.004	0.352
Within Pops	29	289.27	9.97	99.55		
Transfer 3: S3 vs. R3.1						
Among Pops	1	11.73	11.73	1.98	0.020	0.076
Within Pops	28	252.13	9.00	98.02		
Transfer 3: S3 vs. R3.2						
Among Pops	1	13.25	13.25	2.81	0.028	0.023
Within Pops	29	265.46	9.15	97.19		
Transfer 3: S3 vs. R3.3						
Among Pops	1	18.70	18.70	6.15	0.061	0.001
Within Pops	28	264.13	9.43	93.85		
Transfer 3: S3 vs. R3.4						
Among Pops	1	20.58	20.58	7.39	0.074	0.001
Within Pops	27	257.83	9.55	92.61		
Transfer 3: S3 vs. R3.5						
Among Pops	1	14.34	14.34	3.78	0.038	0.004
Within Pops	27	246.76	9.14	96.22		

Table S 3.3: Molecular variance within and among populations of *P. lanceolata* calculated in different analysis of molecular variance (AMOVA) based on 122 AFLP fragments. Levels of significance (p-values) are based on 999 iteration steps. (Df: degree of freedom; SS: sum of squares, MS: mean squares. %: proportion of genetic variance).

	df	SS	MS	%	ΦPT	p-value
Molecular variation among all populations						
Among Pops	14	191.76	13.70	2.21	0.022	0.001
Within Pops	213	2172.93	10.20	97.79		
Molecular variation between populations at source and restoration sites						
Among Site Class	1	11.78	11.78	0.00	0.021	0.001
Among Pops	13	180.37	13.87	2.32		
Within Pops	213	2172.54	10.20	97.68		
Molecular variation among populations at source sites						
Among Pops	3	40.95	13.65	2.05	0.021	0.008
Within Pops	59	605.40	10.26	97.95		
Molecular variation among populations at restoration sites						
Among Pops	10	139.04	13.90	2.38	0.024	0.001
Within Pops	154	1567.53	10.18	97.62		
Molecular variation among single transfers						
Transfer 1: S1 vs. R1.1						
Among Pops	1	10.98	10.98	0.12	0.002	0.385
Within Pops	29	309.02	10.66	99.80		
Transfer 1: S1 vs. R1.2						
Among Pops	1	8.62	8.62	0.00	0.008	0.690
Within Pops	29	284.22	9.80	100.00		
Transfer 2: S2 vs. R2.1						
Among Pops	1	15.41	15.41	3.04	0.030	0.014
Within Pops	30	307.94	10.26	96.96		
Transfer 2: S2 vs. R2.2						
Among Pops	1	13.59	13.59	2.22	0.022	0.048
Within Pops	30	299.31	9.98	97.78		
Transfer 2: S2 vs. R2.3						
Among Pops	1	17.88	17.88	4.81	0.048	0.001
Within Pops	30	296.38	9.88	95.19		
Transfer 3: S3 vs. R3.1						
Among Pops	1	18.45	18.45	4.54	0.045	0.001
Within Pops	26	288.55	11.10	95.46		
Transfer 3: S3 vs. R3.2						
Among Pops	1	16.63	16.63	3.42	0.034	0.012
Within Pops	28	304.27	10.87	96.58		
Transfer 3: S3 vs. R3.3						
Among Pops	1	16.49	16.49	3.28	0.033	0.018
Within Pops	27	298.61	11.06	96.72		
Transfer 3: S3 vs. R3.4						
Among Pops	1	14.36	14.36	2.19	0.022	0.061
Within Pops	26	284.71	10.95	97.81		
Transfer 3: S3 vs. R3.5						
Among Pops	1	16.51	16.51	4.02	0.040	0.004
Within Pops	29	290.46	10.02	95.98		
Transfer 4: S4 vs. R4.1						
Among Pops	1	12.69	12.69	1.97	0.020	0.057
Within Pops	30	288.00	9.60	98.03		

Figure S 3.1: Results of Bayesian cluster analysis for a) *Knautia arvensis and* b) *Plantago lanceolata*. Different shades of grey classify the received groups. Given are Ln P(D) variance, Delta K and bar plot diagram. K for the tested groups is a) K = 1-16 (*K. arvensis*) and b) K = 1-20 (*P. lanceolata)*.




100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
S1
R1.1
R1.2
S2
R2.1
R2.2
R2.3
S3
R.3.1
R.3.2
R.3.3
R.3.4
R.3.5
S4
R4.1

SUPPLEMENTARY MATERIAL – CHAPTER FOUR

Additional supporting information may be found in the online version of the published article at the publisher's website.

Table S 4.1: List of species from seed mixtures, site characteristics and species traits

Table S 4.2: Vegetation surveys

Appendix S 5.1: Material and Methods for Flow Cytometry (FCM)

FCM analysis was performed in accordance with the protocol of Dolezel, Greilhuber et al. (2007) to test for multiple DNA ploidy levels. We used the same dried plant material as we used for the AFLP analyses. Due to the low amount of nuclei in the dried leaf material, analyses were performed with an external standard reference. For *Knautia arvensis* we chose *Pisum sativum* and for *Silene vulgaris* and *Plantago lanceolata* we chose *Petunia x hybrida* as standard reference. For each study species, one individual per populations was investigated.

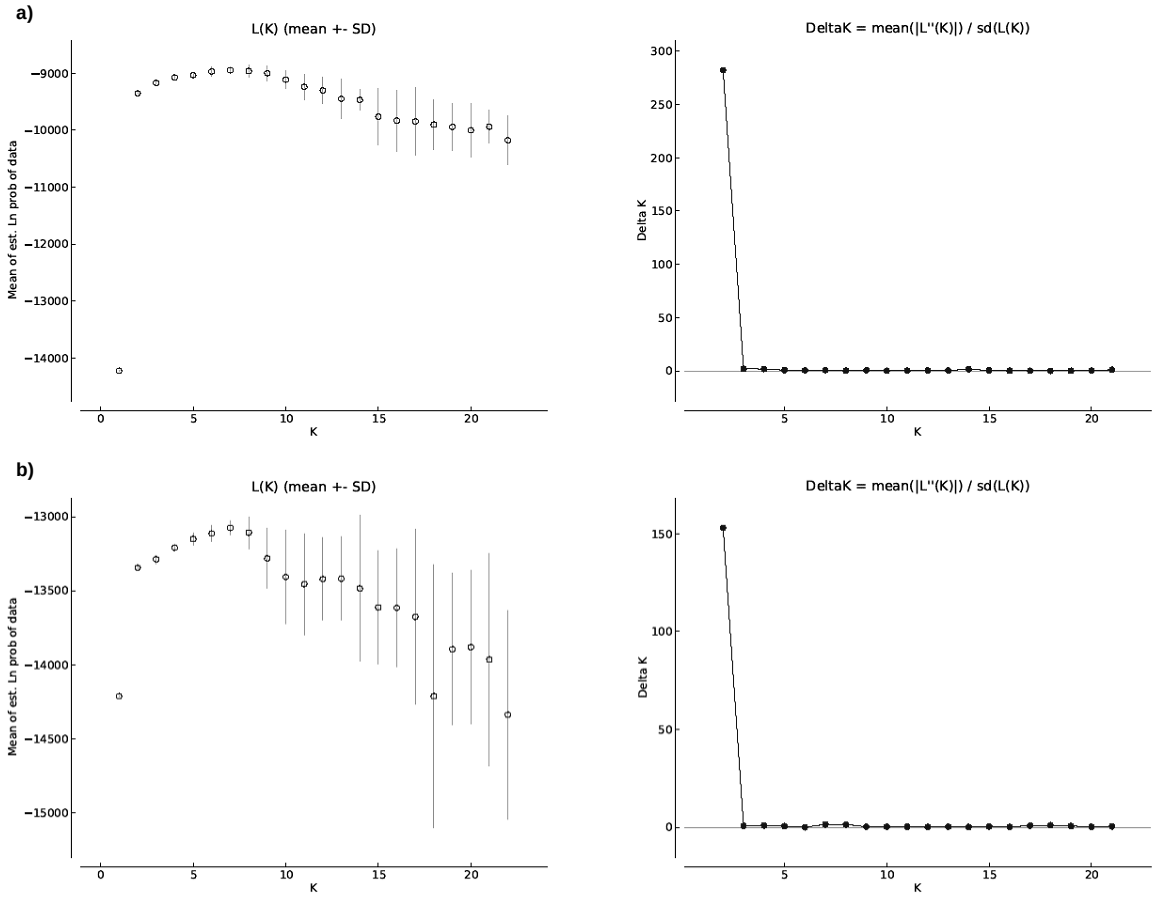
To isolate the nuclei, the plant material was placed in a plastic Petri dish, OTTO 1 buffer was added and then the dried leafs were chopped immediately within the buffer with a sharp razor blade. As the concentration of nuclei in dried leaf material is not as high as in fresh material, we used three cm^2 of dried leaf material and added 1.5 ml OTTO 1 buffer. The chopped material was washed several times with the buffer from the petri dish and filtered through 50 µm nylon mesh (CellTrics filter) into a labeled 1.5 ml sample tube on ice. Nuclei were pelleted via centrifugation for 5 min at 1400 rpm. The supernatant was removed carefully leaving 50 µl of it above the pelleted nuclei. Then 1 ml of LB01 buffer was added and each sample was incubated for 10 minutes on ice before analysis.

Samples were measured on a CyFlow Ploidy Analyser (Sysmex GmbH, Germany) and results were estimated employing the program FCS Express 5 Flow Research Edition (DeNovo). For each sample and standard, an average number of 8500 nuclei was counted and the relative florescence intensity of the DAPI-stained nuclei was measured. Because of the fact, that it is hardly possible to achieve CVs below 5 % with dried plant material (Dolezel, Greilhuber et al. 2007), CV values of ≤ 10 were accepted. To infer polyploidy levels, the DNA content was calculated using the notation “DNA content standard [pg] * geometric mean sample / geometric mean standard”.

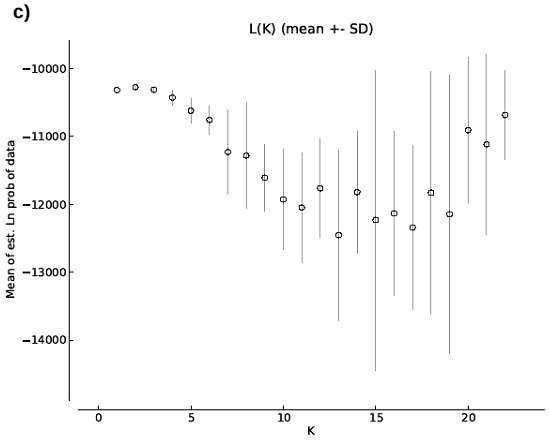
Table S 5.1: Primer combinations used in AFLP analysis for the three study species. Also given are the number of generated loci for each species.

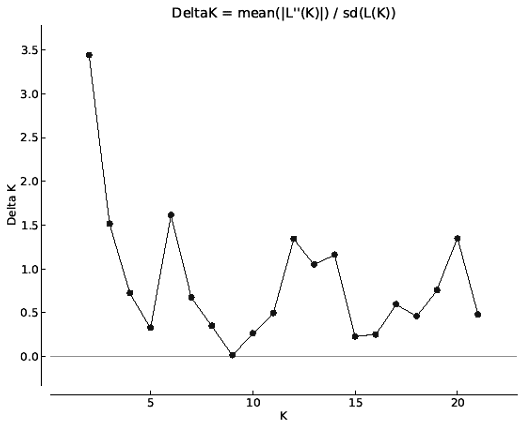
Species	Primer combination D2	Primer combination D3	Primer combination D4	Number of loci
Knautia arvensis	CAG-ACC	CTT-AGG	CTT-ACT	137
Silene vulgaris	CTA-AAC	CTA-AAG	CTT-ACT	121
Plantago lanceolata	CTG-AGC	CAA-AGG	CAG-ACA	127

Figure S 5.1: Supplementary results of Bayesian cluster analysis for a) *Knautia arvensis*, b) *Silene vulgaris* and c) *Plantago lanceolata*. Given are Ln P(D) variance and Delta K for the tested groups K = 1-22.



c)


L(K) (mean +- SD)
Mean of est. Ln prob of data
−10000
−11000
−12000
−13000
−14000
0
5
10
15
20
K


DeltaK = mean(|L''(K)|) / sd(L(K))
Delta K
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
5
10
15
20
K