The geographic scale of genetic variation in common plant species – Implications for genebanks and restoration

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Für meine zwei Deifln

und meine Eltern

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SUMMARY

Genebanks for plant seeds are a necessary tool to support the conservation of biodiversity. In the last decades also the importance of intraspecific diversity within the genebank collections was emphasised. Even though background information on the distribution of genetic diversity within and between populations was scarce for common plant species, seed provenances have been delineated for seed collection and production. Seeds have to be used in the same provenance, where they were collected for restoration projects, agriculture and forestry.
The present study aims to elucidate distribution of intraspecific genetic diversity in three common plant species over different geographic scales. In the last part, further intraspecific differences in seed traits were investigated.

Chapter 2 dealt with the distribution of genetic diversity within and between populations of *Sedum album* in its distribution range in Europe and the history of Central European populations. With a combination of neutral and chloroplast specific markers, we reconstructed postglacial processes that lead to the present distribution pattern in *S. album*. Distinct haplotypes could be detected on the Iberian Peninsula and in Eastern and Southern Europe. Central European populations have their origin in Southern Europe and show meanwhile high influence of Eastern lineages. A clear East/West subdivision was found with hybrid zone in Western Europe. Glacial refugia have been detected on the Iberian Peninsula and in the mountains of Liguria (Northern Italy). Further refugia can be assumed in south-eastern Europe.

In chapter 3 and 4 we used AFLP markers to examine the distribution of genetic diversity in and between populations of *Lathyrus pratensis* and *Hepatica nobilis* in Bavaria. Correlation of genetic diversity with seed provenances was tested. The studies of two perennial, cross-pollinated plants revealed differences in genetic diversity within and between populations. *In L. pratensis* within population diversity was ordinary compared to other plants with similar life history traits. However, differentiation between populations was very high, what can be ascribed to the limited dispersal of the heavy seeds. Moreover, the influence of nearby anthropogenic populations can, therefore, be excluded. Gene flow was present within 110km, resembling the northern and the southern part of Bavaria (delineated by the river “Danube”). We found that the group of populations in southern Bavarian is located in and delineated by one of the official seed production areas. For *L. pratensis* the production zone is sufficient. Nevertheless, material for restoration should be taken as near as possible due to high differentiation between populations. Collections for genebanks should be undertaken from both northern and southern

Bavaria, with an emphasis on southern parts, where genetic variation within populations was higher.

For *H. nobilis*, there were no correlations with official provenances or production zones. Gene flow was very limited, obviously due to the seed dispersal by ants, which agitate within few square metres. Genetic diversity within populations was high, while differentiation between populations was moderate. The pattern can be explained by random, long-distance dispersal, e.g. of plants, which have been used in horticulture for many centuries. Because ex-situ storage of seeds is difficult for *H. nobilis*, it is important to conserve high variation in in-situ populations in old forests.

Intraspecific differences in seed traits were examined in chapter 5. Seed quality and longevity were tested in two experimental setups. We measured percentage of filled seeds with x-ray analysis, 1000-seed-weight, initial and maximum germination rates to infer seed quality. To measure longevity seeds were conducted to accelerated aging. We collected data from wild lowland and alpine populations and from common garden populations, which were cultivated from seeds of the same populations. Percentage of filled seeds and seed weight was not different for lowland and alpine, or wild and common garden populations. Nevertheless, initial and maximal germination was higher in seeds from wild populations. Further, longevity tended to be higher in seeds from lowland than alpine and common garden than wild populations, respectively. Nevertheless, we could show that differences in longevity are environmentally influenced to some extent, because common garden populations of different origin differed less than wild populations.

In a second setup, seeds were collected in consecutive years from the same populations. Seed data were compared with climate data. We used model approaches based on results of former studies. Mean annual temperature and total annual rainfall, and mean annual temperature together with total rainfall and total sunshine within 90 days prior to harvest explained some variation in our data.

In chapter 6, I conclude that history of populations and plant traits have more influence than recent anthropogenic events on the distribution of genetic diversity in common plants. For conservation of intraspecific diversity in common plants, plant traits should be considered in collections for genebanks and in the usage of seeds for restoration. The collection of more data concerning quality and longevity of wild plant species would improve future collections. Costs for testing, regrowth and recollection in genebanks would be reduced.

Chapter 1

General Introduction

Ex-situ conservation of plants has a long history. Monasteries and botanical gardens have cultivated ex-situ populations since the 16th century (Hurka *et al.* 2008). Besides living cultures, they stored and exchanged seeds of rare or medicinal species. The oldest institution that intentionally practiced seed storage ("genebanking") is situated in St. Petersburg, where N. Wawilow started to store seeds from worldwide collections in the early 20th century (Hammer & Diederichsen 2009; Laarz 2014; Nick 2014). In the 1960s Cesar Gomez-Campo has opened "Banco de Semillas de la Escuela Tecnica Superior de Ingenieros Agronomos de Madrid" – the first seed genebank for wild species (Pignone & Hammer 2010).

The enhanced longevity of dry and cold stored seeds is helpful for institutions all over the world and cost-effective genebanks were established (see below: Excursus). Nowadays, genebanks provide a general backup for mankind – the last chance of survival for threatened species, the insurance against global warming, environmental disasters and wars - and a possibility to solve the nutrition of a growing world population (CBD 1992; FAO 2010; Schoen & Brown 2001). It seems easy to restore the biodiversity of plants with storage of some small seeds, it gets difficult when it comes to details.

1 BIODIVERSITY

Three levels of diversity, namely diversity of ecosystems, of species, and of genes have been defined and are often quoted by research and politics (CBD 1992; Gugerli *et al.* 2008). When conservation of plants' biodiversity is considered, priorities and conservation strategies have to be set in all levels.

1.1 Ecosystem level

Whole ecosystems worldwide are under enormous pressure. Arable land is economically and for immediate survival often more relevant than ecologic diversity (Mangel *et al.* 1996; Negri *et al.* 2009). Ecosystem ingredients, like fresh water, soil fertility, and fibre are often

removed, while other factors like input of waste, increase of certain chemicals, and the fragmentation of landscapes perturb important processes (Pagiola *et al.* 2004). It is clear that ecosystem conservation has to consider flora and fauna, and, moreover, all the interactions and processes agitating in and between ecosystems. Nevertheless, too few things are known about those complicated networks (Heywood & Iriondo 2003; WallisDeVries *et al.* 2002).

For conservation priorities of ecosystems different points of view and interests set their focus on different values. Economic or ecologic factors can be prioritised for the evaluation of ecosystems (Pagiola *et al.* 2004; Williams 2007; Wilson *et al.* 2007). Ecosystem services, including the climate regulation, fresh water purification, detoxification, recirculation of waste, providing of fodder, fibre, and biomass are meanwhile highly evaluated aspects of ecosystems (Wilson 2003) p. 106). Further, species richness is a widely accepted evaluation factor (Williams *et al.* 1996; Wilson 2003). The more species ecosystems harbour, the more stable and productive they are (Wilson 2003) p. 108). Species hotspots are, therefore, conservation priorities, e.g. the 25/34 plant hotspots, where 44% of all vascular plant species of the world are residing on only 1.4% of the earth's surface (Li & Pritchard 2009). Besides, rarity of species inhabiting an ecosystem can be a deciding factor for conservation (Williams *et al.* 1996). Within specific areas, furthermore, the principle of complementary sets is applied for conservation, in which most of the species should be present at least in one of the protected ecosystems (van Jaarsveld *et al.* 1998).

Seed genebanks can help to maintain plant biodiversity in ecosystems by conservation of threatened species or certain ecotypes (see 1.2 and 1.3), which can be used for restoration and recolonisation of ecosystems. Nevertheless, conservation of ecosystems is only possible in-situ. Ex-situ conservation, like seed genebanks, can start in the next level: the species level.

1.2 Species level

There is a large number of threatened plant species. Those have been in focus for ex-situ conservation for a long time as life-sustaining measure to prevent their total extinction: Based on IUCN Red List (2014) out of 268000 flowering plants 9905 (7%) are critically endangered, endangered or vulnerable (IUCN 2014). CBD stated that 75% of threatened species should be stored in ex-situ collections until 2020 (CBD 2010; GSPC 2012). In future, those accessions can be irreplaceable, when it comes to restoration or recolonisation.

Independent of the degree of threatening “living material that includes genes of present and potential value for humans” should be preserved (CBD 1992). Plants or parts of plants that can be propagated (generatively or vegetatively) are called plant genetic resource (PGR). This group comprises a wide range of species (Fig. 1), including plants – classified as non-foods - with high economical relevance (e.g. *Bambus*, *Brassica napus oleifera*, *Elaeis guineensis*, and *Gossypium*), worldwide recognized medical used species (*Aloe*, *Chamomilla*, *Eukalyptus*, and *Echinacea*), and many species used in distinct regions for traditional medicine.

Economy and pharmacy are constantly in search of new raw material and substances. It is often stated that there are numerous species containing still unknown substances and ranges of application worthwhile in future (Farnsworth & Soejarto 1991; Mendelsohn & Balick 1997). Those species – not acknowledged as PGR by date – could be highly relevant in future (e.g. see the case of *Fontainea picrosperma*: (Campbell *et al.* 2014)

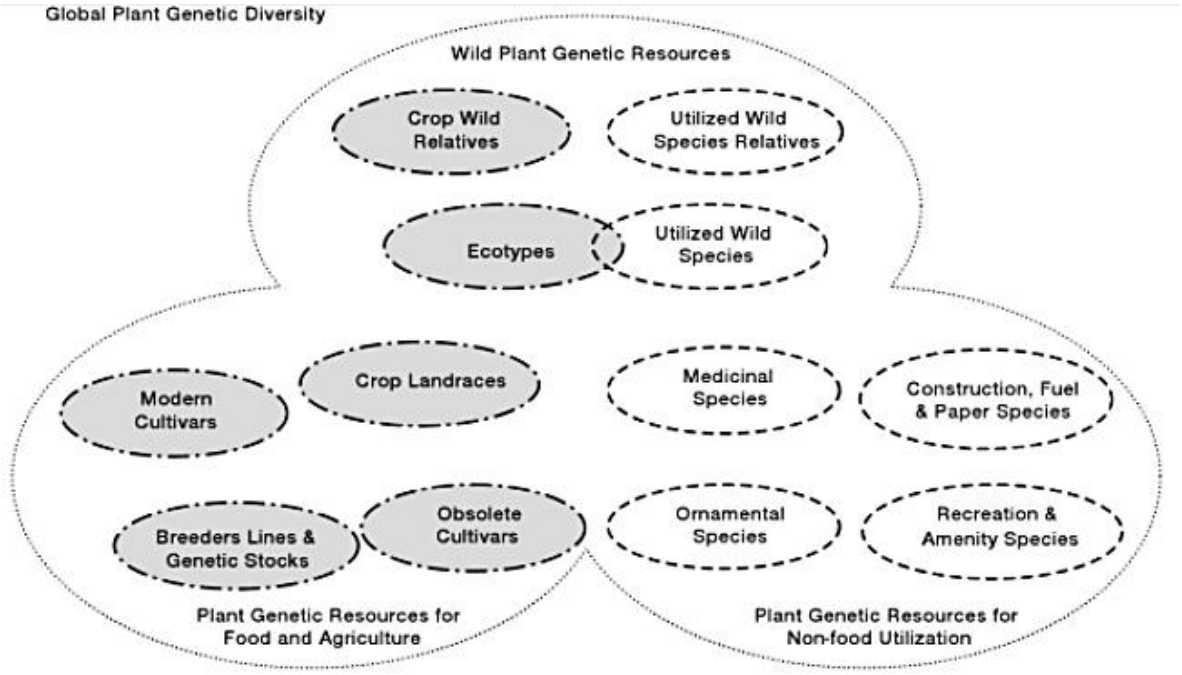


Fig. 1: Plant genetic resources comprise a wide range of global plant diversity, from Maxted (2008).

The main part of PGRs are plants for food and agriculture. Even, if only 15 species account for 90% of human nutrition (rice, maize, wheat and potato alone for about 65%) and only some hundred contribute significantly, more than 30.000 are edible (FAO 1994; FAO 1995).
Next to obvious PGR plants, there is a high number of wild plant genetic resources with possible use, for example, plants that are closely related to crop plants. Those are called “crop wild relatives” (CWR) (Maxted *et al.* 2006; Maxted *et al.* 2012; Parra-Quijano *et al.* 2012). By definition of Maxted *et al.* (2006) „a crop wild relative is a wild taxon that has an indirect use derived from its relatively close genetic relationship to a crop…“. Those species “have particular values because of their potential to contribute beneficial traits to crops, such as biotic and abiotic resistance, leading to improved yield and stability" (Maxted *et al.* 2006). It was shown that CWRs already have high economic influence as they account for 37.3*10^6 € of the agricultural production of 29 priority crops in the Millennium Seed Bank (PwC 2013).
A main part of wild species can not only be of use because of their relation to important PGR plants, but they are considered as PGRs themselves. Some are not widely used at the moment, but they have potential to be useful in future. In Germany out of about 3600 wild species about 1000 are evaluated as PGRs in this sense (BLE 2014; Hurka *et al.* 2008). Moreover, 1.800 plants with high horticultural or ornamental use can be added to the number of PGR species in Germany.
Most wild species have not only one usage, but they can be taken for several advantages. When wild species have to be prioritised the number of usages can be a deciding factor. Based on Schlosser et al. (1991) wild species in Germany are classified in 13 categories: agricultural and food plants, bee food plants, carbohydrate suppliers, fodder crop, forest trees, fruit and vegetable, horticultural and ornamental plants, medicinal and spice plant, oil suppliers, plants with breeding purposes, plants with technical use (e.g. as dye, energy supplier, fibre plants), protein suppliers and windbreaking and shadowing plants are conducted for the species status as PGR (BLE 2014; Poschlod *et al.* 2014; Schlosser 1991). *Symphytum officinale*, for example, is considered in six categories as medical, fodder and edible plant, as bee food plant and for horticulture and breeding purposes.

> Excursus: Genebanks worldwide – a short overview

The long-term ex-situ conservation of seeds has started with the help of worldwide genebank initiatives with different collection strategies. Starting with Wawilow genebank (320000

species, mainly CWRs, Russia), nowadays about 1750 genebanks are officially registered worldwide, which contain about 7.4 million accessions (FAO 2010). The most popular storage place is Svalbard Global Seed Vault, managed by the Global Crop Diversity Trust with 820000 accessions from all over the world (Breen 2015). Established seed genebanks that deposit their "backup" seeds in the "Trust" are e.g. the international agitating Millennium Seed Bank with 34.000 species (GB, www.kew.org/) and the Leibniz-Institut für Pflanzengenetik und Kulturpflanzenforschung (IPK) with >150000 accessions of 3200 crops species (Germany, http://www.ipk-gatersleben.de/).

Nevertheless, most genebanks are species or region specific. This makes perfect sense as it is efficient - especially for small institutions - to restrict collections to certain areas and/or the management to certain species.

Staple foods are for many genebanks the priority group of collections, e.g. the International Crops Research Institute for the semi-arid Tropics (ICRISAT) holds 123.000 accessions of sorghum, pearl millet, chickpea, pigeon pea and groundnut from 144 countries (Asia and Africa, http://www.icrisat.org/) and in the International Rice Research Institute (IRRI) 177.000 types of rice are deposited (Philippines, www.irri.org/).

Broader collections, but still with focus on staple foods are the National Small Grains Collection (NSGC) with the biggest collections of wheat (>856.000 accessions), barley (>466.000 accessions) and oat (>130.000 accessions) (South Africa, www.arc.agric.za/arc-sgi/Pages/Germplasm%20Development/National-Small-Grain-Collection.aspx), and the International Institute of Tropical Agriculture (IITA) holdings >28 000 accessions of major food crops of Africa (Nigeria, www.iita.org/).

Even though, only a few genebanks have their priority in wild species, the number is growing with a growing interest from conservation, seed producers, pharmacy, and breeders. The intention to collect region-specific, endemic, and threatened wild species have Western Australia Seed Technology Centre (WASTC), which harbours > 3500 Australian species (Australia, http://www.bgpa.wa.gov.au/about-us/research/wa-seed-tech-centre) and Genbank Bayern Arche with a collection of 500 threatened wild species from Bavaria (Germany, www.genbank.ur.de). Collections, which contain wild species accounting as PGR are National Plant Germplasm System (NPGS), which collects crop and CWR species worldwide (USA, www.ars-grin.gov/npgs/), Plant Genetic Resources of Canada (PGRC) with 110000 accessions of all kind of bred and wild plants (Canada, http://pgrc3.agr.gc.ca/index_e.html), European Native Seed Conservation Network (ENSCONET) with only wild species (Europe,

http://ensconet.maich.gr/), and Genbank für Wildpflanzen für Ernährung und Landwirtschaft (WEL) for unthreatened and wild PGR species in Germany (Germany, www.genbank-wel.de).

1.3 Intraspecific/ Gene level

Conservation of genetic diversity and, therefore, intraspecific diversity is a main level in plant conservation and worldwide politically acknowledged since ratification of the CBD (1992). Intraspecific diversity of plant species comprises all levels of evolutionary divergence, subspecies and adaptations (CBD 1992; Moritz 2002). Nevertheless, conservation of biodiversity mainly focuses on ecosystems and species (Moritz 2002). In the last years, it has been shown that landraces of some species have become enormously threatened by genetic erosion due to the replacement by high productive breeding lines in many regions (Negri *et al.* 2009).

Many cases of adaptations in wild populations have been described that can be useful for breeding.

- Cassava (*Manihot esculenta*) harvests in several parts of Africa were decimated by cassava mosaic disease since 1988. Naturally resistant lines were identified in different genebanks and could help to overcome the crisis partly (FAO 2014b; Otim-Nape *et al.* 1994).
- With a local variety of *Triticum* that was collected in 1948 in Turkey, it was possible to gain resistance against pathogens (Laarz 2014).
- In Germany a project is conserving the last wild populations of *Vitis vinifera* ssp. sylvestris, the wild relative of vine (*Vitis vinifera* ssp. vinifera), which already helped breeders to acquire natural resistances against several diseases (Nick 2014).
- An accession of soy from the Wawilow genebank in St. Petersburg showed resistance against a pathogen that infested US soy beans. A new soy breed was created and is cultivated all over US America today (Laarz 2014).
- The Alb-lentil was cultivated until the middle of the 20th century in the Swabian Alb. Two of the main breeds were lost, after the cultivation was quit due to more rentable cultivation of wheat and sugar beet, or cattle breeding (Poschlod 2015). In 2006 some seeds of these cultivars were found in the Wawilow genebank. At first some hundred seeds were cultivated in 2007 to increase the number of seeds. Nowadays the lenses are sold as speciality from the Swabian Alb-region (Fideghelli & Engel 2008).

It was further shown that plant traits, chemical compound included, differ between habitats (Forwick *et al.* 2003; Rieger *et al.* 2008) (see also chapter 5). In wild populations of the pharmaceutical relevant *Arnica montana* chemical compounds differed with ecological parameters (Seemann *et al.* 2010). For pharmaceutical and medicinal use of the plants the chemical composition of these compounds is an important factor. In future, as much diversity in plant traits as possible should be accessible in PGRs.

For collections of PGRs the prioritisation in intraspecific diversity needs further attention: basis of the "gene pool concept" is the degree of kinship to the "main crop", the possibility of gene transfer and the production of fertile offspring (Harlan & de Wet 1971). The high priority crop *Hordeum vulgare* was used as example by Von Bothmer & Seberg (1995) (see also Fig. 2). The primary gene pool consists of all breeding varieties and landraces (farmers' races or traditional breeds). Secondary gene pool would be the wild forms of the species *H. vulgare* ssp. *spontaneum* and the directly related species *H. bulbosum*, which are possible to crossbreed with breeding lines of *H. vulgare*. Furthermore, other wild related species should be collected for future breeding purposes as tertiary gene pool. With this classification a main part of the whole gene pool, namely secondary, tertiary gene pool and even the wild form in the primary gene pool, have to be collected in wild populations.

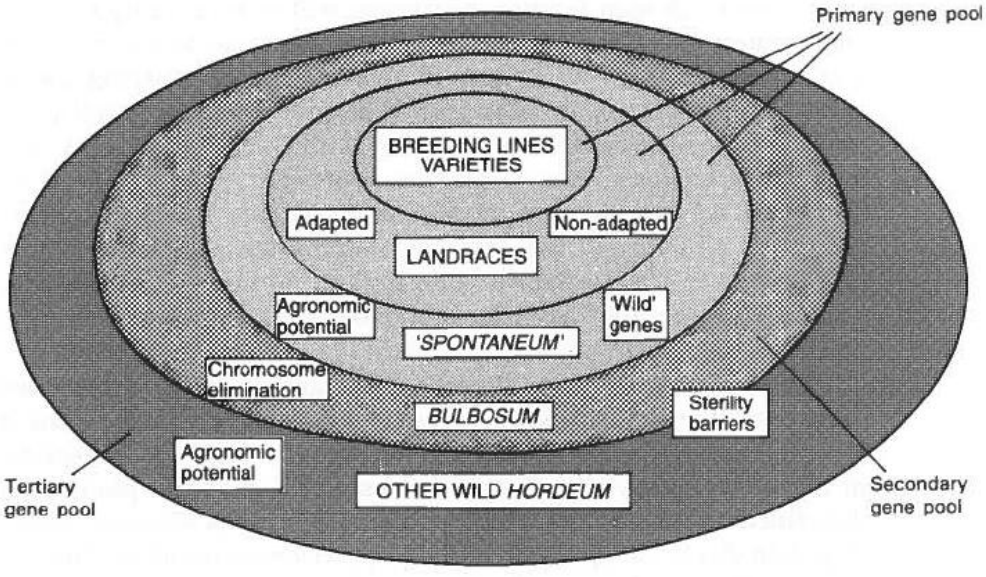


Fig. 2: Gene pool of *H. vulgare*, from Von Bothmer & Seberg (1995) derived from gene pool concept ofHarlan & de Wet (1971).

From breeding lines to landraces it is obvious, which “populations” have to be chosen for conservation collections. It gets more difficult, when it comes to CWRs, which are represented by tertiary, secondary and part of the primary gene pool. In Germany 4211 species are CWRs by definition (Kell *et al.* 2008). For rare or geographically restricted species all populations should be included in conservation purposes. For common species a prioritisation has to be made (see 3). Genebank guidelines specify and laws define limitations for collections of wild species, but those regulations are often ambiguous or lacking in background information.

Collections with high intraspecific diversity contain high diversity in seed traits, too. In wild species all life cycles (germination, flowering time, time of seed dispersal, dormancy) are adapted to the environmental conditions and differ between habitats and populations. It is important for collections to restore those adaptations, when they are genetically determined. Nevertheless, different environmental conditions also cause differences in seed quality and longevity (Mondoni *et al.* 2014; Mondoni *et al.* 2011; Probert *et al.* 2009). High quality and long lived seeds can be stored over long time periods without the necessity of testing, recollection or regrowth. Especially the knowledge about longevity of seeds is crucial to avoid the loss of the accessions. In cultured plants, also seed quality is optimised and overall longevity is well known. Several long-term storage data have been analysed over the last years e.g. (Hong & Ellis 1996; Nagel & Börner 2010). They all found that longevity under genebank conditions is species or family specific. Those studies also emphasise intraspecific differences in longevity that should be acknowledged, when it comes to determine the time of renewing the accession. In wild species, which are characterised by high intraspecific variation in other traits, also longevity and quality shows high differences between accessions (FAO 2014a).

2 GENEBANK STANDARDS

The knowledge about genebank management is growing with each collection. Several genebanks in different regions of the world described their findings for their range of species or collection area. General procedures are similar for all genebanks (Fig. 3). Most genebanks refer their strategies for collection and management to certain standards that describe all steps of genebank management, like ENSCONET (ENSCONET 2009b; FAO 2014a; ISTA 1999). Advises of standards for field collections are inconsistent, especially, when it comes to

wild species that are not restricted to certain regions and populations, but common and widely distributed. Concrete instructions concerning numbers of collected populations and even delineation of populations are very ambiguous (Neel & Cummings 2003).

For cleaning and preparation (drying included), as well as for long-term storage conditions of orthodox seeds guidelines are concordant. Quality and germination tests should be done by the genebanks regularly, but this depends on their species pool and their available resources. Intraspecific variation in the time, when seed quality steps beyond a certain percentage of viability, is hardly acknowledged. The adjustment of regrowth or recollection, therefore, is sometimes difficult.

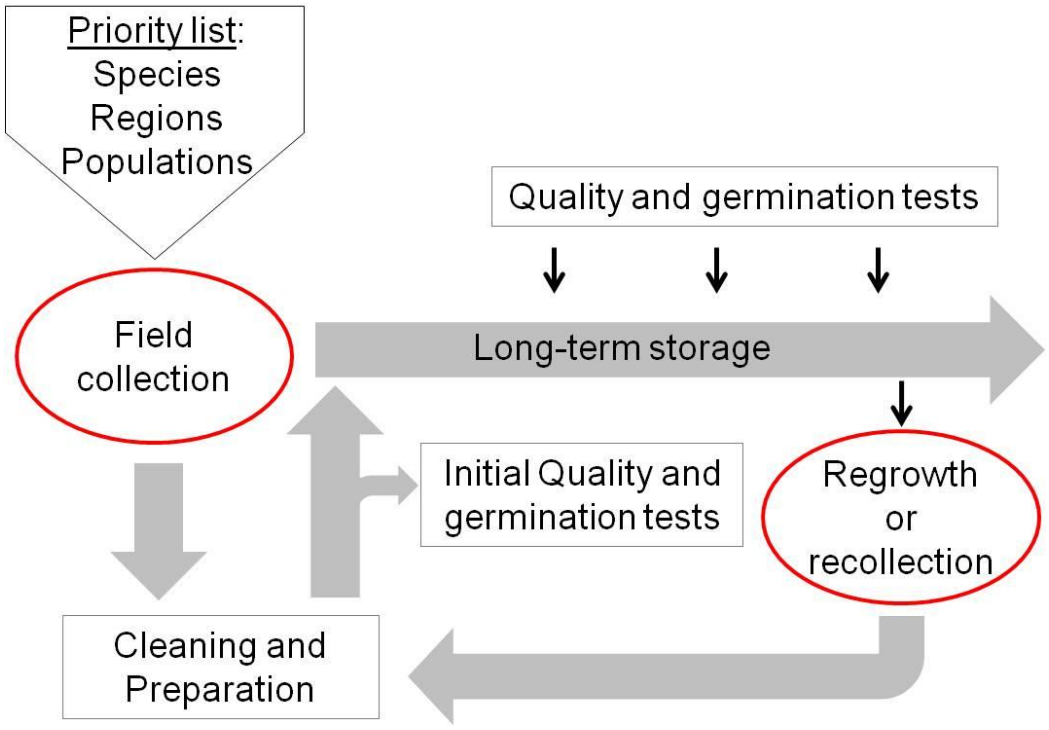


Fig. 3: Overview over important steps in genebank management. Red circled steps are objectives of the thesis at hand.

Guidelines for planning of field collections have to serve different aspects and, therefore, differ a lot. Overall, suggestions are made for regions and the number and extent of populations that should be collected (ENSCONET 2009b; FAO 2014a). On one hand bias should be avoided, i.e. collections should not be made too close (Hijmans *et al.* 2000) to prevent a wasting of resources (storage place, labour time and money). On the other hand

as many adaptations as possible should be collected to restore the main genetic diversity of each species. Important gene pools or areas should be represented. A limited sample of populations should represent a maximum amount of useful genetic variation in the species (Crossa & Venkovsky 2011; Marshall & Brown 1975).

One example, based on phenotypic diversity, is given by Ortiz *et al.* (1998). The authors indicate to restore 103 populations as „core"-collection of *Chenopodium quinoa*. Since the late last century genetic studies can be found in literature. Nevertheless, the intention of these studies and, therefore, the collection strategy and number of collected populations differ: 1-5 populations of endangered species are recommended, but differentiation and ecotypes have to be acknowledged (Briggs & Walters 1984; Brown & Briggs 1991; Guerrant *et al.* 2004). Alike, Falk & Holsinger (1991) suggest that 5 populations over the geographical range should be sufficient, which account for 67-83% of all alleles of four globally rare taxa (Neel & Cummings 2003). In contrast Guerrant *et al.* (2004) advise to collect in 50 populations over the whole geographical range. 53%-100% of all populations must be sampled to get all alleles with frequencies of > 5% according to Neel & Cummings (2003), who tested four rare species.

First and basic problem for wild and common species is the definition of a population. It is sometimes hardly possible to define strict borders of populations for common and widely distributed species. Different theories have been developed, of which the "gamodeme" theory (Briggs & Walters 1984) has to be mentioned, which is very similar to the Mendelian or panmictic populations (Silvertown & Lovett Doust 1993). Hereby, a population is the landscape unity in which free gene flow is possible. The distances of seed and pollen dispersal, at best in combination with genetic studies, can help to define such unities. It has been shown that populations classified by this definition are quite small and restricted for most plants (Briggs & Walters 1984; Ehrlich & Raven 1969). Even, if species are distributed quite homogenously over a wide area, samples should be taken within relatively small distances (isolation by distance effects) and held separate (Von Bothmer & Seberg 1995).

Only few genebanks have the possibility to operate globally. Most projects are conducted within restricted landscape areas or within political boundaries. The priority of collection areas and populations has to be defined within these areas. At least ecogeographic surveys should always be supplied before collection trips are planned (Von Bothmer & Seberg 1995). However, background studies are not always possible or reasonable: For common wild species, there is a problem to get funding for background studies, because there is no

obvious threat and no need to investigate population diversity in central distribution areas. Nevertheless, based on former explanations, we see that collections without prior knowledge are not advisable.

Guidelines for seed testing are based on certain family or species-specific traits. Long-term studies and artificial aging give a good idea of the time range, in which a collection should be renewed (Nagel & Börner 2010; Nagel *et al.* 2010).
Nevertheless, it was also described that quality and longevity of wild species showed high intraspecific differences (Probert *et al.* 2009). Differences are often caused by conditions at collection or prestorage, which are the most sensitive phase for damages of the seeds (Rao et al. 2006). Seed collection should be conducted at the time of natural dispersal (ENSCONET 2009a), what is difficult for wild species as seeds differ in ripening time within populations and even within one plant (Guttermann 2000). It is further advised to collect at dry and hot conditions, what cannot always be synchronised with ripening times of the seeds. However, intraspecific differences in quality and longevity are based on seed traits, which differ between areas and populations (Abedi 2013; Guttermann 2000). Few studies deal with the question, if intraspecific variance is caused by maternal effects or genetic adaptation (Donohue 2009; Kochanek *et al.* 2011; Mondoni *et al.* 2014). For effective genebank management it should be determined which environmental factors are decisive for quality and longevity of seeds to narrow down the time range of renewal of accessions.

4 THESIS OUTLINE

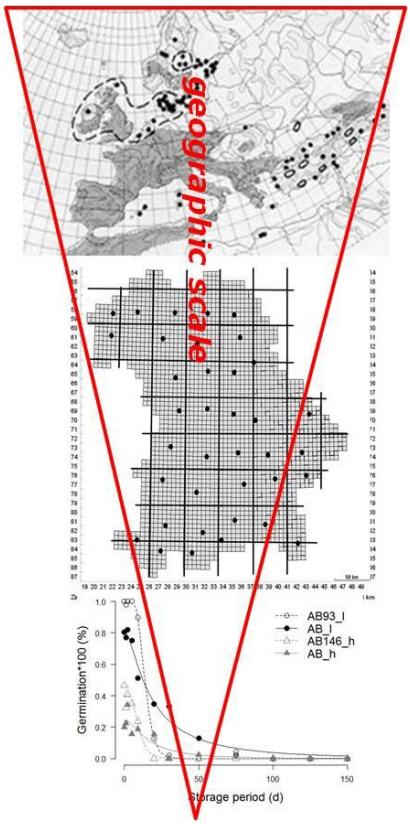
In times of globalisation the worldwide transfer of “perfected” and unified seeds is day-trade. With these standardisations local adapted races and genotypes – and even locally used species will disappear (Biber-Klemm & Cottier 2006; FAO 2010). Loss of arable land and fast environmental changes rise new challenges to plant breeders. The importance of PGRs for breeding is undeniable. It is necessary to restore backup seeds of wild PGRs with high levels of genetic diversity that can be used for agriculture, medicine, horticulture - or in any other aspect- for future purposes (FAO 2010). While collections focus on rare or geographically restricted species, it is rather difficult to define areas or populations of common and widely distributed species, which should be prioritised. Investigations of distribution of genetic

diversity within and between populations of common wild species can help to define economically and ecologically reasonable “collection zones”.
For effective genebank management it is further crucial to restore high quality seeds. The influence of environment al conditions on quality and longevity of seeds – and the impact of genetic adaptation are still unknown.
The present thesis deals with the distribution of genetic diversity in three common and widely distributed plants (chapter 2, 3, 4), and the influence of genetic and environment on seed quality and longevity (chapter 5). The thesis aims to provide basic information about possible collection strategies for common species (Tab. 1).

In chapter 2 the distribution of genetic diversity, postglacial history and human influence on populations of *Sedum album* L. was analysed over its distribution range in Europe. To infer population genetic diversity and differentiation we used amplified fragment analyses (AFLP). Supported by chloroplast microsatellite analysis and sequences of small non-coding chloroplast regions possible glacial refugia and migration routes after glaciations could be revealed. The knowledge about the ancient and current history of a species’ populations is crucial to define priorities for genebank collections.

Chapter 3 and chapter 4 aim to describe population genetic and differentiation of common species in a limited region. *Lathyrus pratensis* L. (chapter 3) is widely used as fodder and restoration plant, *Hepatica nobilis* Schreb. (chapter 4) was mainly used for horticultural purposes in Bavaria. The species are both hemicryptophytic and perennial, but differ strongly in level of usage, habitat and life traits, particularly type of seed dispersal. Of both species populations were collected at comparable distances all over Bavaria (18.4km to 388.5km for *L. pratensis* and 19.6km to 363.0km in *H. nobilis*). AFLP analyses were used to describe within population diversity and differentiation between population and regions. Priorities for collections should be determined. To evaluate the regions, which build the statutory frame for regionalised seed production, I compared those regions with the results of differentiations for both species.

Tab. 1: Intraspecific diversity of plant species in different geographic scales and the related chapters of the thesis at hand.



	region	chapter	title
scale 1	Europe	**2**	Phylogeography of *Sedum album* L.
scale 2	Bavaria	**3**	Diversity and differentiation of populations of *Lathyrus pratensis* L.
		4	& *Hepatica nobilis* Schreb.
scale 3	within populations	**5**	Intraspecific variability in seed quality & longevity

Genetic determination and environmental influence on quality and longevity of seeds were investigated with two experiments in chapter 5. For the first experiment seeds from 3 species were collected in lowland and alpine populations. Out of all collectioned populations, plants were grown in a common garden. All seeds from wild and common garden populations were tested for quality with x-ray, combined germination and Tetrazoliumtests – and for longevity with controlled artificial aging. For the second experiment seeds were collected in different years from the same populations and tested for quality and longevity with the same methods as mentioned above. Both lowland and alpine collection areas were chosen nearby a weather station. Climatic data of different years were tested for their influence on quality and longevity of the seeds. Chapter 5 aims to evaluate, if genetic adaptation or maternal effects are most influential to quality and longevity of seeds – and which environmental factors have to be acknowledged for collection of high quality and long-living seeds in genebank.

Finally, in chapter 6 I reviewed the results of former chapters in the context of planning collection trips and quality management in genebanks. Strengths and limitations of the conducted studies are discussed and general perspectives for common species are given.

Chapter 2

Phylogeography of a tough rock survivor in European dry grasslands

ABSTRACT

Phylogeography of plants in Europe already revealed common glacial refugia and migration routes for many trees and herbs with arctic-alpine distribution. Postglacial history of dry grassland species could be representative to understand the history of a threatened, extremely species-rich habitat in Central Europe. *Sedum album* (Crassulaceae) is a common inhabitant of rocky sites in dry grassland in Central Europe. We inferred phylogeography of *S. album* over its distribution range in Europe.

Genetic diversity within and differentiation between 34 populations of *S. album* was revealed with AFLPs. Isolation of populations was indicated by rarity of fragments and isolation-by-distance effects. We sequenced the trnL-trnF region in 32 populations and additionally used chloroplast microsatellites to analyse chloroplast haplotype distributions.

Two distinct lineages of *S. album* were detected. Eastern, Central Europe and the Italian Peninsula were related, a second lineage comprising populations from the Iberian Peninsula, Western and Northern Europe was found. Accumulation of chloroplast haplotypes, high diversity of AFLP fragments within populations and high levels of rare fragments could be detected in Liguria, the Italian and the Iberian Peninsula - indicating zones for glacial refugia. Isolation by distance was present all over the distribution range and in south-western and Central Europe separate, but missing in Western Europe, where a contact zone for the two lineages can be expected.

Our results suggest migration routes of *S. album* north-eastward from glacial refugia in southern Iberia, northward from the Italian Peninsula, and north- and westward from Eastern Europe. Central European grasslands were recolonised from source populations in Eastern Europe and on the Italian Peninsula.

Keywords: Central Europe; Crassulaceae; dry grasslands; phylogeography; rocks; *Sedum album*

INTRODUCTION

The geographical distribution of plant species has always been influenced by climatic and edaphic factors. Most influential were Quaternary ice ages that dramatically reduced habitats of most species (Hewitt 2000; Mithen 2011; Taberlet *et al.* 1998). Large parts of northern Europe, the Alps and the Pyrenees were covered by ice. Steppe-tundra with cold and dry conditions was the dominant vegetation type over main parts of Western and Central Europe. Most species were only able to survive in Southern and Eastern Europe, where climatic conditions were not as harsh as in the Northern parts.

Next to archaeobotanical findings (most notably pollen depositions, but also macrorest findings) genetic techniques are the methods of choice to reveal postglacial history of plant species (Avise 2009). Possible scenarios of colonisation are based on high haplotype diversity in glacial refugia. In areas that have been recolonised after the glacial maximum (LGM), a loss of diversity due to founder effects during rapid expansion is suggested (Grivet & Petit 2002; Hewitt 2000). High genetic diversity within and strong differentiation between populations in southern areas (compared to northern areas) is particularly adducted for the northward expansion of species (Demesure *et al.* 1996; Després *et al.* 2002; Dumolin-Lapègue *et al.* 1997; Ferris *et al.* 1998; Grivet & Petit 2003; Hedrèn 1997; Hewitt 1999; Rendell & Ennos 2002). Nonetheless, high genetic diversity can also be found in contact zones, where different lineages from distinct genetic sources mix up (Petit *et al.* 2003; Walter & Epperson 2005). Therefore, history of the landscape has to be taken into account, when genetic pattern of plant populations are interpreted. The accumulation of rare haplotypes is further suggested for very old and stable populations in glacial refugia. Due to stochastic processes it is more unlikely to find rare haplotypes in recently colonised habitats (Schönswetter & Tribsch 2005).

Comparative phylogeographic studies already found patterns in the distribution of genetic diversity that could explain collective areas of glacial refugia and migration routes for the Eurasian area (Comes & Kadereit 1998; Taberlet *et al.* 1998). Mountain ranges, in particular the Alps and the Pyrenees, form natural barriers in migration routes of European plant species (Ferris *et al.* 1998; Grivet & Petit 2003; Palmé & Vendramin 2002). Rivers can build barriers or – more likely for most plants – serve as transportation routes for their dispersal (King & Ferris 1998).

A centre zone for potential glacial refugia of Eurasian species is the Iberian Peninsula, from where trees and shrubs were heading northward and westward after the LGM (Cottrell *et al.*

2005; Dumolin-Lapègue *et al.* 1997; Palmé & Vendramin 2002; Rendell & Ennos 2003). Potential glacial refugia were identified for the genus *Helianthemum* in southern France and near the Pyrenees from where the species migrated north- and westward (Soubani 2010). Refugia were further suggested in Italy and on the Balkan Peninsula for several species (Cottrell *et al.* 2005; Dumolin-Lapègue *et al.* 1997; Heuertz *et al.* 2004; Konnert & Bergmann 1995; Magri 2008), from where the species expanded north- and westward to Central Europe. Based on aforementioned studies, it is assumed that Central Europe was recolonised after the LGM mainly from source populations in the South-east, on the Iberian Peninsula or in the South-west (Taberlet *et al.* 1998).

Next to contradiction-expansion theories, a survival of plants and animals in small areas with suitable microclimate is rather thinkable (Kajtoch *et al.* 2016). For many species cryptic refugia in areas with sheltered topography, e.g. river valleys and caves in river valleys has been described (Stewart & Lister 2001; Stewart *et al.* 2010).

For migrations during interglacials and after the LGM mankind was an influential factor for most plant species in Central Europe (Poschlod 2015; Poschlod & Bonn 1998). Natural postglacial reforestation was restrained by land use (grazing, agriculture) and suitable habitats for thermophilic plants were formed, e.g. dry grasslands that evolved on sites with grazing livestock. Several plants further dispersed via migrations and settlement of humans after the LGM, where they took plants or seeds with them – conscious or unconscious with pastoralism (Bonn & Poschlod 1998; Di Castri 1989; Poschlod *et al.* 2013). For several anthropochorous species (plants and animals) migration routes can be referred to migration of humans, e.g. with livestock (Hanotte *et al.* 2002; Meindl *et al.* 2016; Negrini *et al.* 2007; Poschlod 2015).

So far, postglacial history of Central Europe was investigated for many trees (Dumolin-Lapègue *et al.* 1997; Ferris *et al.* 1998; Grivet & Petit 2003; Heuertz *et al.* 2004; Konnert & Bergmann 1995; Magri 2008; Palmé & Vendramin 2002), arctic-alpine or rare species (Abbott & Comes 2004; Alsos *et al.* 2007; Després *et al.* 2003; Kropf 2008; Kropf *et al.* 2002; Reisch 2008; Reisch *et al.* 2003a; Reisch *et al.* 2003b; Ronikier *et al.* 2008; Sanz *et al.* 2014; Schönswetter *et al.* 2006b; Skrede *et al.* 2006), whereas studies about postglacial history of herbaceous lowland plants are scarce (Bylebyl *et al.* 2008; Grivet & Petit 2002; Meindl 2012; Pérez-Collazos *et al.* 2009; Rendell & Ennos 2002; Soubani 2010).

Especially the investigation of common dry grassland species in Europe can reveal information on the history of an extremely species-rich and important habitat for plant and

animal conservation – and add more information on general pattern of glacial refugia and postglacial migration in plants.

The succulent *Sedum album* L. (Crassulaceae) is a typical inhabitant of dry grasslands on alkaline and calcareous soils in Central Europe. It can be found on rocks and shallow soils within the vegetation type Festuco-Brometalia and in pioneer vegetation on rocks within the Sedo-Scleranthetalia- group (Sedo albi-Veronicion dillenii) (Ellenberg 1996; Polunin 1980; Tutin 1980). *S. album* can also be found on crevices on stony riverbanks and in forests with oak or olea in the Mediterranean. Further, it immigrates to man-made habitats, like stone walls of buildings and gardens in cities and villages (Bonn & Poschlod 1998; Brandes 1995; Smith & Figueiredo 2010; Wittig 2004). Nowadays, *S. album* is common and distributed over wide regions of temperate Europe and the Mediterranean. Similar to many other plants, it was possibly introduced to Central Europe after the LGM from glacial refugia in the South-west or South-east. But what can be told about the postglacial history of *S. album* and its migration to dry grasslands in Central Europe?

In this study we investigated 34 populations of *S. album* in Europe with AFLPs, chloroplast sequences (trnL-trnF) and chloroplast microsatellites to answer the following questions:

Can we infer the postglacial history of a common plant like *S. album* with standards methods (AFLPs, chloroplast markers)?

Was it introduced to Central European after the LGM from glacial refugia in the South-west or South-east – or did it spread from cryptic refugia in Central Europe?

What can be told about its migration routes to dry grasslands in Central Europe?

MATERIALS AND METHODS

Species and Collection

Sedum album L. (Crassulaceae) is a perennial herb, with succulent woody stem and succulent linear-cylindrical to ovoid leaves. Seeds are long-term persistent and distributed by rain and water. Shoots and leaves are very dry resistant and can develop roots, when they attain proper habitat. Flowers have white to pink petals and are pollinated by insects or self-pollinated.

S. album was used as ornamental, medical and edible plant, and was proven to grow near human settlements (Bonn & Poschlod 1998; Cakilcioglu & Turkoglu 2010; Jman Redzic

2006; Özgen & Kaya 2004; Simkova & Polesny 2015; Tardío *et al.* 2006; Wittig 2004). The natural distribution range is all over Europe except for parts of the north and east (Meusel *et al.* 1965; Tutin 1980). In the East borders of distribution within Poland, , Slovakia, Hungary, Romania, Bulgaria and Turkey have been described (Polunin 1980; Tutin 1980) . In the South *S. album* can be found until northern Morocco, Algeria and Tunisia. In the North the natural distribution is described in the South of Sweden and Norway, while it is introduced in main parts of the UK and Ireland, as well as in parts of Sweden (Tutin 1980).

Chromosome numbers of 2n=2x=32, 34 and 38, as well as tetraploid individuals with 2n=4x=64, 68 have been described for *S. album* (Albers 1998; Dobes & Vitek 2000; t' Hart 1991), which are not congruent with segregates (*S. athoum, S. micranthum*) that have been described based on leaves (size and shape), flowers (size of flowers and shape of petals), distribution area and habitat conditions.

Fig. 4: *S. album* in one of its typical habitats: on a rocky site within a meadow of dry grassland in Germany.

For our study 34 populations of *S. album* were sampled in dry grassland (Festuco-Brometalia) and rock slopes (Sedo albii-Veronicion dilenii) all over Europe (Table 1, Appendix Tab. S1) except for northern and eastern parts, where populations are described as introduced (Meusel *et al.* 1965). *S. album* is not protected and we did not collect in protected areas (Appendix Tab. S1). We avoided populations with characteristics of segregates, as well as populations near gardens and roof greenings to exclude the direct influence of horticultural S*. album* breeds. Fresh and young branches of 6 (GB) to 14 (S12) plants per population were sampled (Table 1) and stored in filter bags with silica gel until the material was frozen at -18°C. Voucher specimens are deposited at the Institute of Plant Sciences, University of Regensburg.

Tab. 2: 34 populations of *S. album*, given are identification (*ID*), country, number of individuals for AFLP (*nr*), microsatellite haplotypes (*ccmp*), trnL-trnF sequence haplotype (*seq*), groups revealed by AFLPs (*AFLP*): Central Europe East (CE-E) and West (CE-W), Western Europe (WE), Iberian Peninsula (IP), Southern Italy and western Alps (S-It & Al), Liguria (Lig); further, percentage of polymorphic fragments (*%PL*), diversity as Shannon Index (*SI*) and SSWP/n-1 (*SSWP*), down-weighted rare fragments (*DW*), private fragments (*priv*) and fragments restricted to two populations (*with*) are shown.

ID	***country***	***nr***	***ccmp***	***seq***	***AFLP***	***%PL***	***SI***	***SSWP***	***DW***	***priv***	***with***
A	Austria	11	a	H1	CE-E	20.22	0.10	12.92	9.85	0	SK
AND	Andorra	11	a	H12	IP	30.75	0.16	21.06	11.59	0	
BE	Belgium	11	a	H8	WE	22.71	0.12	15.08	7.61	0	
CZ	Czech Republic	11	a	H1	CE-E	26.87	0.15	4.75	10.09	0	
D1	Germany	11	a	H6	CE-W	9.70	0.04	7.36	9.87	2	
D2	Germany	12	a	H1	CE-W	10.35	0.06	6.27	7.25	0	
D3	Germany	12	f	H17	CE-W	13.3	0.05	15.78	12.31	1	SRB2
D4	Germany	12	a	H1	CE-W	17.73	0.07	8.39	7.70	0	
D5	Germany	11	a	H5	CE-W	19.94	0.09	12.06	9.17	0	
D6	Germany	11	a	H1	CE-E	19.39	0.10	13.66	8.06	0	
E1	Spain	10	c	H13	IP	22.71	0.12	12.99	9.45	0	
E2	Spain	12	a	H12	IP	33.24	0.17	22.49	11.88	0	
E3	Spain	13	a	H12	IP	33.24	0.17	21.17	14.22	2	E4
E4	Spain	12	a	H14	IP	26.87	0.14	17.62	12.07	0	E3

F1	France	12	a	H10	WE	26.32	0.12	15.76	10.32	0	F3
F2	France	11	a	H11	WE	23.82	0.12	15.75	7.40	0	
F3	France	11	a	H9	WE	27.7	0.14	18.96	10.53	1	F1, F4
F4	France	12	a	H12	WE	13.3	0.06	7.64	7.83	1	F3
F5	Spain	12	a	*	IP	21.88	0.10	6.00	7.62	0	
GB	Great Britain	6	a	*	WE	15.79	0.09	13.37		0	
I1	Italy	9	a	H3	S-It & Al	13.57	0.07	6.46	9.70	0	I8
I2	Italy	11	a	H4	CE-E	13.57	0.05	13.63	6.87	0	
I3	Italy	12	a	H1	CE-E	21.33	0.11	15.20	9.31	0	
I4	Italy	10	a	H1	CE-E	21.88	0.11	15.24	10.97	0	
I5	Italy	12	d	H16	Lig	13.3	0.05	6.56	14.74	3	2*I6
I6	Italy	11	e	H12	Lig	22.71	0.11	14.60	17.19	4	2*I5, I8
I7	Italy	14	a	H7	S-It & Al	23.55	0.12	14.68	10.04	1	
I8	Italy	12	a	H1	S-It & Al	29.64	0.15	20.28	13.82	2	I1, I6
PO	Portugal	11	b	H15	IP	9.42	0.04	5.00	15.68	6	
S1	Switzerland	12	a	H7	CE-W	22.71	0.12	12.54	8.97	0	
S2	Switzerland	12	a	H2	S-It & Al	15.79	0.06	7.33	10.52	1	
SK	Slovakia	12	a	H1	CE-E	21.05	0.10	19.71	10.02	0	A
SRB1	Serbia	11	f	H18	CE-E	22.99	0.11	14.87	12.70	0	SRB2
SRB2	Serbia	12	f	H18	CE-E	20.22	0.09	10.69	11.24	0	SRB1, D3

* no sequence data available

DNA extraction

5-8 leaves of each plant were homogenised with liquid nitrogen and DNA was extracted using CTAB method (Rogers & Bendich 1994) with minor adaptations (Reisch *et al.* 2003a). DNA concentrations of all extractions were measured as transmission of optical density. A standard DNA concentration (7.8ng/µl) was diluted, which was taken for all further investigations.

AFLPs

A screening of 3 x 12 fluorescence labelled primer combinations was carried out with 8 individuals of 4 populations. Three high resolving primer combinations were selected (D2: MseI-CAA/EcoRI-AAC, D3: MseI-CAT/EcoRI-AAG, D4: MseI-CAA/EcoRI-ACT).

AFLP analysis was conducted with 388 individuals in total following the protocol of Vos et al. (Vos *et al.* 1995) (Vos *et al.* 1995) with minor variations (Reisch 2008). About 50ng of DNA was digested with EcoRI and MseI restriction enzymes (Fermentas) and ligated to adaptors (MWG) with T4 DNA Ligase (Fermentas) at 37°C for 2h with a final step of 70°C for 15min. Restriction-Ligation and all following polymerase chain reactions were carried out in automated Thermocycler (Eppendorf) with Taq Polymerase (PeqLab). The first amplification step was conducted with a pair of one-base primers (MseI-C/EcoRI-A, MWG) in 30 cycles. Fluorescence labeled Mse primers were used for the final amplification with three-base primers (MseI-C/EcoRI-A, MseI-C/EcoRI-A, MseI-C/EcoRI-A, MWG); 25 cycles were run of which 10 cycles had a 1°C touchdown profile. DNA was precipitated with NaAC, EDTA, Glycogen and cold EtOH (96%, -20°C) and cleaned with cold EtOH (70%, -20°C). After the DNA pellet was dried, it was dissolved in Sample Loading Solution and charged with CEQ Size Standard 400 (both Beckman Coulter). The capillary gel electrophoresis was run on an automated sequencer (CEQ 8000, Beckman Coulter) and raw data were automatically analysed with CEQ 8000 software (Beckman Coulter) aligning fragments according to CEQ Size Standard 400.

Clear and well-defined fragments were scored manually in the program BIONUMERICS (Applied Maths, v. 3.0). Fragments with sizes of 60 to 420bp were processed and scored across all individuals as either present or absent. Both individuals and fragment sizes that did not give clear, defined and reproducible fragments were excluded from the analysis. The resulting binary matrix was used for further statistical analysis.

To reassure good quality of fragments 55 individuals were replicated, which gave an error rate of 1.36% (Bonin *et al.* 2004).

Genetic diversity within populations was calculated based on all markers as polymorphic fragments, percentage of polymorphic fragments and Shannon Information Index ($I = \sum pi \ln pi$) for each population (Parisod & Christin 2008), using the program POPGENE version 1.32 (Yeh *et al.* 1997). Sum of squares within populations (SSWP) was derived by analysis of molecular variance in GENALEX version 5 (Peakall & Smouse 2001). SSWP was divided by n-1 for each population to describe within population diversity independent from the number of individuals (Fischer & Matthies 1998b).

Rarity of fragments can indicate the level of isolation and differentiation (Slatkin 1985). Therefore, we tested populations for private fragments or shared fragments between two populations. We detected rarity as frequency down weighted markers (DW) for each

population (Schönswetter & Tribsch 2005) with AFLPdat in R (Ehrich 2006). We randomly chose 9 individuals per population in five iterations. Due to few individuals Population GB was excluded from this analysis.

Among population distances were calculated with the program AFLP-SURV (Vekemans 2002) as Nei's standard (Ds) with non-uniform prior distribution of allele frequencies. Based on Ds distances we constructed consensus Neighbour-Net graphs with Splitstree (Huson & Bryant 2006).

The allocation of differentiation within populations, among populations and among main groups revealed by Neighbour-Net graphs and Bayesian clustering was calculated in GENALEX version 5 (Peakall & Smouse 2001). Based on pairwise genetic distances between individuals, population differentiation was inferred as PHiPT, which is an analogue of Wrights F-statistic (Wright 1965). Further a hierarchical AMOVA was used to estimate differentiation between populations within groups and between groups (Excoffier *et al.* 1992). Based on pairwise PHiPT values from AMOVA and pairwise geographic population distances (km) we conducted Mantel-Test (Mantel 1967) in GenAlEx version 5 (Peakall & Smouse 2001). Correlations of genetic and geographic distance were tested using 999 permutations.

Genetically homogenous groups were inferred with Bayesian clustering in the program STRUCTURE version 2.2 (Pritchard *et al.* 2000). Allele frequencies were used as correlated in an admixture model (Evanno *et al.* 2005). 10^4 iterations for estimating the number of groups with a burn-in-period of 10^4 were set. For each predefined number of K (1-35) 10 iterations were run. From the resulting values of L(K) standard deviations and ΔK were calculated to affirm the most likely number of groups (Evanno *et al.* 2005).

cpDNA sequence analysis

The plastid trnL intron and trnL-F spacer sequences were chosen, because they were most variable in a preliminary test using primers Tab c and Tab f (Taberlet *et al.* 1991). TrnS–G intron (Hamilton 1999) and rpl32–trnL(UAG) (Shaw *et al.* 2007) were tested, but excluded for further analysis due to few variability or difficult amplification.

Polymerase chain reactions contained the following reagents: 3 µl DNA (7.8ng/µl), 0.4 µl of each primer (10 mM), 1 µl BSA, 1 µl dNTPs (5 mM), 1 µl PCR Buffer (10x), 1 µl MgCl2 (50 mM), 0.08 µl Taq (PeqLab, place, land) and 5.52 µl water for molecular biology (Roth). Reactions were run on a thermal cycler (Eppendorf) with 35 cycles of denaturing at 95°C

(30s), annealing at 52°C (1.30min) and extension at 72°C (4min) were run, closing with final extension step at 72°C (7min) and hold at 4°C.

PCR products were purified using 4µl of a mixture of Exonuclease (0.08µl, 20U/µl), SAP (0.8µl, 1U/µl) and water (3.12µl). Purification was conducted at 37°C (30min) and finished with a step at 95°C (5min).

Cycle sequencing was performed with 3µl purified product, 0.6µ Buffer (20x, Beckman Coulter), 0.6µl primer (10µM), 1.4µl DTCS Master Mix (Beckman Coulter) and 4.4 µl water for molecular biology (Roth). PCR products were precipitated with Glycogen (20mg/ml), NaAc (3M, pH5.2), Na_2EDTA (100mM, pH8) ice-cold EtOH (96%) and cleaned with EtOH (70%). Products were separated with capillary gel electrophoresis on an automated sequencer (CEQ 8000, Beckman Coulter).

Two individuals per population were conducted. All samples were amplified in both directions (with Tab f and Tab c), but an overlap could not be achieved for all samples. Though, to reassure the stability of the sequences, individuals were sequenced twice and consensus sequences were used for further analyses. Populations F5 from France and GB from Great Britain were excluded from the sequence analysis because we failed to produce two appropriate sequences per population.

We compared resulting sequences to GenBank using Blast (Altschul *et al.* 1994). Alignment was conducted using Clustal Omega (McWilliam *et al.* 2013) and adjusted manually with BioEdit Sequence Alignment Editor 7.2.0 (Hall 1999). Sequences were submitted to Genbank (Appendix Tab. S1).

Concatenated cpDNA sequences were used to build a network under statistical parsimony (as implemented in Clement *et al.* (2000)) in TCSv1.21 with gaps coded as missing. The connection limit was set at 120 steps to include the out-group *Sedum dasyphyllum* from Barcelona (BCN) (van Ham & 't Hart 1998).

Chloroplast microsatellites

Universal conserved chloroplast microsatellite primers (ccmp1, ccmp2, ccmp3, ccmp5, ccmp6, ccmp7, ccmp8, ccmp9, ccmp10) were tested in an initial screening of four individuals (Weising & Gardner 1999). Ccmp2, ccmp3, ccmp6, ccmp7, ccmp8 and ccmp10 yielded consistent products. Only ccmp2, ccmp3 and ccmp6 were variable and used for further investigations comprising 2 individuals per population. Universal M13-tailed primers were labeled with fluorescent dyes DY-571, Cy 5.5, Cy 5 (MWG). Amplifications were conducted in

10µl volumes containing following reagents: 2µl DNA (7.8ng/µl), 0.1µl ccmp-forward (1 mM), 0.15µl universal ccmp-forward, fluorescence labelled (10 mM), 0.15µl ccmp-reverse (10 mM), 0.4 µl dNTPs (5 mM), 1 µl PCR Buffer (10x), 0.05 µl Taq (PeqLab) and 6.25 µl water for molecular biology (Roth). Reactions were run in a thermal cycler (Eppendorf) with a denaturation step at 94°C (5min), followed by 34 cycles of denaturing at 94°C (1min), annealing at 50°C (1min) and extension at 72°C (1min), closing with final extension step at 72°C (8min) and hold at 4°C.

1 µl of each primer reaction of one individual was mixed and added to Sample Loading Solution, charged with an internal standard (CEQ Size Standard 400) and run on an automated capillary sequencer (CEQ 8000, Beckman Coulter). Chloroplast haplotypes were defined by fragment lengths using CEQ 8000 software. A haplotype network was built under statistical parsimony in TCSv1.21 (Clement *et al.* 2000). Geographic sampling regions were used to analyse group structure of sequence and microsatellite haplotypes.

RESULTS

AFLP analysis of 385 individuals out of 34 populations resulted in 361 fragments (D2: 135, D3: 116, D4: 110), of which 96.4% were polymorphic. 24 fragments were only represented in single populations (private, Tab. 2). Genetic diversity ranged from SI = 0.1724 (E3) to SI=0.391 (D1). In general, populations from southern regions showed higher diversity than populations from Northern and Central Europe (Fig. 5). Likewise, the rarity of fragments was high in southern populations (max. DW=17.19 in I6 and max. private fragments = 6 in PO) and lower in northern and central populations (min. DW=6.87 in I2) (Fig. 5). Private fragments were accumulated in populations D1 (Germany), I5 and I6 (Liguria), PO (Portugal), E4 (northern Spain) and I8 (Central Italy).

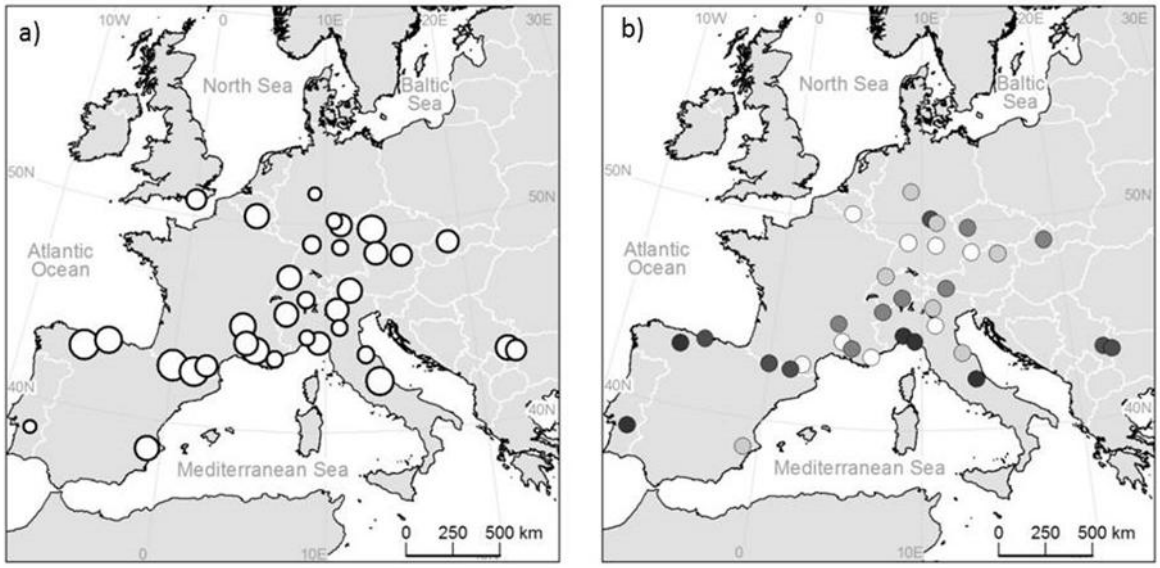


Fig. 5: Investigated populations of *S. album*: Level of genetic diversity (SI) is indicated by circle size (a), higher levels of rarity (DW) for each population are indicated by darker colour (b).

In the parsimony network (Fig. 6) the separation of south-western Europe (Spain and Portugal), Western Europe, Serbia, Liguria and Central Europe with Italy was evident. Central Europe and Italy formed one group in the AFLP network and a further division in three subgroups was possible: Central Europe-west comprises populations from western Germany and Switzerland, Central Europe-east comprises populations from Austria, Czech Republic, Germany, Italy, Slovakia and Great Britain. With the exception of the population from Great Britain, populations formed one lineage from north-east Italy northward to Germany and eastward to Czech Republic and Slovakia. Populations from the middle of the Italian Peninsula and the western Alps (Italy and Switzerland) built a third group. Populations I5 and I6 from north-western Italy (Liguria) were segregated from the rest of the Italian group and clustered between populations from Serbia and the populations from Iberia. D3 from Central Europe was separated in the network from its geographic neighbours and situated between the populations from Serbia and western Central Europe.

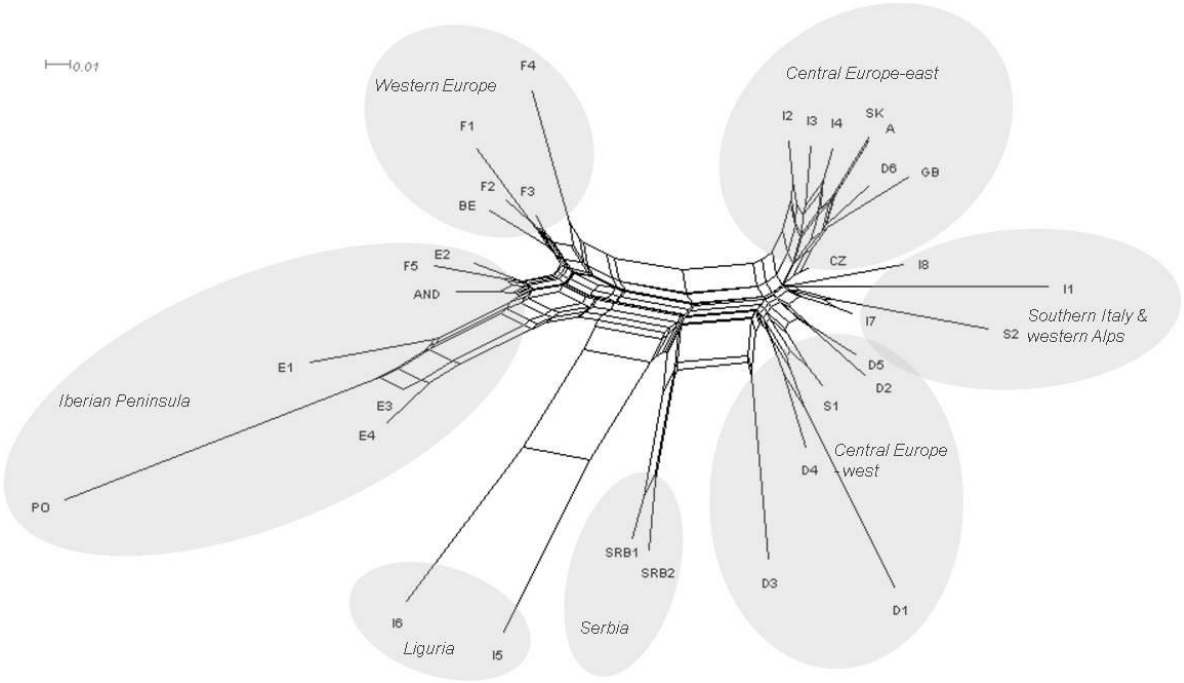


Fig. 6: Phylogenetic network based on 385 *S. album* individuals analysed with AFLPs.

Differentiation was high between populations in general (72.3%,Tab. 3). We found 32.7% of differentiation between groups, which have been determined by neighbour-net analysis and were mainly congruent with geographic regions. We omitted the regions Liguria and Serbia (incl. D3) from AMOVA with STRUCTURE groups, because those regions contain high levels of both STRUCTURE groups and the assignment to only one group is, therefore, not accurate. The AMOVA of STRUCTURE groups revealed similarly high differentiation between both groups (29.8%).

To reveal the influence of other regions on Central European populations, we tested differentiations of all combinations of the regions against each other (Tab. 3). The groups "Western Europe" and "Iberian Peninsula" versus "Central Europe" with "Serbia" and "Southern Italy & western Alps" showed the highest differentiation. Even without the population D3, which was closely connected to Serbia in all kind of analyses, a close connection of populations from Central Europe to eastern and southern parts of Europe could be shown. It is notable, that differentiation between populations was extraordinary high in the groups from the Iberian Peninsula (61.0%) and between populations from Liguria (75.0%; data analysis of single neighbour-net regions not shown).

Tab. 3: Analysis of molecular variance (AMOVA) was conducted for main geographic regions (neighbour-net regions) and STRUCTURE groups. Differentiation of the regions Iberian Peninsula, Serbia, Western and Central Europe were tested in different combinations; populations from Liguria were excluded from the analysis.

	d.f.	sum of squares	est. var	total variance (%)
Overall analysis				
among populations	34	13379.442	34.337	72.3
among individuals within populations	353	4653.576	13.183	27.7
Analysis of neighbour-net regions				
among regions	6	6700.3	16.29	32.7
among populations within regions	27	6555.33	20.36	40.8
among individuals within populations	351	4641.58	13.22	26.5
Analysis of STRUCTURE groups (without Liguria and Serbia)				
among regions	1	2922.16	16.07	29.8
among populations within regions	28	8066.74	24.33	45.2
among individuals within populations	309	4157.1	13.45	25.0
Iberia <-> Western E & Central E & Serbia				
among regions	1	1895.484	11.976	21.6
among populations within regions	32	11360.145	30.218	54.5
among individuals within populations	351	4641.576	13.224	23.9
Iberia & Western E <-> Central E & Serbia				
among regions	1	2734.036	13.553	24.8
among populations within regions	32	10521.593	27.894	51.0
among individuals within populations	351	4641.576	13.224	24.2
Serbia (without D3) <-> Central E & Iberia				
among regions	1	846.14	10.583	18.7
among populations within regions	31	11841.109	32.638	57.6
among individuals within populations	340	4572.66	13.449	23.7

We found significant correlation of genetic and geographic distances with Mantel-Test over the whole dataset (r=0.362, p=0.001) indicating isolation by distance among populations.

Isolation by distance was further present in two of the main network groups (Central Europe, Iberian Peninsula), but absent between the populations from Western Europe (data not shown).

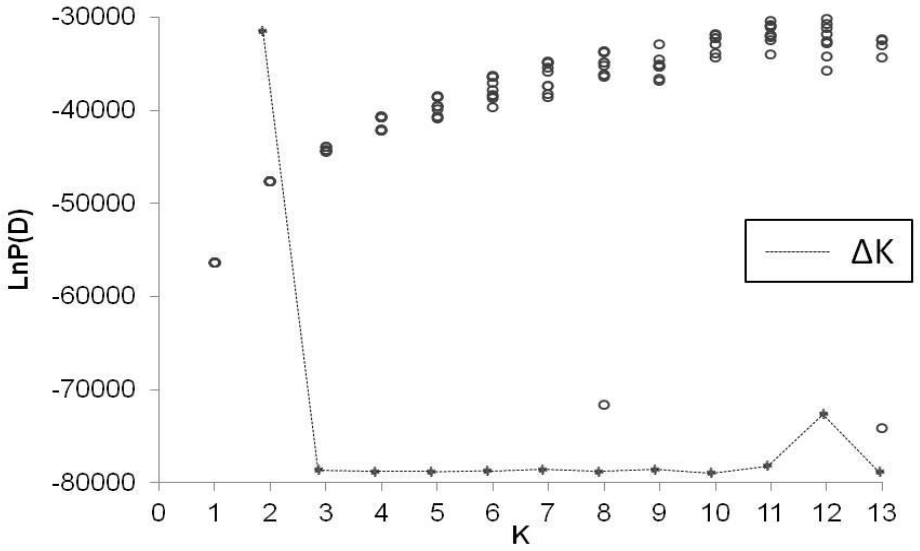


Fig. 7: Bayesian clustering of AFLP data with STRUCTURE revealed most possible partition of the *S. album* into two groups

Bayesian analysis distinguished the most probable partition in two lineages (Fig. 7). The first lineage was dominant in populations from southern, western and northern Europe including Spain, Portugal, Andorra, France and Belgium (Fig. 8). The second lineage was found in all Central European populations (Germany, Austria, Switzerland, UK, Italy -north and east of the Appenin mountain range-, Czech Republic, Serbia). A high content of both lineages could be seen in populations I5 and I6 from Liguria, I1 from Central Italy, D4 from western Germany and both populations from Serbia.

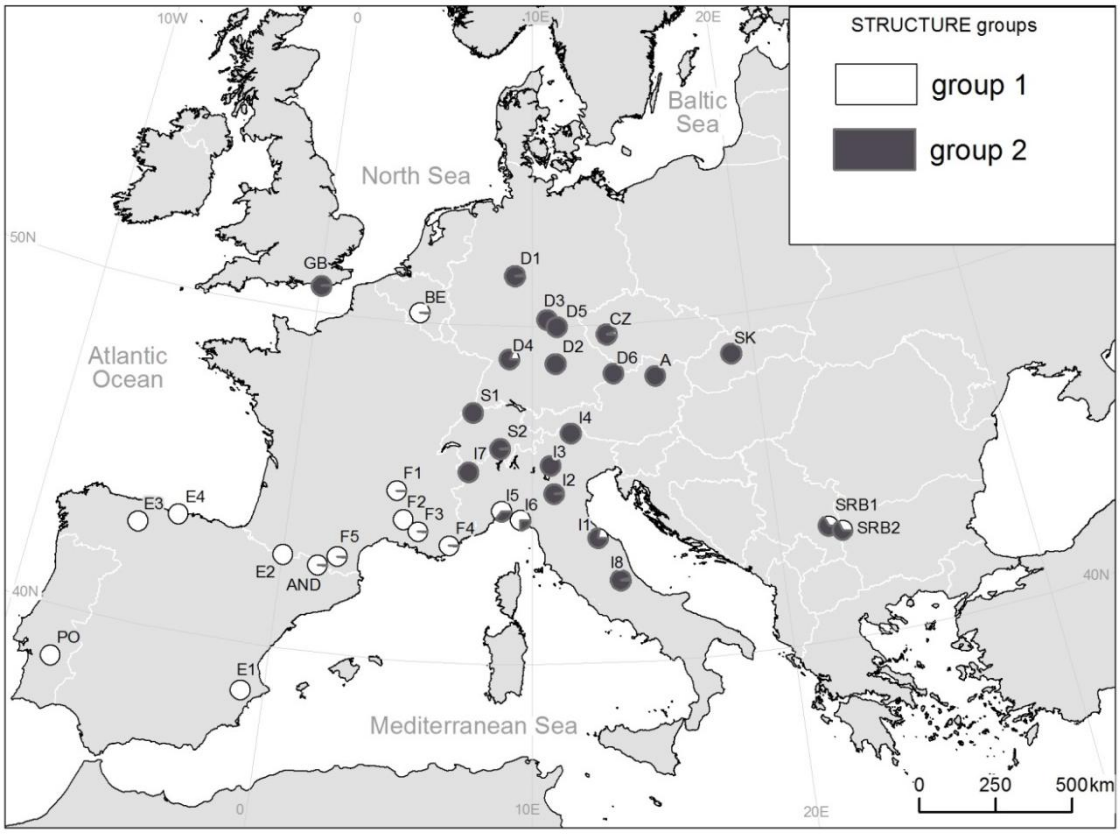


Fig. 8: Geographic distribution of Bayesian groups (K=2).

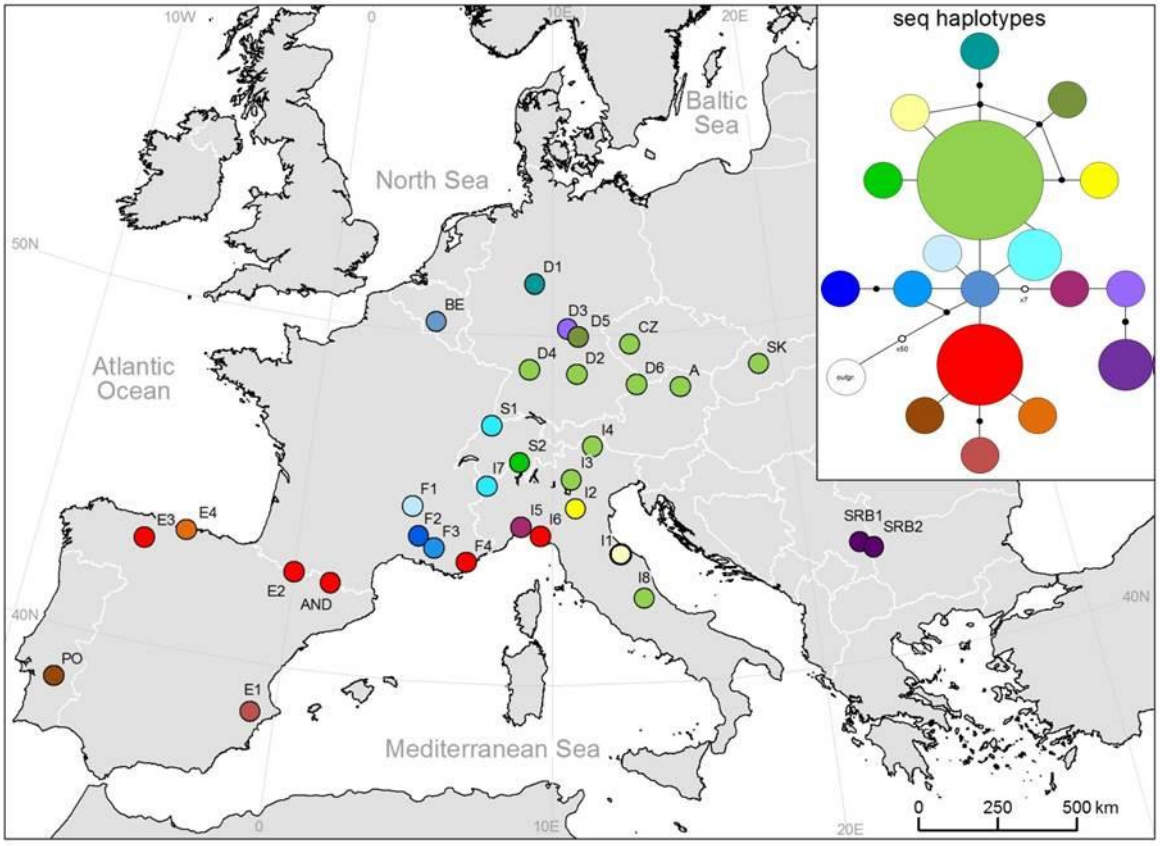


Fig. 9: cpDNA haplotypes revealed by sequence analysis of trnL-trnF chloroplast region: relationships between haplotypes are shown in the statistical parsimony network; filled nodes explain two steps difference; unfilled nodes explain differences according to nearby numbers.

Chloroplast sequences of regions trnL-trnF in 33 individuals of *S. album* resulted in concatenated sequences of 750 bases (gaps and missing data excluded). Forward strand (revealed by Tab c) was 324 bases long and had 14 mutation sites (7 single-nt polymorphisms, two single-nt indels, two 2-nt indels, two 3-nt indels and one 8-nt indel). Reverse strand (revealed by Tab f) resulted in 426 bases with 16 variable positions (12 single nucleotide polymorphisms, one single-nt indel, two 2-nt indels and one high variable 12-nt indel). Overall we detected 19 single-nt polymorphisms and 11 indels resulting in 18 sequence haplotypes (see Appendix, Tab. S1 and S2).

A group of nine populations from north-east Italy (I3, I4, I8), Austria (A1), Czech Republic (CZ), Slovakia (SK) and Germany (D2, D4, D6) shared haplotype H1 (Fig. 9; For our study 34 populations of *S. album* were sampled in dry grassland (Festuco-Brometalia) and rock slopes (Sedo albii-Veronicion dilenii) all over Europe (Table 1, Appendix Tab. S1) except for northern and eastern parts, where populations are described as introduced *(Meusel et* al.

1965*). S*. album is not protected and we did not collect in protected areas (Appendix Tab. S1). We avoided populations with characteristics of segregates, as well as populations near gardens and roof greenings to exclude the direct influence *of* horticultural S. album breeds. Fresh and young branches of 6 (GB) to 14 (S12) plants per population were sampled (Table 1) and stored in filter bags with silica gel until the material was frozen at -18°C. Voucher specimens are deposited at the Institute of Plant Sciences, University of Regensburg.

Tab. 2). Furthermore, single populations from north-western Italy (I6), southern France (F4) and the Iberian Peninsula (AND, E2, E3) were represented by haplotype H12. A clear separation of populations from Serbia (SRB1, SRB2), Germany (D3) and Ligura (I5) was detected in the network (Fig. 6), too.

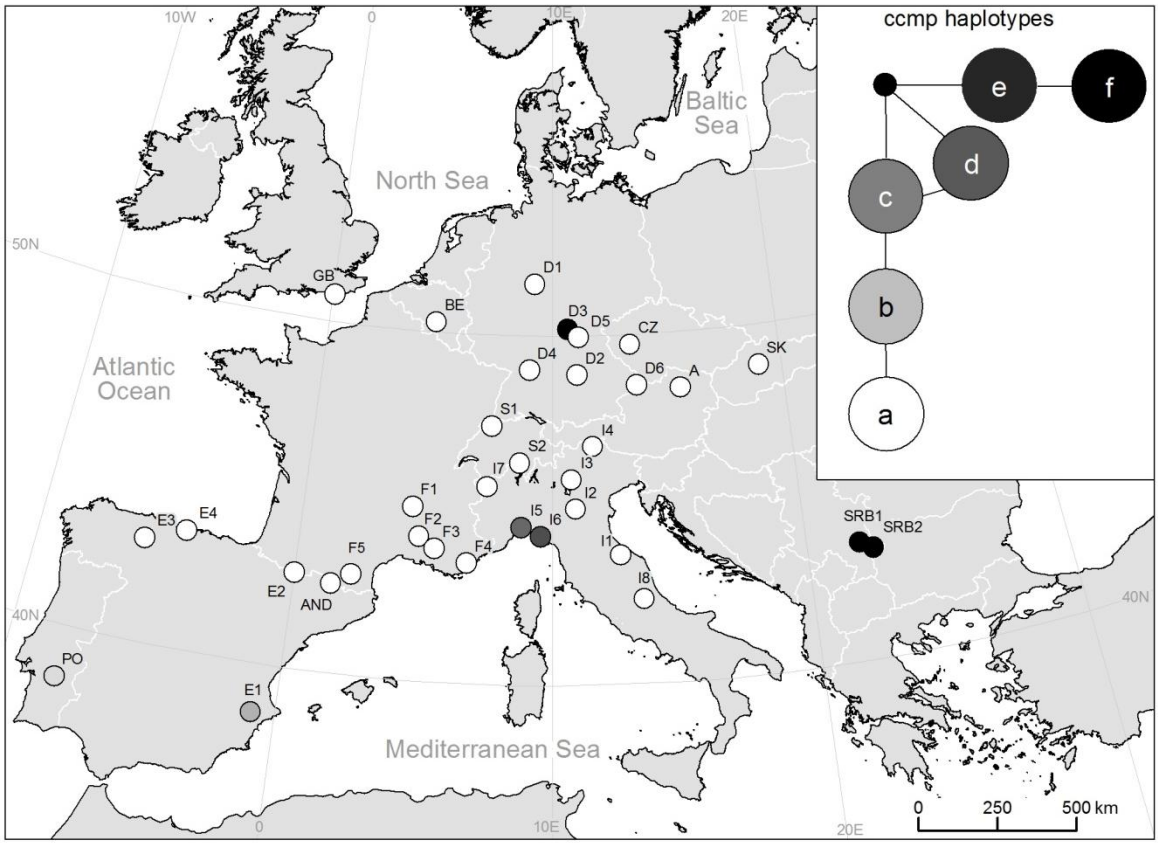


Fig. 10: cpDNA haplotypes revealed by ccmp analysis, relationship between haplotypes is shown in the network.

Chloroplast microsatellites resulted in six haplotypes (Fig. 10, For our study 34 populations of *S. album* were sampled in dry grassland (Festuco-Brometalia) and rock slopes

(Sedo albii-Veronicion dilenii) all over Europe (Table 1, Appendix Tab. S1) except for northern and eastern parts, where populations are described as introduced (Meusel *et al.* 1965). *S. album* is not protected and we did not collect in protected areas (Appendix Tab. S1). We avoided populations with characteristics of segregates, as well as populations near gardens and roof greenings to exclude the direct influence of horticultural S*. album* breeds. Fresh and young branches of 6 (GB) to 14 (S12) plants per population were sampled (Table 1) and stored in filter bags with silica gel until the material was frozen at -18°C. Voucher specimens are deposited at the Institute of Plant Sciences, University of Regensburg.

Tab. 2), whereas we could detect four polymorphisms at locus ccmp1, three at locus ccmp2 and two at locus ccmp3. Haplotype a was present in the main group of 28 populations. Haplotype f was observed in both Serbian populations (SRB1 and SRB2) and the population D5. Four haplotypes represented single populations (E1, I5, I6, and PO). Relations of the haplotypes are shown in a network (Fig. 10).

DISCUSSION

We found high resolution of AFLPs and clear geographic structure in *S. album* populations in Europe. Isolation by distance was present over the whole dataset indicating gene flow between populations. Sequence analysis resulted in 18 haplotypes, which were also clustering according to geographic regions. AFLPs were supportive to chloroplast microsatellite and sequences (single exceptions discussed below), as it was shown in other studies for plants and animals (Gaudeul *et al.* 2004; Negrini *et al.* 2007).

Iberian Peninsula

Populations on the Iberian Peninsula showed high differentiation and high genetic diversity within populations. Both values suggest that populations are more isolated than in other regions. High levels of rarity were likewise indicating strong isolation. Microsatellite and sequence analysis further revealed distinct and high differentiated chloroplast haplotypes of populations E4 from northern Spain and PO from Portugal. Distinct haplotypes have been found in Spain for black alder (King & Ferris 1998) and *Helianthemum* sp. (Soubani 2010) supposing glacial refugia for plants on the Peninsula.

Populations AND and E2 are situated in the Pyrenees, together with population F5. All were clearly assigned to the Iberian Peninsula group, but AND and E2 also had extraordinary high genetic diversity and rarity, indicating glacial refugia. The Pyrenees have been described as zone of glacial refugia for other species before (Petit *et al.* 2003; Taberlet *et al.* 1998).

Western Europe (France, Belgium)

Populations east of the Pyrenees were still strongly differentiated from the rest of the *S. album* range and clustered close to populations from the Iberian Peninsula. Populations west of the Alps showed a strong connection and obviously share one history. This northward line in Western Europe represents the genetic border to populations in Central Germany. Based on high genetic diversity within populations, it is possible that *S. album* has survived the glacial in lowland Central Europe refugia as suggested for *Helianthemum nummularium* (Soubani 2010). However, we could not detect isolation by distance within this group. Differentiation between the populations in general was low and rarity was moderate. Therefore, we suggest that populations in Western Europe build a contact zone for lineages from Iberia and Central Europe-east, with definitely more influence from Iberia.

Central Europe and Italy

Central European and Italian populations share microsatellite haplotype a, except for I5 and I6 from Liguria. Nine populations from Central Europe and Italy shared one sequence haplotype; further haplotypes had a close relation (except for I5 and I6 from Liguria). In the AFLP network populations from Central Europe and Italy built one main group, which could be divided in three subgroups (Central Europe-east, Central Europe–west and Italy with western Alps). *S. album* presumably has a young colonization history in Central Europe as it was shown for most of the investigated plant species before (Taberlet *et al.* 1998). Due to low differentiation, low rarity and low levels of diversity, a young colonization history can be assumed for most populations from Austria, Switzerland and Germany.

Nevertheless, high diversity and rarity have been found in populations from the Czech Republic and Slovakia. Cryptic refugia have been shown for many plants surviving the ice ages in scattered areas with more suitable microclimate (Stewart & Lister 2001; Stewart *et al.* 2010). Both populations show connections westward to German and southward to Italian population north of the Appenin (I2, I3, I4).

Mountain ranges have been detected as contact zones for different animal and plant species (Barton & Hewitt 1985; Flanagan *et al.* 1999; Hewitt 1975; Lugon-Moulin *et al.* 1999). The region Liguria in particular is known to be a potential contact zone for lineages from different source populations of *Anthyllis montana* (Kropf 2008; Kropf *et al.* 2002). Notwithstanding, populations I5 and I6 showed extraordinary high rarity and differentiation, which both indicates strong isolation, contradictory to the theory of a contact zone. Survival in small-scaled bedrocks near the Alps was already supposed for different alpine species (Médail & Diadema 2009; Schönswetter & Tribsch 2005; Soubani 2010). Supposedly, Liguria also harboured glacial refugia in the mountain ranges or nearby valleys for *S. album*.

Population I5 from north-eastern Italy (Liguria) had a strong connection to populations from Serbia. In AFLP analysis it was also related to the group on the Iberian Peninsula. In *Anthyllis montana* (Kropf *et al.* 2002) a strong connection of populations from Liguria with eastern populations - from Greece and the Balkans - has been shown before. This connection was also present in red deer (Vernesi *et al.* 2002). A similar picture can be drawn for *S. album* in the nearby population I6 in Liguria. It has a strong connection to the south-western group in Spain/Portugal, but also some similarities with the Serbian populations. The strong relation of populations along the Mediterranean coast, from Spain and France to the middle of the Italian Peninsula, and further eastward to Serbia was not detected in other grassland species so far. It is possible that this reflects the northern distribution limit of *S. album* that first expanded west- and eastward after the LGM, before the ice und steppe regions moved further north.

The dispersal along with human migrations has to be considered, regarding the ethnobotanical use of *S. album* and the longevity of seeds and shoots. Therefore, another influence for the plants distribution could be seen by human migrations that were detected from South-eastern Europe along the Middle of the Italian Peninsula and the Mediterranean coast (Poschlod 2015).

Populations situated near the Alps (I7 and S2, from Italy and Switzerland, respectively) have a strong relation to populations from the middle of the Italian Peninsula. We suggest that at least these parts of the Alps are colonised from lowland southern Italian source populations after the LGM (Ronikier *et al.* 2008). Alpine populations further have a strong connection to Central Europe and, therefore, share a colonisation history with the northern parts of Europe.

Serbia

With AFLP and chloroplast DNA analyses we detected a clear differentiation of Serbian populations, together with the German population D3. Population D3 was most likely founded by long-distance dispersal of *S. album* from a source in South-eastern Europe. The south-eastern regions of Europe (Greece/ Balkans) are known to harbour glacial refugia (Dumolin-Lapègue *et al.* 1997; Heuertz *et al.* 2004; Hewitt 1999; Konnert & Bergmann 1995). In our data both Serbia populations were more related to Central Europe than populations from Iberia and Western Europe. Nevertheless, Serbian populations show few rarity and low differentiation among populations with AFLP analysis. It is rather possible that Serbia was colonised from source populations further the East after the LGM. The influence of the eastern lineage on Central Europe can still be seen in the strong relation to population I6, and the close relation to central and northern populations in AFLP analysis.

Conclusion

A combined analysis of conserved chloroplast regions and AFLP markers with high resolution made it possible to infer historical processes of *S. album* populations in Europe. *S. album* survived in glacial refugia on the Iberian and the Italian Peninsula, and probably in Eastern Europe - as shown for several other species (Dumolin-Lapègue *et al.* 1997; Koch & Bernhardt 2004; Taberlet *et al.* 1998). We detected candidate areas for glacial survival in the Pyrenees and the mountains of Liguria. Small areas like closed valleys in mountains can harbour glacial refugia (Feliner 2011) and, therefore, glacial survival of *S. album* in the Pyrenees and Liguria is most likely. Further cryptic refugia in Germany, the Czech Republic and Slovakia are rather thinkable.

It was shown that *Hippocrepis comosa* migrated to Central Europe from source populations on the Iberian Peninsula after the LGM (Leipold 2015, pers. communication). In contrast, we could show that *S. album* migrated to western and north-western parts of Europe from Spain/Portugal, but Central Europe was recolonised from source populations in the Balkans and southern Italy. A very similar picture has been drawn for postglacial history of black alder (Ferris *et al.* 1998), common holly (Rendell & Ennos 2003) and silver birch (Maliouchenko *et al.* 2007).

Based on our results for *S. album,* we suggest that dry grasslands in Central Europe developed under the influence of source populations coming from South (Italy) and East,

while the influence of source populations from South-western and Western Europe for some species is rather is low.

Chapter 3

The geographic scale of genetic variation in a common species – a case study on *Lathyrus pratensis* L.

ABSTRACT

With the increased use plant species collected in wild populations for breeding, restoration and conservation purposes the collection of plant material from wild populations is getting more important. The intraspecific distribution of genetic diversity is imminent for genebanks and for the usage of plant material for restoration. As genetic data are missing for most plant species, edaphic and climatic factors are used to delineate conservation units. Nevertheless, for common species it is hardly known which regions should be delineated and considered as evolutionarily significant units (ESUs) for conservation.

We studied genetic diversity and population differentiation of *Lathyrus pratensis*, a common and agriculturally used species with AFLPs. 706 individuals were sampled in 37 populations based on the official grid system in Bavaria with distances of 18.4km to 388.5km. We compared our results with the regionalised seed production practices and searched for regions and populations to prioritise in seed collections.

We found moderate genetic diversity within populations (25.25% to 48.51% polymorphic loci) and high differentiation between all populations (mean PHIPT=0.427). Two main genetic groups were delineated by a north-south boarder along the river “Danube”. The southern group matched with one of the production zones that regionalised seed production refers to. Seed provenance regions were not reflected at all. Genetic diversity and rarity of fragments were higher in southern than in northern populations.

Seed collections of L. pratensis should take into account genetic differences between northern and southern populations in Bavaria. For genebank collections populations from southern regions should be prioritised, because they are genetically more diverse. Considering the limited gene flow between populations, we suggest using *L. pratensis* material for restoration as locally as possible.

Keywords: AFLPs; genebank collections; seed provenance; seed production area; restoration

INTRODUCTION

Collections from wild plant populations are important for plant breeders, commercial seed producers and genebanks. Collections should cover the genetic diversity of the species in the collection area and represent the differentiation of evolutionarily significant units (ESUs) (Moritz 2002).

The distribution of genetic diversity within and between populations and regions in rare and endangered plants was topic of different studies over the last decades (Fischer *et al.* 2000; Reisch 2007; Reisch 2008; Ronikier *et al.* 2008; Schönswetter *et al.* 2006a; Schönswetter *et al.* 2002). In recent years more collections from wild populations are made for genebanks and restoration projects. Species are not necessarily restricted to certain areas, but common and occur over wide distribution ranges. For common species the distribution of genetic diversity within and between populations is hardly known and the delineation of ESUs is difficult (Bucharova *et al.* in press; Durka *et al.* in press; Michalski & Durka 2012).

High genetic diversity is imminent for material that is used in restoration to ensure the potential for adaptations to a changing environment and support fitness of the populations (Bischoff *et al.* 2010; Reed & Frankham 2003; Robichaux *et al.* 1997). On one side, gene flow between populations is outstanding to sustain high genetic diversity within populations. On the other side, ecotypic adaptations in plant populations can evolve from spatial isolation or differences related to the habitat (Bischoff *et al.* 2010; Hufford & Mazer 2003; Reisch & Poschlod 2009; Zopfi 1998).

The introduction of material that is genetically too distant may bear a lot of risks for plant populations and ecosystems. Studies of common species have shown that the input of genes from geographically and ecologically distant source regions can devitalize populations. Adaptations to the special requirements of the habitat can be lost and maladapted genes can be imported to local populations (Bischoff *et al.* 2006; Fenster & Dudash 1994; Hufford & Mazer 2003; Joshi *et al.* 2001; Keller *et al.* 2000; Krauss *et al.* 2005; Schoen & Brown 2001; Vander Mijnsbrugge *et al.* 2010). *Lotus scoparius* seedlings planted in genetically or environmentally distant populations have shown reduced fitness (Montalvo & Ellstrand 2001). Crossbreeds between local and introduced *L. scoparius* plants performed even weaker.

Without artificial introductions gene flow in and between populations often is skewed over a certain region or following the stepping-stone-model (Mantel 1967; Sackville Hamilton 2001). The distance in which gene flow is prominent and populations are highly related to each

other can be defined as ESU (Moritz 2002) in common species, too (Allendorf *et al.* 2013; Krauss *et al.* 2013).

Without prior knowledge of genetic diversity in a certain species, the general assumptions for genebank collections are that

- populations with high genetic diversity should always have higher priority in genebank collections, because they reflect differences in frequencies of common alleles (Culley *et al.* 2002),
- Collecting in ecologically and geographically most differing areas is advisable (Diniz-Filho & De Campos Telles 2002; Parra-Quijano *et al.* 2012).

Nevertheless, guidelines concerning numbers of collected populations and delineation of populations and provenance regions are very ambiguous (Neel & Cummings 2003). For common species it is suggested to sample 172 individuals to capture most of genetic diversity of the species (Lawrence *et al.* 1995). The most cited approach comes from Brown & Marshall (1995), who suggested to collect 50-100 populations over the whole distribution area to achieve 95% of alleles that are locally common (>5%).

With the restoration in arable land, road- and railroad sides with special seed mixtures the discussion of genetic admixture started again recently in Europe (Kiehl 2010; Kirmer & Tischew 2006; Vander Mijnsbrugge *et al.* 2010; Walker *et al.* 2004). Zahlheimer & Schuhwerk (2006) explained a general necessity to preserve autochthony of plant populations. The sawing of seed mixtures from ecologically and geographically distant regions in restoration projects has to be handled with care. To avoid the input of seed material, which is genetically too different a delineation of provenances for commercial seed producers was defined in several countries, e.g. Switzerland and the UK (Vander Mijnsbrugge *et al.* 2010).

In Germany demands for autochthony in seed mixtures were met with the delineation of provenances regions in 2011 that are based on geomorphologic and climatic data (Erhaltungssortenverordnung 2014; Prasse *et al.* 2010b; Rieger *et al.* 2014). Seeds of wild populations should be reintroduced within the same provenance, where they were collected. Furthermore, production areas were specified that consist of several provenances each (Prasse *et al.* 2010b). Regeneration should take place within the production area of which the provenance is part of.

Theoretically, seed provenances should reflect genetic differentiation in all common plant species. Collections of plant material based on the provenance delineations should reflect a maximum of genetic differentiation and, on the other hand, include most of the genetic diversity in the whole sampling region.

Provenance delineations are appropriate for most of the investigated common species, so far (Bucharova *et al.* in press; Durka *et al.* in press). Nevertheless, the sampled populations are agglomerated in small areas within provenance regions. Therefore, the results for differentiation between provenance regions may be affected by the study design.

To avoid the influence of the study design we investigated 37 populations of the common meadow species *Lathyrus pratensis* L. in Bavaria based on a grid system with AFLPs.

Distribution of genetic diversity within and between populations and provenance regions was analysed to answer the following questions:

Does the distribution of genetic diversity in a common and widely sown herb fit in the predefined provenances or production areas?

Which regions are qualified as provenances based genetic diversity?

Are there regions with high genetic diversity and rare fragments in common plants that should be preferred in collections?

MATERIAL AND METHODS

Study species

The Meadow Pea (*Lathyrus pratensis* L., Fabaceae, Fig. 11) is a perennial wild pea and a common part of seed mixtures that are widely used e.g. for restoration of roadsides. Flowers are pollinated by bees and bumblebees. Seeds are compressed pods with 6-8 seeds ripening from July to October (Brunsberg 1977). They are not dispersed over wide distances by themselves because they are relatively heavy, but mainly propagated by birds (chicken), cattle, sheep and goats or with the transportation of hay (Bonn & Poschlod 1998).

Fig. 11: Habitus of *L. pratensis* L. with flowers and pods, photo taken at Brauneck (foothills of the Bavarian Alps) in September 2010.

L. pratensis is native to Europe, temperate Asia and North Africa. It was introduced to several countries in North and South America, as well as East Asia, mainly for production of forage. As plant genetic resource (PGR) it is highly rated (4 of 5 points) in the list for German PGR plants (Hammer & Schlosser 1995; Schlosser 1991). *L. pratensis* is also known as nutrition for bees, for breeding purposes und as foliage plant.

Two chromosomic races are described for *L. pratensis*, diploid and tetraploid (2n=14 and 2n=28, respectively), which cannot be distinguished by morphological traits (Brunsberg 1977). In the mediterraneo-atlantic zone tetraploid populations dominates, in north-east

continental Europe mainly diploids can be found (Cartier & Blaise 1981; Simola 1964). To make sure our analyses are independent from chromosomic races (Greiner *et al.* 2013), i.e. only diploid populations have been chosen, DNA content of a subset of control samples was tested with flow cytometry. For analysing the DNA content we were following the protocols of Doležel & Bartoš (2005) and Doležel *et al.* (2007).

Study design

We collected leaf tissue of 16-20 individuals per population. 37 undisturbed and not introduced populations in the whole area of Bavaria were sampled. Choice of populations was made with a grid system that was based on topographical maps (1:100000). Each grid comprised 4x4 quadrants according to the ordnance maps of Bavaria (Bresinsky 1966) (Tab. 4, Fig. 12). Populations were situated in nine provenances and four seed production areas that are both numbered according to Prasse et al. (2010).

Tab. 4: Information on populations of *L. pratensis*: Identification (*Id*), official name (*Name*), corresponding name and number of seed provenance region (*pvr_name* and *pvr_nr*), name and number of seed production area (*pa_name* and *pa_nr*) (according to Prasse et al. 2010) and STRUCTURE group (*sg)* are given for each population. Number of ordnance map (*Om*), and number of individuals (*No*) are provided. Further results of genetic analysis are given as percentage of polymorphic loci (*PL*), Shannon information index (*SI*), averaged down-weighted rare fragments (*DW*), number of rare fragments, e.g. present in less than 10% of all populations (*RF)* and unique fragments (UF).

Id	*Name*	*pa_nr*	*pa_name*	*pvr_nr*	*pvr_name*	*sg*	*Om*	*No*	*PL*	*SI*	*DW*	*RF*	*UF*
1	Wiesen	4	Westdeutsches Berg- und Hügelland	21	Hessisches Bergland	1	5822	19	37.62	0.218	3.69	0	0
14	Heimbuchenthal	4	Westdeutsches Berg- und Hügelland	21	Hessisches Bergland	1	6121	20	33.66	0.186	4.38	0	0
25	Hammelburg	4	Westdeutsches Berg- und Hügelland	21	Hessisches Bergland	1	5825	20	32.67	0.184	3.97	0	0
2	Münchberg	5	Südost- und ostdeutsches Bergland	15	Thüringer Wald, Fichtelgebirge und Vogtland	1	5836	18	37.62	0.217	3.67	0	0
4	Sulzbach-Rosenberg Süd	5	Südost- und ostdeutsches Bergland	19	Bayerischer und Oberpfälzer Wald	1	6536	20	34.16	0.196	3.92	0	0
7	Zwiesel	5	Südost- und ostdeutsches Bergland	19	Bayerischer und Oberpfälzer Wald	2	6945	19	39.11	0.214	4.67	0	0
16	Tännesberg	5	Südost- und ostdeutsches Bergland	19	Bayerischer und Oberpfälzer Wald	1	6439	16	25.25	0.144	4.5	1	0
18	Waldkirchen	5	Südost- und ostdeutsches Bergland	19	Bayerischer und Oberpfälzer Wald	1	7247	20	36.63	0.196	4.25	0	0
26	Kemnath	5	Südost- und ostdeutsches Bergland	19	Bayerischer und Oberpfälzer Wald	1	6137	20	36.14	0.196	4.67	0	0
3	Ebrach	7	Süddeutsches Berg- und Hügelland	11	Südwestdeutsches Bergland	1	6128	20	31.19	0.181	4.01	0	0

5	Heidenheim	7	Süddeutsches Berg- und Hügelland	12	Fränkisches Hügelland	1	6930	20	36.14	0.191	6.7	2	2
13	Burgkunstadt	7	Süddeutsches Berg- und Hügelland	12	Fränkisches Hügelland	1	5833	18	34.65	0.184	4.65	1	1
15	Buttenheim	7	Süddeutsches Berg- und Hügelland	12	Fränkisches Hügelland	1	6132	20	41.09	0.214	4.82	0	0
27	Markt Erlbach	7	Süddeutsches Berg- und Hügelland	12	Fränkisches Hügelland	1	6529	20	38.12	0.212	4.52	0	0
28	Röthenbach a.d.Pegnitz	7	Süddeutsches Berg- und Hügelland	12	Fränkisches Hügelland	1	6533	19	39.6	0.224	4.63	0	0
37	Hofheim i.UFr.	7	Süddeutsches Berg- und Hügelland	12	Fränkisches Hügelland	1	5829	17	27.72	0.165	3.79	0	0
6	Kelheim	7	Süddeutsches Berg- und Hügelland	14	Fränkische Alb	2	7037	20	41.09	0.228	5.56	0	0
17	Thalmässing	7	Süddeutsches Berg- und Hügelland	14	Fränkische Alb	1	6933	20	38.12	0.192	6.53	2	1
8	Schrobenhausen	8	Alpen und Alpenvorland	16	Unterbayerische Hügel- und Plattenregion	2	7433	19	48.51	0.277	5.84	1	1
9	Altenerding	8	Alpen und Alpenvorland	16	Unterbayerische Hügel- und Plattenregion	2	7737	19	46.53	0.257	6.45	3	1
19	Aham	8	Alpen und Alpenvorland	16	Unterbayerische Hügel- und Plattenregion	2	7440	17	38.12	0.217	4.23	0	0
21	Rotthalmünster	8	Alpen und Alpenvorland	16	Unterbayerische Hügel- und Plattenregion	2	7645	18	34.16	0.186	4.72	0	0
29	Mintraching	8	Alpen und Alpenvorland	16	Unterbayerische Hügel- und Plattenregion	2	7039	20	31.19	0.169	5.21	0	0
30	Höchstädt a.d.Donau	8	Alpen und Alpenvorland	16	Unterbayerische Hügel- und Plattenregion	2	7329	20	36.63	0.206	4.24	0	0
31	Bruckberg	8	Alpen und Alpenvorland	16	Unterbayerische Hügel- und Plattenregion	2	7437	18	38.61	0.214	5.91	1	1

32	Aidenbach	8	Alpen und Alpenvorland	16	Unterbayerische Hügel- und Plattenregion	2	7444	18	34.16	0.184	4.86	0	0
33	Türkenfeld	8	Alpen und Alpenvorland	16	Unterbayerische Hügel- und Plattenregion	2	7832	18	36.63	0.207	5.39	0	0
34	Neumarkt-Sankt Veit	8	Alpen und Alpenvorland	16	Unterbayerische Hügel- und Plattenregion	2	7641	19	34.65	0.193	6.2	2	1
10	Wildpoldsried	8	Alpen und Alpenvorland	17	südliches Alpenvorland	2	8228	20	40.1	0.233	5.1	1	0
20	Jettingen	8	Alpen und Alpenvorland	17	südliches Alpenvorland	1	7628	20	39.6	0.225	5.11	1	1
22	Wangen im Allgäu Ost	8	Alpen und Alpenvorland	17	südliches Alpenvorland	2	8325	18	45.05	0.255	5.13	2	0
23	Marquartstein	8	Alpen und Alpenvorland	17	südliches Alpenvorland	2	8240	20	40.59	0.231	4.9	0	0
36	Iffeldorf	8	Alpen und Alpenvorland	17	südliches Alpenvorland	2	8233	19	43.07	0.25	5.25	1	0
11	Lenggries	8	Alpen und Alpenvorland	18	Nördliche Kalkalpen	2	8335	19	39.6	0.23	4.45	0	0
12	Hoher Göll	8	Alpen und Alpenvorland	18	Nördliche Kalkalpen	2	8444	19	44.55	0.253	4.92	0	0
24	Zugspitze	8	Alpen und Alpenvorland	18	Nördliche Kalkalpen	2	8531	20	40.59	0.235	4.18	0	0
35	Hinterstein	8	Alpen und Alpenvorland	18	Nördliche Kalkalpen	2	8528	19	33.17	0.185	5.95	3	1

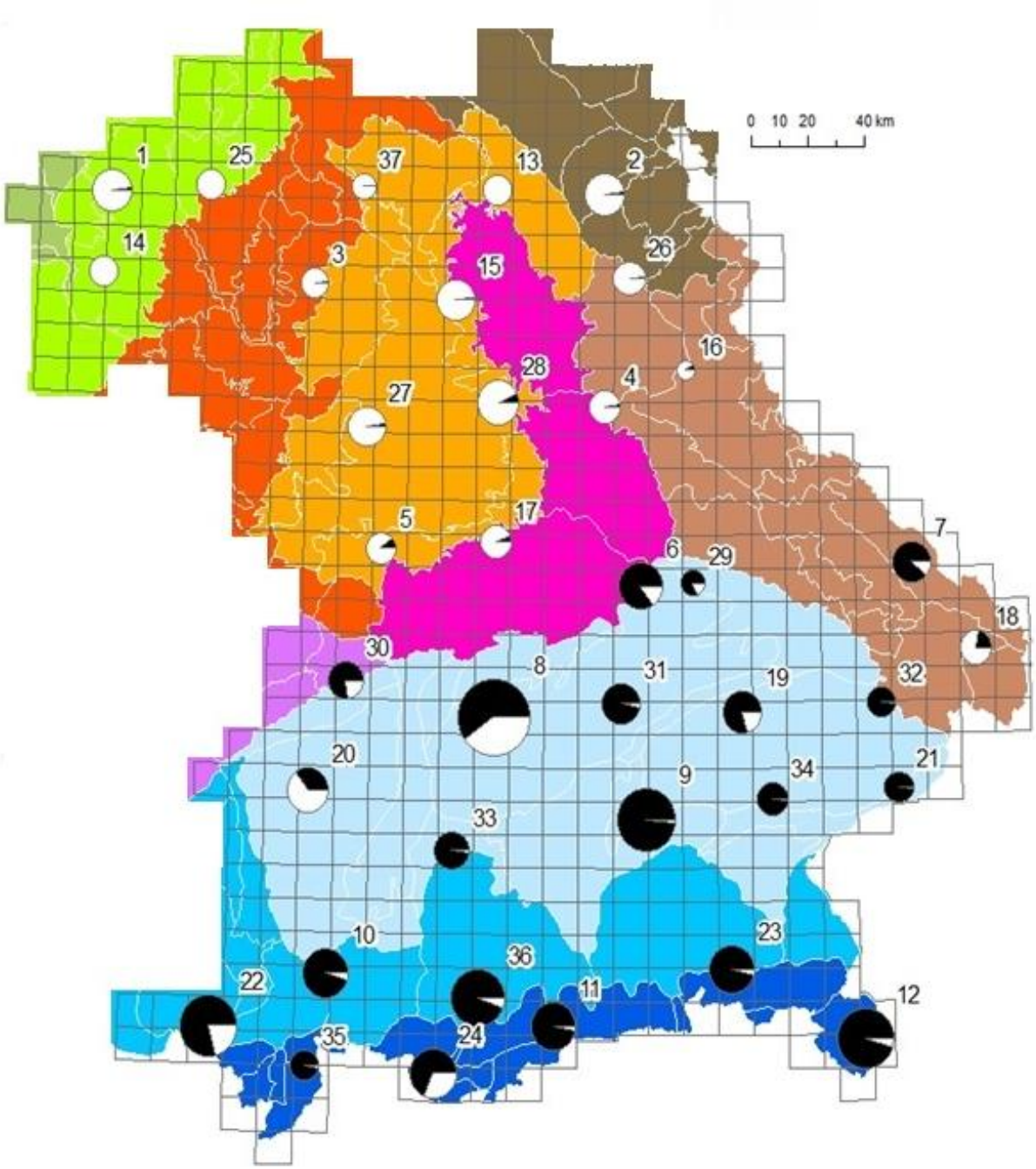


Fig. 12: Distribution of sampled populations of *L. pratensis* L. within the grid system in Bavaria, background according to ten seed provenances (colour) and four seed production areas (greenish, reddish, brownish, blueish). Affiliation of populations to two main groups revealed by STRUCTURE analysis, size reflects genetic diversity (SI)

Molecular Analysis

Leaves were collected in plastic bags and stored cool until they were frozen at -18°C at the University Regensburg. DNA was extracted of 25 - 35g frozen and pulverized plant material using CTAB method (Rogers & Bendich 1994) with minor adaptations (Reisch *et al.* 2003a). DNA concentration of all extractions was measured as transmission of optical density and extracts were diluted to a standard of 7.8ng/µl.

We decided to use AFLPs, because it was already shown that variation in neutral markers mainly correlates with the variation in markers under selection pressure (Leinonen *et al.* 2008). On the long term, genome-wide neutral markers reflect the evolutionary potential even better than quantitative traits (McKay & Latta 2002).

A screening of 3 x 16 fluorescence labelled primer combinations was carried out with 8 individuals of 4 populations. Three highly resolving primer combinations were selected (D2: MseI-CAT/EcoRI-ACC, D3: MseI-CTT/EcoRI-AGG, D4: MseI-CTG/EcoRI-ACT).

AFLP analysis was conducted with 706 individuals in total following the protocol of Vos *et al.* (1995) with minor changes (Reisch 2008). About 50ng of DNA were digested with EcoRI and MseI restriction enzymes (Fermentas) and ligated to adaptors (MWG) with T4 DNA Ligase (Fermentas) at 37°C for 2h with a final step of 70°C for 15min. For the following polymerase chain reactions Taq Polymerase (PeqLab) was used. The first amplification step was conducted with a pair of one-base primers (MseI-C/EcoRI-A, MWG) in 30 cycles. For the final amplification three-base primers were used (MseI-CAT/EcoRI-ACC, MseI-CTT/EcoRI-AGG, MseI-CTG/EcoRI-ACT, MWG Biotech/Proligo) with fluorescence label on Mse primers. 25 cycles were run of which 10 cycles had a 1°C touchdown profile. DNA was precipitated with NaAC,EDTA, Glycogen and cold EtOH (96%, -20°C) and cleaned with cold EtOH (70%, -20°C). After the DNA pellet was dried, it was dissolved in Sample Loading Solution and charged with CEQ Size Standard 400 (both Beckman Coulter). The capillary gel electrophoresis was run on an automated sequencer (CEQ 8000, Beckman Coulter) and raw data were automatically analysed with CEQ 8000 software (Beckman Coulter) aligning fragments according to CEQ Size Standard 400.

Clear and well-defined fragments were scored manually in the program BIONUMERICS (Applied Maths, v. 3.0). Each fragment was scored across all individuals as either present or absent.

To reassure good quality of fragments 48 individuals were replicated, which gave an error rate of 1.33% (Bonin *et al.* 2004). Both individuals and fragment sizes that did not give clear, defined and reproducible fragments were excluded from the analysis.

Statistical Analysis

Fragments with sizes of 60 to 420bp were included in the analysis. The presence of a fragment was scored as 1, while the absence of the fragment was coded as 0 in the data matrix. The resulting binary matrix was used for all further statistical analyses.

Genetic diversity within populations was calculated based on all markers as percentage of polymorphic fragments (PL) and Shannon Information Index ($SI = \sum pi \ln pi$) for each population (Parisod & Christin 2008), using the program POPGENE version 1.32 (Yeh *et al.* 1997).

Rare alleles can reflect special adaptations to habitat conditions and, therefore, even represent new variants (Bengtsson *et al.* 1995). The rarity for each population is given by frequency down weighted marker values (DWs), which are calculated according to Schönswetter & Tribsch (2005). To even out sample sizes we randomly chose 16 individuals (smallest samples size) of each population for the calculation of DWs. We repeated random drawing of lots and calculation five times and took the mean value for each population.

The detection of unique fragments is very sensitive, because one wrong detected fragment accounts for one unique or rare fragment. Therefore, we cleaned our dataset of all fragments that occur lower frequented as the calculated error rate. Based on this new matrix we searched for unique fragments (UF) and rare fragments (RF). The latter were defined as appearing in less than 10% of the populations (adapted from (Stehlik 2002).

We tested, if there is an increased amount of genetic diversity (SI) or rare fragments (DW) in certain production areas, provenances or STRUCTURE groups (see analysis below) with Kruskal-Wallis non-parametric tests and Dunn's post-tests with Bonferroni-correction in R (Dinno 2015).

The allocation of genetic diversity within and between populations was calculated in GENALEX version 5 (Peakall & Smouse 2001) based on Wrights F-statistic (Wright 1965) with Meirmans standardisation (Meirmans 2006) with analysis of molecular variance (hierarchical AMOVA) (Excoffier *et al.* 1992) for the whole dataset. Further, variance components and their significance levels for diversity within populations, between populations and between groups were calculated (three-level AMOVA). Seed provenances

regions (pv), seed production areas (pa) and the results from STRUCTURE analysis were investigated as groups.

Genetically homogenous groups were inferred with Bayesian clustering in the program STRUCTURE version 2.2 (Pritchard *et al.* 2000). Allele frequencies were used as correlated in an admixture model (Evanno *et al.* 2005). 10^4 iterations for estimating the number of groups with a burn-in-period of 10^4 were set. For each predefined number of K (1-38) 10 iterations were run. From the resulting values of L(K) standard deviations and ΔK were calculated to affirm the most likely number of groups (Evanno *et al.* 2005).

We constructed a consensus Neighbour-Net graphs with Splitstree (Huson & Bryant 2006) based on Nei's standard among population distances (Ds) with non uniform-prior distribution of allele frequencies (Vekemans 2002).

Isolation by distance was tested with Mantel test in GenAlEx version 5 (Peakall & Smouse 2001) using pairwise PHiPT values derived from AMOVA and pairwise geographic population distances (km) (Mantel 1967). Correlations of genetic and geographic distance were studied using 999 permutations. The whole dataset and the two most possible STRUCTURE groups were tested.

Further the smallest distance identifying a group of not significantly different individuals was analysed with a method adapted from Diniz-Filho & De Campos Telles (2002). We calculated distance kinship coefficients for AFLPs according to Hardy (2003) with the program SPAGEDi (Vekemans & Hardy 2004). We used 11 distance classes from a minimum distance between populations of 18.4km (between population 6 and 29) to a maximum of 388.5km (between population 1 and 12).

RESULTS

Genetic diversity and rare fragments

Out of three primer pairs 195 fragments were generated, 183 of which were polymorphic. Genetic diversity within populations varies from 25.25% to 48.51% (percentage of polymorphic fragments) and from 0.144 to 0.277 (Shannon index) (Fig. 12, Tab. 4). Further, values of rarity calculated as down-weighted rare frequency (DW) ranged from 3.67 to 6.7, rare fragments (RF) from 0 to 3 and unique fragments (UF) from 0 to 2.

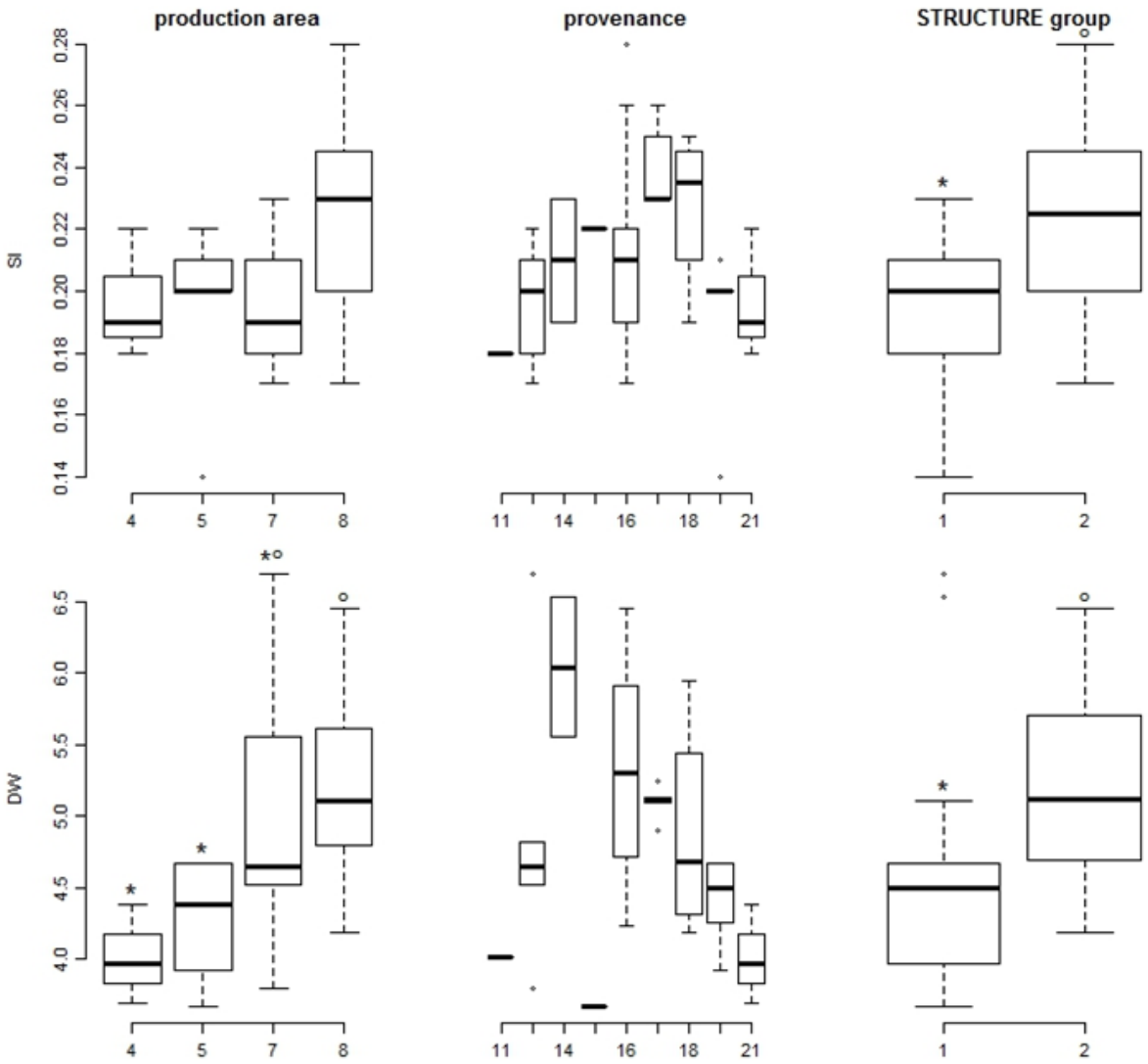


Fig. 13: Genetic diversity (SI) and rarity of fragments (DW) compared between seed production areas, seed provenance regions and main STRUCTURE groups in Kruskal-Wallis tests with Dunn's Bonferroni-corrected post-tests. Significance levels <0.05 are indicated as different groups by asterisks and circles, respectively.

No significant differences were found in genetic diversity (SI) between production areas and between provenances, and in rarity of fragments (DW) between provenances (Fig. 13). Nevertheless, the southern STRUCTURE group had significant higher levels of genetic diversity and rarity of fragments. The seed production area Bavarian Alps (8) had significant higher levels of rarity compared to production areas West-German Hills and Southeast- and East-German Hills (4 and 5), but was not different to the South-German Hills (7).

Differentiation and spatial genetic structure

The most likely number of groups in the dataset revealed by STRUCTURE analysis was n=2, dividing the dataset into a northern and a southern group (Fig. 14). When we compared seed production areas, provenances and groups revealed from STRUCTURE analysis, the STRUCTURE group dominating the southern part of Bavaria resembled the alpine production area. Only two populations within the alpine provenance showed more influence of the northern STRUCTURE lineage (Pop. 20, Jettingen and Pop. 18, Waldkirchen), Nevertheless, both populations clustered near southern populations in the network (Fig. 14).

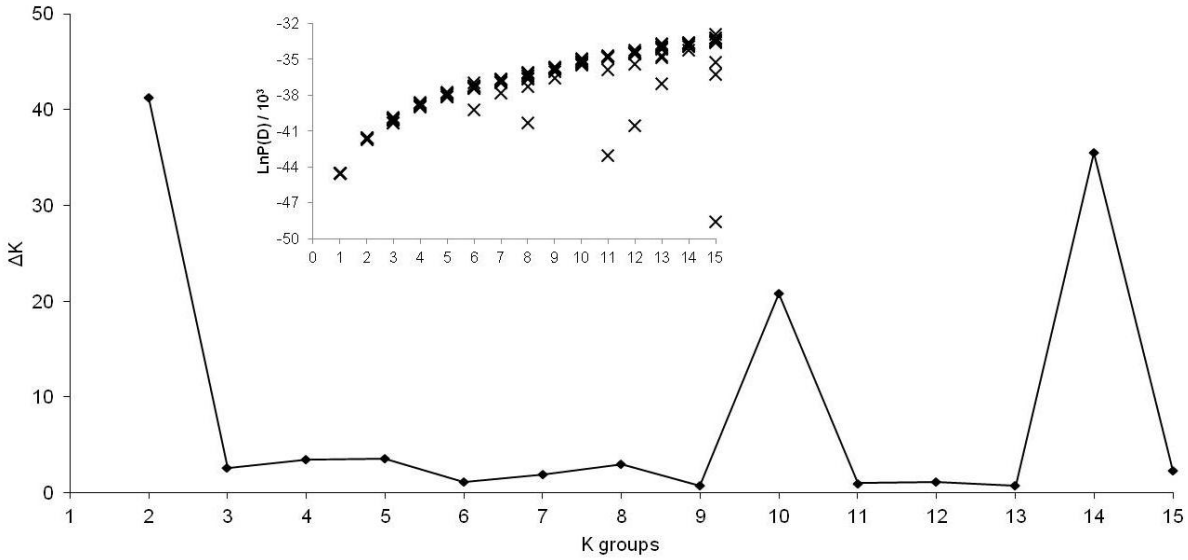


Fig. 14: Bayesian analysis with STRUCTURE revealed the most possible partition of 37 of *L. pratensis* populations into two subgroups.

Neighbor Net network (Fig. 15) further showed a clear connection of populations from the prealpine and alpine region. Populations 5, 17 and 18 were clustering between populations from the prealpine and alpine region. Those populations are also geographically near to the prealpine region.

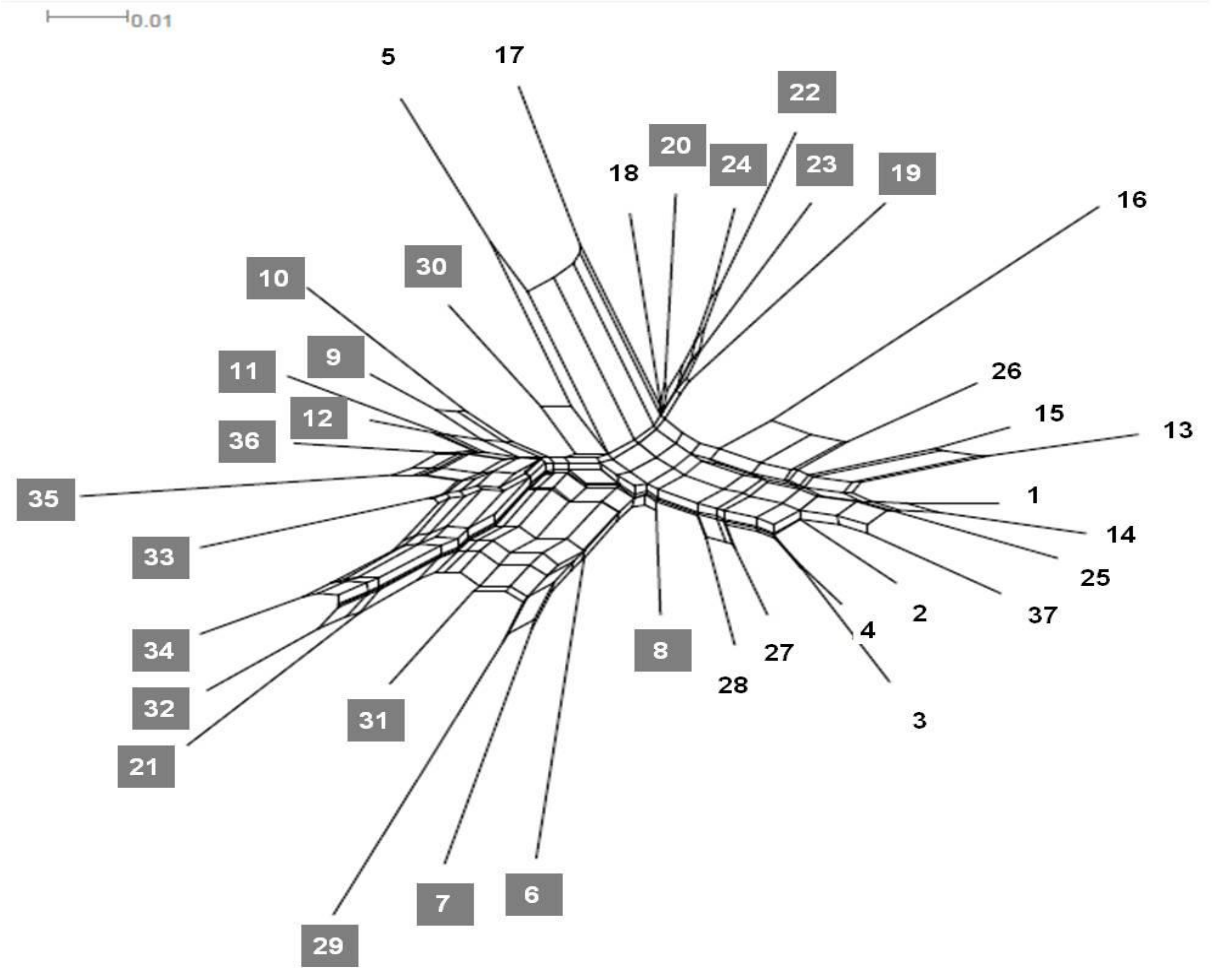


Fig. 15: Network of 37 populations of *L. pratensis* in Bavaria; populations are marked according to STRUCTURE groups, for affiliations see Tab. 4.

High differentiation between populations was revealed by AMOVA (AMOVA-PHIPT = 0.427, Tab. 5). To reassure group assignments, we further analysed provenances, production areas and STRUCTURE groups. A hierarchical AMOVA showed significant differentiation (PHiRT = 0.103) between the two STRUCTURE groups, and significant differentiation between seed production areas and seed provenances (PHiRT = 0.097 and 0.105, respectively).

Tab. 5: Analysis of molecular variance (AMOVA) of 706 individuals from 37 populations of *L. pratensis*. Three-level AMOVAs were conducted for seed provenance regions, seed production areas and STRUCTURE groups (2, 10 and 14).

	d.f.	Sum of squares	est. variance	% of total variance
Overall analysis				
among populations	36	6331.877	8.613	42.7
among individuals within populations	669	7725.305	11.548	57.3
Seed provenance regions				
among provenance regions	8	2412.876	2.157	10.5
among populations within provenance regions	28	3919.000	6.749	33.0
among individuals within populations	669	7725.305	11.548	56.5
Seed production areas				
among production areas	3	1309.860	2.015	9.7
among populations within production areas	33	4435.050	6.392	30.7
among individuals within populations	669	8312.272	12.425	59.6
STRUCTURE groups				
among groups	1	923.874	2.190	10.3
among populations within groups	35	5408.003	7.494	35.3
among individuals within populations	669	7725.305	11.548	54.4

We found no significant association between genetic and geographic distances over the whole dataset of *L. pratensis* in Mantel test (Fig. 16c). Nevertheless, divided in two STRUCTURE groups, significant positive correlation of genetic and geographic distances in both groups was detected, with r=0.320, p=0.026 for the northern group (Fig. 16a) and r=0.396, p<0.001 for the southern group (Fig. 16b), respectively.

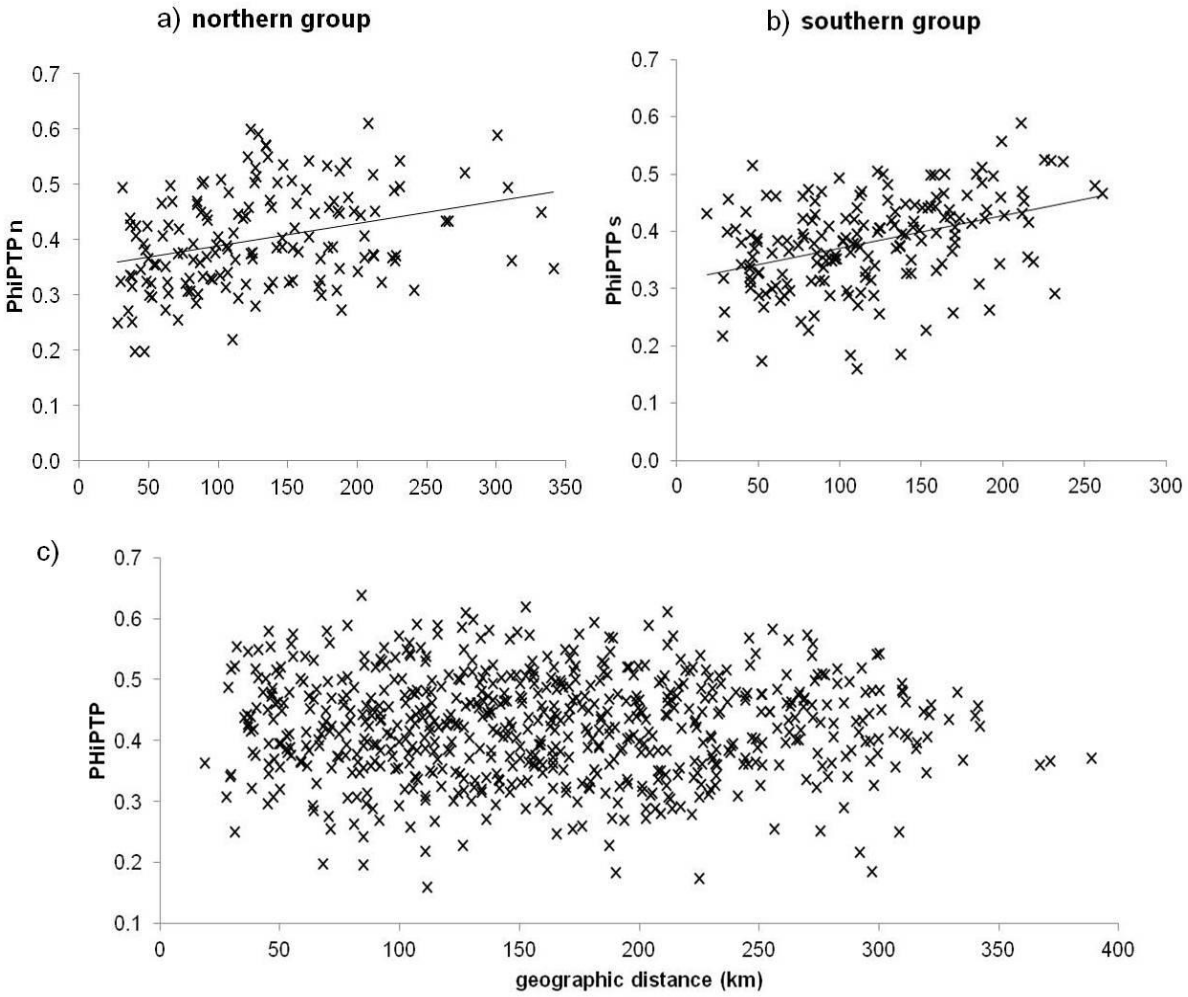


Fig. 16: Correlation of genetic (PhiPT) and geographic distances are significant positive for the northern (a) and the southern (b) Bayesian group, but not significant in the whole dataset (c) in Mantel tests.

The correlogram of spatial genetic structure (Fig. 17) revealed a significant positive kinship coefficient at distance intervals till110km in the whole dataset. For the intervals from 155km

to 210km no significant kinship could be found. For distance classes above 210km relatedness was smaller than random..

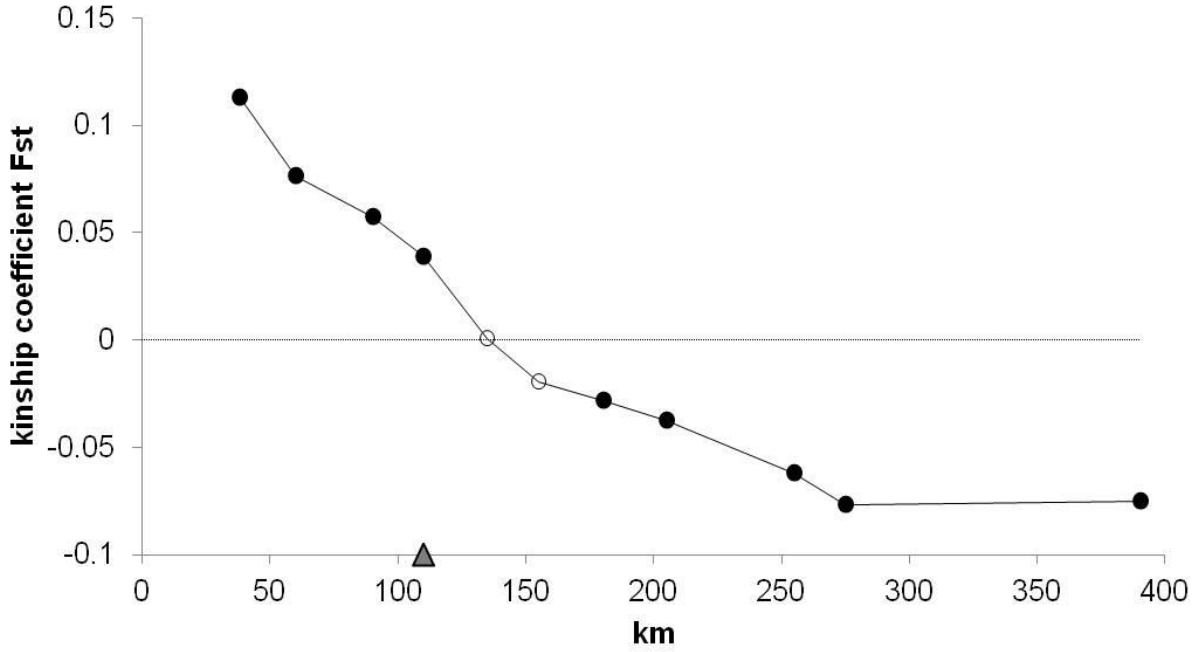


Fig. 17: Analysis of spatial genetic structure in eleven distance classes of *L. pratensis* in Bavaria. Kinship coefficients are significant positive (black) for the classes up to 110km, not significant (circles) or negative for the other classes.

DISCUSSION

Genetic diversity and rare fragments

Genetic diversity within *L. pratensis* populations lays within classifications for common perennials (Hamrick & Godt 1996; Reisch & Bernhardt-Römermann 2014).. Life form, breeding system, seed dispersal and frequency of populations has influence on the distribution of genetic diversity within and between populations. Therefore, the combination of traits in *L. pratensis* can help to explain the discrepancy in genetic diversity.

Diversity was significantly higher in southern populations. Variable values of diversity within populations can show differences in history and current status (number of individuals, gene flow) of single populations. Bottleneck effects, strong isolation and small population size can

lead to low genetic diversity (Ellstrand & Elam 1993). High genetic diversity can be a hint for populations consisting of many individuals and populations that have a long persistence on the site.

Furthermore, rarity of fragments was higher in southern than in northern parts of Bavaria. Isolation can be the reason, why rare fragments can be accumulated in a population. Again, we found that the production area “Foothills of the Alps and Alps” is richer in rare fragments than northern production areas, i.e. “west German hills” and “southeast and east German hills”. Both, genetic diversity and rarity of fragments can be ascribed to the history of *L. pratensis* populations, which have presumably a longer history in southern than in northern regions. This pattern was already shown for many other plants in broader geographic scales (Hewitt 1996; Taberlet *et al.* 1998).

Differentiation and spatial genetic structure

Differentiation between populations is caused by restricted gene flow and a certain level of isolation (Kimura & Weiss 1964; Slatkin 1987; Slatkin 1993; Wright 1943) (by distance). Transfer of pollen or seed material ensures gene flow and reduces differentiation between populations (Nybom 2004). Extenuated levels of differentiation for common and constantly disseminated species can be expected, because of frequent gene flow between sites (Bradshaw 1975; Hufford & Mazer 2003). *L. pratensis* is further described as outbreeding further indicating enhanced gene flow (within and between populations). Nevertheless, we found high differentiation between-populations (PHIPT = 0.427). Our findings even exceeded compilation data based on RAPDs and microsatellite data for outcrossed (F_{ST} = 0.22) short-lived perennials (F_{ST} = 0.31-0.32) in a regional scale (F_{ST} = 0.28) (Nybom 2004; Reisch & Bernhardt-Römermann 2014) and for other grassland species at a comparable scale (Honnay *et al.* 2006) (see Michalski & Durka 2012 for more examples). Nevertheless, high differentiation among populations was also found in 2 widespread *Cyclamen* species on the island of Corsica with enzyme electrophoresis (F_{ST} =0.131- 0.415, (Affre & Thompson 1997), supporting our own findings for *L. pratensis*.

Seeds of *L. pratensis* vary between 750-1650mg (Brunsberg 1977) and have no additional specification for dispersal with wind or water. If there is no seed dispersal over long-distance with other vectors (grazing livestock, birds or transportation with hay), the dispersal would be restricted to the surrounding of the mother plants (Bonn & Poschlod 1998). It was shown that gravity dispersed species have higher differentiation between populations compared to other

dispersal types, even when the species are widespread (Hamrick & Godt 1996). In *Geranium pratense* gene flow at distances larger than 10km is not relevant (Michalski & Durka 2012). In wind-pollinated *Festuca ovina* even pollination only effects genetic diversity within populations (11m), because of limited extensions of pollen clouds (Bengtsson *et al.* 1995). Seen from these studies, differentiation between wild populations in common species is depending much more on biological traits than on single sowing events.

Seed production areas and provenances

For the investigated dataset of *L. pratensis* in Bavaria a clear genetic delineation with two ESUs in the biggest geographical scale over the whole area of Bavaria was detected. The most possible partition of the dataset was a northern and a southern region. The southern region resembles exactly one provenance of regionalised seed production. In concordance to a former study (Michalski & Durka 2012) we found no significant differences in differentiation of populations between provenances.

L. pratensis is frequently produced and used in “seed mixtures” (Firma Saaten Zeller GmbH & Co KG 2015; Rieger-Hofmann GmbH 2016/2017). We sampled only populations without apparent anthropogenic influence. As differentiation between populations was very strong even within provenances, we can definitely exclude the direct influence of sown seed material.

On the other side, natural barriers hamper gene flow in all geographic scales (Slatkin 1987). The concordance of the alpine provenance and southern group of *L. pratensis* populations can as well be the result of the river “Danube”, which demarcates the regions from each other.

When IBD is present, the kinship of individuals and populations tend to decrease with increasing distance (Kimura & Weiss 1964; Slatkin 1987). At a certain distance neither gene flow by pollen nor seeds do account any more. Only random gene flow and distinct kinship can be detected in those distances. The distance classes can be designated as independent or at least not connected by obvious gene flow (Diniz-Filho & De Campos Telles 2002; Escudero *et al.* 2003; Krauss & Koch 2004). Associations between genetic and geographic population distances could not be found in the whole dataset of *L. pratensis*, but by separating the data in a northern and a southern group.

This is further supported by a significant positive (more than random) correlation in the distance intervals until 110km, whereas populations at distances greater than 110km are not

significant or only distantly related compared to the whole dataset. Compared to distances classes revealed with similar methods, 20km, 20km, 26km for Australian species was found byKrauss & Koch (2004) ,110km are rather great distances. However, we could detect gene flow with clear isolation by distance pattern in northern and a southern group separately. Populations within those regions have mean distances of 129km and 118km, respectively. The resulting delineation of ESUs resembles the northern and southern lineages. Gene flow is restricted by barriers or within a certain distance (Krauss *et al.* 2013). It is, therefore, essential to perpetuate the north – south barrier along the river "Danube" for *L. pratensis.*

Based on AFLPs, our study revealed high differentiation between populations of *L. pratensis* in Bavaria. Gene flow is limited within a scale of 110km, what is congruent to two main groups (ESUs) divided by a north-south boarder. Only the official seed production area ""foothill of the alps and alps" matches the distribution of genetic diversity and distances of gene flow. Based on these results collections should be undertaken in both ESUs, in northern and in southern Bavaria, to represent the differentiation of Bavarian populations. Nevertheless, southern regions of Bavaria should be prioritised as they tend to have higher levels of genetic diversity and rarity of fragments. To prevent outbreeding effects, we suggest to use material for restoration as locally as possible, because differentiation between populations is very high in the common species *L. pratensis*.

Chapter 4

Genetic diversity in populations of *Hepatica nobilis* Schreb. in Bavaria against the background of seed transfer guidelines in forestry and restoration

ABSTRACT

Forests in Bavaria consist of different compositions of abiotic factors, trees and successional stages. Herbs with specific habitat requirements, e.g. soil chemistry or light, are therefore restricted to small regions or patches. *Hepatica nobilis* (Ranunculaceae) is a perennial, understorey herb limited to calcareous rich soils in beech and oak forests. In Bavaria, it has four main areas of distribution: Franconia, the Franconian-Swabian Jura, the prealpine moraine belt and the Alps. Provenance delineations are used in German forestry since 1987. Those resemble the provenances and production areas of commercial seed production and restoration in herbs. We asked: Can provenance delineations or geographic regions better reflect genetic diversity of *H. nobilis*?

Distribution of genetic diversity in 24 populations of *H. nobilis* was examined with AFLPs. Relations within and between geographic regions of distribution and seed provenances/production areas were tested.

We found high diversity within and moderate differentiation between populations. Differentiation between seed production areas and seed provenances was very low, slightly higher between geographic regions. Genetic diversity or rare fragments did not differ between production areas, provenances or geographic regions. Significant positive relations of genetic and geographic distances were present within 100km.

Pollination and seed dispersal happens within populations in myrmecochorous *H. nobilis*, long-distance dispersal is occasional and random. We can reinforce the influence of biological traits for all considerations in conservation. In the case of *H. nobilis* the in-situ conservation of old forests, type Querco-Fagatea, is exceedingly important to conserve *H. nobilis* populations in Bavaria.

Keywords: AFLPs; beech forest; myrmecochory; seed provenances; understorey herb

INTRODUCTION

Forests in Central Europe formed anew after the last glacial maximum. The postglacial history of *F. sylvatica* forests, which would be the main natural forest type in Central Europe today, has been investigated in many studies (Magri 2008). However, in the last centuries forest distribution was strongly influenced by human activities, like agriculture and housing, and a long history of forestry (Hosius *et al.* 2006; Poschlod 2015; Zerbe & Brande 2003). Excessive forest use was followed by afforestation with monocultures in the late 20th century, and the reinvention of sustainable forestry in the last decades. Thereby, increasing demands on forestry resulted in the delineation of provenances to ensure autochthony in forest trees (BMVEL 2003; Degenbeck 2012; FoVG 2002; FoVHgV 2003; Hosius *et al.* 2006; Schmidt & Krause 1997). Provenances are based on historical and geomorphological data. Plant material, e.g. seeds and seedlings of wild populations should only be transferred inside the same provenance, where they were collected.

Similar provenances were defined for herbs in 2011 satisfying the growing demand for collecting and planting herbs and shrubs for restoration (Erhaltungssortenverordnung 2014; Prasse *et al.* 2010a). Therefore, tree and herb collections for seed producers, breeders and seed bank collections are based upon similar provenance delineations. However, only few species have been tested for the conformity of natural genetic units and seed provenances.

Forests of Central Europe are characterized by mosaics of forest patches with varying compositions of trees, shrubs and herbs, and successional stages. Plant species are often restricted to certain soil and climate conditions and the distribution areas are large-scaled patches. This can be seen in the distribution of the forest herb *Hepatica nobilis* Schreb. (Ranunculaceae, Fig. 18) in Bavaria. The perennial, outbreeding understorey herb is common in beech and oak forests (Querco-Fagatea), but only where soil is calcareous rich. *H. nobilis* has always been in focus of breeders and gardeners. In some regions it was endangered because of frequent cutting of flowers and transplantation of whole plants to gardens (Szewczyk-Taranek & Pawłowska 2015). As a consequence, collection of plant material is illegal by German law since 1987 (BNatSchG 2009). Nowadays, the north-western margin of *H. nobilis'* distribution lays within Bavaria. Three main areas of occurrences in Franconia (F), the Franconian-Swabian Jura (J) and alpine area (A), and a transition zone in the prealpine moraine belt (G) can be distinguished (Fig. 19a).

Fig. 18: Habitus of H. nobilis: flowers and first leave in early spring (March).

When habitats are fragmented and the distances between populations are long, regular gene flow is not always guaranteed. It has already been demonstrated that common outbreeding species are even more affected by isolation than rare species (Honnay *et al.* 2006). Gene flow is outstanding to sustain a certain level of diversity. Two levels of gene flow in outbreeding plants can be distinguished. First, gene flow occurs within populations in short distances. Second level of gene flow takes place between populations and regions. Exchange of material between populations and regions ensures high genetic diversity, decreases differentiation between populations and inbreeding. On both levels gene flow is depending on the availability of vectors for pollen and seed dispersal, which are specific for the genus or species.

Especially for insect pollinated species pollination is regionally restricted and depending on the distance radius of the insect (Kwak *et al.* 1998). The distances between populations are decisive for the connectivity of populations by pollination events (Matter *et al.* 2013; Steffan-Dewenter & Tscharntke 1999). The dispersal of seeds is sometimes even more limited (Poschlod *et al.* 2013). Myrmecochorous seeds of *H. nobilis* are dispersed at very small scales only within few meters around the mother plant (Bonn & Poschlod 1998; Fokuhl *et al.* 2012; Smith *et al.* 1989).

For genetic diversity within populations additionally the ratio of clonal to sexual propagation is decisive (Soane & Watkinson 1979; Stehlik & Holderegger 2000). It was estimated that almost 70% of the plants in the European flora are able to propagate clonally (Honnay & Bossuyt 2005). In habitats, where sexual reproduction is hampered e.g. because of the lack of pollinators or, because of unsuccessful germination or seedling recruitment, plants often reproduce clonally (de Witte & Stöcklin 2010). Clonal propagation can reduce the loss of individuals due to disturbances and ensure persistence of a plant throughout a long period of time without sexual reproduction (de Witte & Stöcklin 2010; Sheffield *et al.* 1989). (Honnay & Bossuyt 2005). Over-proportional and prolonged clonal growth can be a severe problem in fragmented habitats. However, the proportion and spatial extension of clonal growth is very species specific (Reisch & Scheitler 2009). In *H. nobilis* ramification of the rhizomes is the prevalent form of clonal reproduction (Persson 1975).

The necessity of gene flow and sexual reproduction is irrevocable. Nevertheless, in the last decades the negative impact of distantly related genetic material to populations has been studied. It has been demonstrated that “too foreign” material can be harmful for the population. Outbreeding depression was noticed, further leading to the loss of adaptations to environmental conditions and reduced fitness (Krauss *et al.* 2005; Montalvo & Ellstrand 2000; Schoen & Brown 2001).

Even though the concept of provenance delineation is widely used by forestry, breeders, seed producers, in restoration projects and for genebank collections, only few studies have compared the delineation of production areas and provenances with spatial genetic structure (Bengtsson *et al.* 1995; Michalski & Durka 2012). Common forest understorey herbs have not been investigated so far.

We tested 24 populations of *H. nobilis* all over Bavaria with AFLP analysis to answer the following questions:

a Do population genetic analyses reflect the disrupted geographic distribution of *H. nobilis* in Bavaria?

b Are provenance delineations suitable for a myrmechorous propagated forest herb?

c What would be the best collection strategy for *H. nobilis* for genebank or breeding purposes?

MATERIAL AND METHODS

Species description

Hepatica nobilis Schreb. (Ranunculaceae) is a diploid (2n=14), perennial woodland herb flowering in early spring (Baumberger 1971; Inghe & Tamm 1985). Three flower variants have been described: blue, deep-purple to pink or white. Flowers are pollinated by bees, beetles, flies and syrphid flies (Hara 1962; Klotz *et al.* 2002). Only few cases of selfing are reported, when pollination fails (Knuth 1898). Rosettes can reproduce vegetatively by rhizomes that also serve as storage organ (Inghe & Tamm 1985). Seeds are apocarpous, have elaiosomes containing sugars and lipids (Bresinsky 1963) and are ant-dispersed (myrmecochorous) (Mark & Olesen 1996). Individuals can gain life spans of at least 6-30 years (Bierzychudek 1982). *H. nobilis* was used as a medical plant in the past and is nowadays widely used as ornamental plant and for breeding purposes (Żuraw *et al.* 2013).

H. nobilis occurs in temperate, sub-meridional and montane vegetation zones in Eastern, Central and Northern Europe and in Central and Western parts of Asia. It is mainly present in mixed forests dominated by beech (*Querco-Fagatea*) with nutrient rich soils. It is sensitive to soil acidity and Al^{3+} availability and, therefore declining in most regions (Tyler *et al.* 2002). As it is restricted to calcareous soils the distribution in Bavaria is disrupted into several areas (Fig. 19a).

We sampled fresh leaf tissue of 7-10 individuals of 24 populations all over Bavaria in summer and late summer 2010 (Fig. 19b, Tab. 6). Material that was sampled in late summer 2010 contained DNA material of fungi, assumedly *Ascochyta dolomitica* (Henricot 2010; Mel'nik 2000). This resulted in exceedingly various fragments of the infected leaves in the AFLP analysis and was not comparable to all other samples. Therefore, another collection was carried out in spring 2011, where fresh, young leaves were sampled. Samples within populations were taken in distances of at least 2m, maximum 30m to avoid clonal shoots. Maximum distance between populations was 363.0km, minimum was 19.6km.

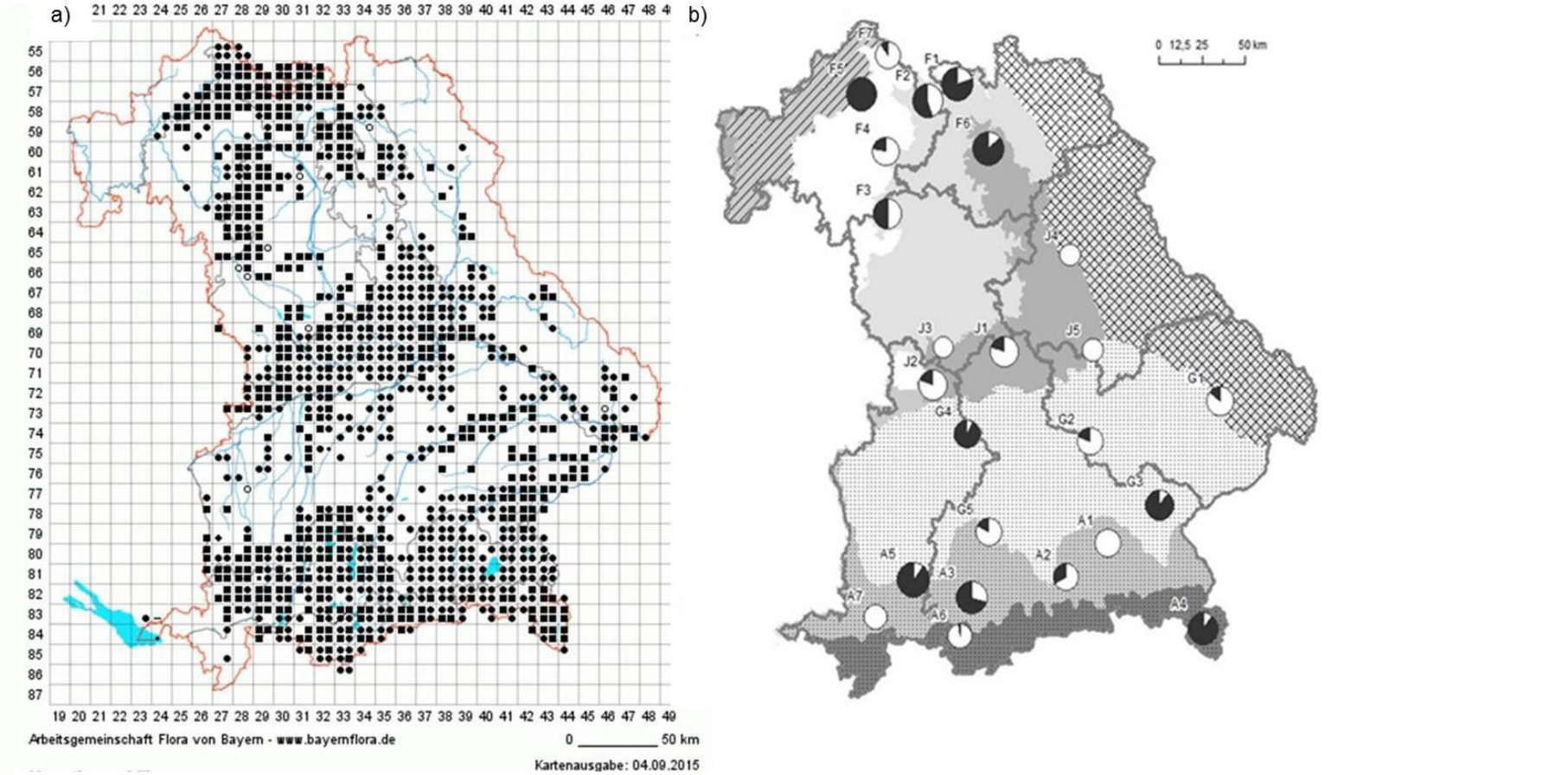


Fig. 19: (a) geographic distribution of *H. nobilis* in Bavaria: squares = populations proven 1945-1983, filled dots = populations proven after 1983, circles = populations proven before 1945 (*Arbeitsgemeinschaft Flora von Bayern, vers. 09/2015*) and the 24 collected populations in Bavaria (b) within their situation in seed production areas (dots, plain, stripes, squares) and seed provenances (different colours and patterns) according to Prasse et al. 2010a; populations are labelled according to Tab. 6, size reflects genetic diversity (SI), black and white gives the partition of STRUCTURE groups within each population.

Tab. 6: Information on populations of *H. nobilis*: Identification (*Id*), Name of the locality nearby (*loc*), number of individuals (*no*) and geographic information as Longitude (*lon*) and Latitude (*lat*), and affiliation to seed provenance (*pv*), seed production area (*pa*) and geographic region (*gr*) are provided. Further results of genetic analysis are given as percentage of polymorphic loci (*PL*), Shannon information index (*SI*), SSWP/(n-1) (*SSWP*), averaged down-weighted rare fragments (*DW*) and rare fragments = present in less than 10% of all populations (*RF*).

Id	*loc*	*no*	*lon*	*lat*	*pv*	*pa*	*gr*	*PL*	*SI*	*SSWP*	*DW*	*RF*
A1	Edgarten	9	12.173306	48.040201	17	8	A	45.78	0.27	21.25	10.27	
A2	Weyarn	8	11.839904	47.870098	17	8	A	42.17	0.25	21.13	9.80	
A3	Untermaxlried	10	11.108300	47.767798	17	8	A	62.25	0.37	31.69	9.54	
A4	Berchtesgaden	10	12.911946	47.615152	18	8	A	58.63	0.35	26.77	8.95	
A5	Kaufbeuren	10	10.649776	47.848775	17	8	A	70.28	0.40	34.33	9.84	1
A6	Linderhof	9	11.017659	47.579205	18	8	A	38.96	0.23	17.69	9.68	
A7	Sulzheim	10	10.358926	47.664692	17	8	A	36.14	0.22	15.83	10.12	
F1	Neuses	10	10.940934	50.282978	11	7	F	64.26	0.39	30.97	10.11	1
F2	Eckartshausen	9	10.697948	50.199049	12	7	F	59.44	0.36	28.94	9.94	
F3	Markt Bibart	10	10.390284	49.641765	12	7	F	52.21	0.31	24.99	9.46	
F4	Mönchstockheim	9	10.366348	49.948740	11	7	F	48.59	0.28	22.28	9.33	
F5	Nüdlingen	10	10.144378	50.228172	11	7	F	64.26	0.37	30.16	9.07	
F6	Steinfeld	8	11.201124	49.968297	14	7	F	63.45	0.38	32.18	10.00	2
F7	Sondheim	10	10.366872	50.417811	11	7	F	41.77	0.25	18.79	9.37	
G1	Hengersberg	9	13.065004	48.727703	19	5	G	46.59	0.29	21.50	9.28	
G2	Gündlkofen	10	12.032099	48.537297	16	8	G	44.58	0.27	19.63	9.96	
G3	Mühldorf am Inn	10	12.578665	48.224694	16	8	G	51.41	0.31	24.62	10.08	2
G4	Pöttmes	9	11.053340	48.567219	16	8	G	44.18	0.27	21.08	9.26	
G5	Gilching	10	11.232273	48.091841	17	8	G	44.58	0.27	19.83	8.88	
J1	Eichstätt	10	11.342176	48.974862	14	7	J	51.00	0.31	22.49	9.82	1
J2	Bibernhof	10	10.777499	48.807220	14	7	J	50.20	0.30	22.89	10.37	1
J3	Fremdingen	7	10.857810	48.993485	12	7	J	26.91	0.17	13.29	9.81	
J4	Pyrbaum	10	11.870346	49.452466	19	5	J	28.11	0.17	12.36	9.54	
J5	Pentling	10	12.050850	48.986569	16	8	J	30.12	0.18	13.43	9.52	
	mean							48.58	0.29	22.84	9.67	
	SE							11.88	0.07	6.22	0.41	

DNA extraction and AFLP analysis

Leaf material was stored in cooled bags until it was frozen at -18°C at the University Regensburg. DNA was extracted from 1-1.5cm² of frozen and powdered leaf material following the protocol of Rogers & Bendich (1994) with minor changes (Reisch 2008). AFLP analysis was conducted in following the protocol of Vos *et al.* (1995) with minor changes (Reisch *et al.* 2003a). DNA content was standardized in solutions with 7.8ng DNA/µl. For restriction and ligation approximately 50ng genomic DNA were used in reaction volumes of 10µl. For restriction EcoRI and MseI enzymes (Fermentas) were used, for ligation EcoRI and MseI adaptor pairs were used with T4 Ligase enzyme (Fermentas). Reaction was run on an automated thermal cycler at 37°C for 2h.

All PCR reactions were run in reaction volumes of 5µl with Taq polymerase. For preselective amplification primer pairs with single selective nucleotides were used (MseI-C and EcoRI-A, both MWG). After one initial denature step (2min at 94°C) 30 cycles of denaturing at 94°C (20s), annealing at 56°C (30s) and extension at 72°C (2min) were run closing with final extension steps at 72°C (2min) and at 60°C (30min). An initial screening on 6 individuals out of 3 populations with 36 combinations of selective primers was conducted. Three primer combinations were chosen for the analysis of all individuals: MseI-CTA/EcoRI-AGC, MseI-CAT/EcoRI-AGG, MseI-CAT/EcoRI-ACT. Selective amplifications were started with initial denature step at 94°C (2min) and a touch-down profile: denaturing at 94°C (20s), annealing at 66°C minus 1°C for the next ten cycles, extension at 72°C (2min). This was followed by 25 cycles of denaturing at 94°C (20s), annealing at 56°C (30s), and extension at 72°C (2min). Final step was elongation at 60°C (30min).

After PCR reactions DNA was diluted according to intensity of fluorescence labelled primers: EcoRI-AGC x2, EcoRI-AGG x-, EcoRI-ACT x5), precipitated with ice-cold EtOH (95%) and vacuum dried. Pellets were resolved in a mixture of Sample Loading Solution and CEQ Size Standard 400 (both Beckman Coulter). Selective products were separated with capillary gel electrophoresis on an automated sequencer (CEQ 8000, Beckman Coulter). Fluorescence signals were automatically detected and aligned to CEQ Size Standard.

Clear and defined fragments were searched by hand in the program BIONUMERICS version 3.0 (Applied Maths) over all individuals and coded as present (1) or absent (0). The resulting binary matrix was basis for all further calculations. To specify the methological error 15 individuals (6.6%) were reproduced (Bonin *et al.* 2004) resulting in an error rate of 1.52%.

Computations

Genetic diversity within and between populations was examined with the program POPGENE version 1.32 (Yeh *et al.* 1997) as percentage of polymorphic loci and Shannon Information Index ($I = \sum pi \ln pi$) for each population (Parisod & Christin 2008). Sums of squares for each population (SSWP) derived from the analysis of molecular variance (AMOVA) were divided by n-1, because it gives a further value for gene diversity independent from sample size (Fischer & Matthies 1998a).

The dataset was tested for rare and unique fragments as they would indicate the level of isolation and differentiation (Slatkin 1985). Rarity was given as frequency down weighted markers (DW) (Schönswetter & Tribsch 2005) and as fragments present in less than 10% of the individuals.

AMOVAs were conducted in GENALEX version 5 (Peakall & Smouse 2001) based on pairwise Euclidian distances between individuals. Differentiation between populations was calculated as estimated Φ_{PT} = diversity between populations/total estimated diversity (equivalent to Fst of Wright's F-Statistic).

Genetic relatedness between individuals was shown by a principle coordinate analysis (PCoA) that was conducted using Bray-Curtis similarities in the program MVSP version 3.12f (Kovach 1999). A majority rule (50%) consensus UPGMA tree based on chord-distances was constructed with non-uniform prior on 10^4 replicates in FAMD to show relations between populations (Cavalli-Sforza & Edwards 1967; Schlueter & Harris 2006; Zhivotovsky 1999).

Hierarchical AMOVAs were conducted for seed production areas, seed provenances and the four geographic regions of distribution of the species, respectively. Provenances and production areas are numbered according to official seed zones (Tab. 6) (Prasse *et al.* 2010a). Provenances of herbs are almost identical to provenances in forestry in Bavaria (BMVEL 2003; FoVHgV 2003). At the sites of *H. nobilis* collections, there is no difference between provenances for herbs and trees. Production areas comprise several provenances. Plant production can be done all over the production area, but the products have to be used in the provenance, where they were collected (Prasse *et al.* 2010a). As geographic distribution areas we used Franconia (F), Franconian-Swabian Jura (J), prealpine transition regions (G) and alpine regions (A) (Tab. 6). ΦRT was calculated as estimated diversity between regions/total estimated diversity. We further compared mean genetic diversity within populations (SSWP/n-1) and rarity (DW) dependent on seed production areas, seed provenances and geographic regions, respectively, with Kruskal-Wallis rank sum test in R.

Correlation of geographic distances (km) and genetic distances (Φ_{PT} from AMOVA) was tested with Mantel test in GenAlEx version 5 (Mantel 1967) with significance levels based on 999 permutations. Historical gene flow (Nm) between populations was calculated with mean Φ_{PT} values using the approach $F_{st} = 4(Nm+1)-1$ (Stehlik & Holderegger 2000; Wright 1965).
Further spatial genetic structure was revealed using the program SPAGeDi (Vekemans & Hardy 2004). Pairwise kinship coefficients for dominant markers (Hardy 2003) were assessed in seven distance classes. The inbreeding coefficient (F_{is}) was estimated using an approach by (Dasmahapatra *et al.* 2008) and, thereupon, set at $F_{is}=0.22$.

RESULTS

AFLP analysis of 227 individuals resulted in 249 fragments of which 94% were polymorphic. Genetic diversity given as Shannon information index and AMOVA-based SSWP/(n-1) ranged from 0.166 to 0.402, with an average of 0.291, and from 12.36 to 34.33, with an average of 22.84, respectively (Tab. 6). 26.91% to 70.28% of the fragments were polymorphic within the populations.
We found fragments occurring in only two or three populations and meanwhile in less than 10% of all individuals, but no fragments were unique to single populations (Tab. 6). Rarity calculated as frequency down-weighted ranged from 8.88 to 10.37 (mean 9.67).
Total genetic differentiation between 24 populations of *H. nobilis* was moderate and significant ($\Phi_{PTp} = 0.269$, $P<0.001$). Most of the diversity could be found within populations (Tab. 7). Hierarchical AMOVA revealed low differentiation between geographic regions and even lower differentiation between production areas and provenances. We could not detect significant correlations of genetic diversity (SSWP/n-1) or rarity of fragments (DW) with seed production areas, seed provenances or geographic regions (data not shown).

Tab. 7: Analysis of molecular variance (*AMOVA*) is shown in a one-level approach, where degrees of freedom (*df*), sum of squares (*SS*), mean squares (*MS*) and percentage of diversity (*%*) between and within populations is given. Φ_{PT} and attached significance level is shown for one-level AMOVA and hierarchical (two-level) AMOVAs. For seed production areas, seed provenances and geographic regions as major regions further Φ_{RT} and Φ_{PR} values are given with attached significance levels (*p*).

one-level AMOVA	***df***	***SS***	***MS***	***%***	Φ_{PT}	
Between Pops	23	2366.2	102.88	27%		
Within Pops	203	4653.84	22.93	73%	0.269	0.001

two-level AMOVAs	Φ_{RT}	***p***	Φ_{PR}	***p***	Φ_{PT}	***p***
Production areas	0.032	0.001	0.255	0.001	0.279	0.001
Provenances	0.016	0.001	0.259	0.001	0.271	0.001
Geographic regions	0.051	0.001	0.239	0.001	0.278	0.001

Group structures in higher dimension were examined using PCoA. Individuals of populations are disarranged along the first three axes of the PCoA (Fig. 20). UPGMA population tree shows that populations are grouped independent of their geographic region or seed provenance (Fig. 21), and we could not infer any geographic pattern.

Gene flow (Nm) was low between populations ranging between 0.34 and 3.60, with an average of 0.76 over the whole dataset. No isolation by distance patterns could be found with Mantel test, which was not significant over the whole area (r=0.121, p=0.075) and not significant within seed production areas, seed provenances and geographic regions, respectively (data not shown).

Further spatial genetic structure with SPAGeDi was based on seven distance levels and revealed significant positive correlation of geographic and genetic distances in the first two distance classes at 19 to 65km and 65 to 100km (Fig. 22).

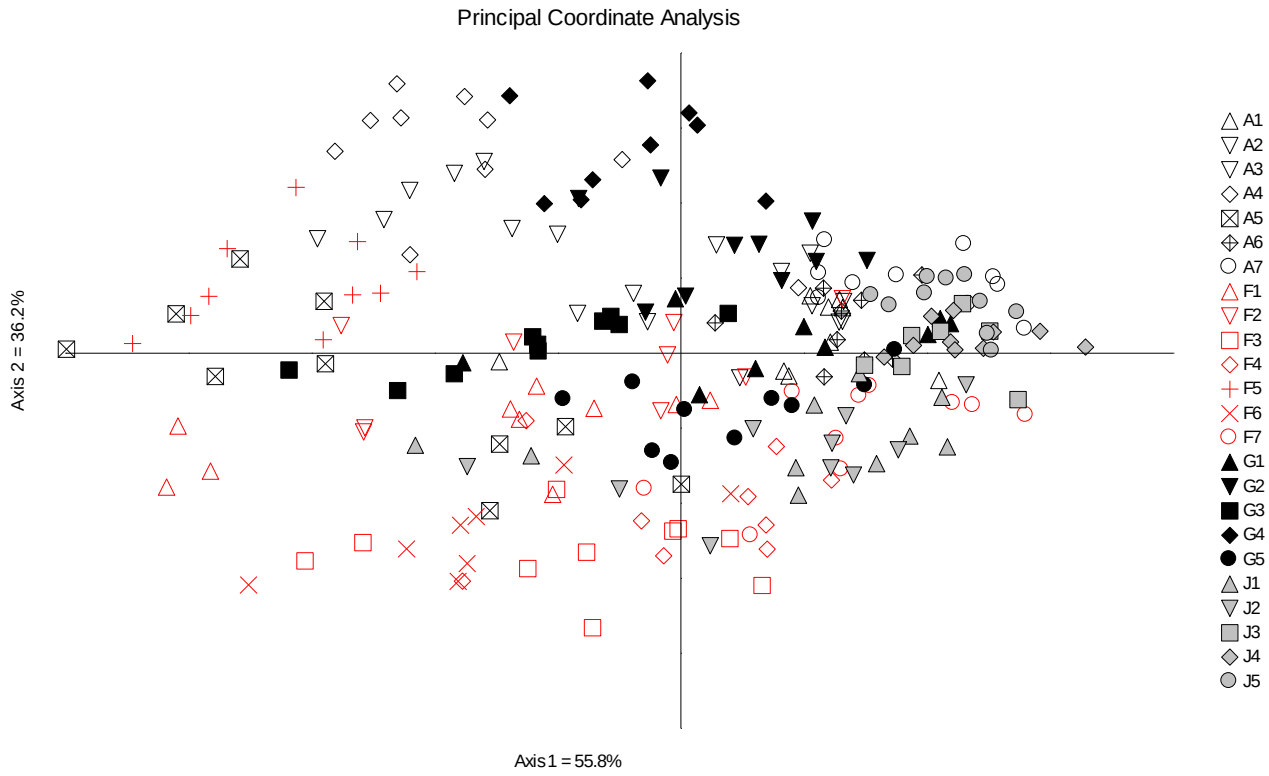


Fig. 20: Principal Coordinate Analysis of 227 individuals of *H. nobilis* from Bavaria. Populations are labelled according to Tab. 6: alpine region (A)= lined black, Franconia (F) = lined red, prealpine areas (G) = filled black, Franconian-Swabian Jura (J) = filled grey.

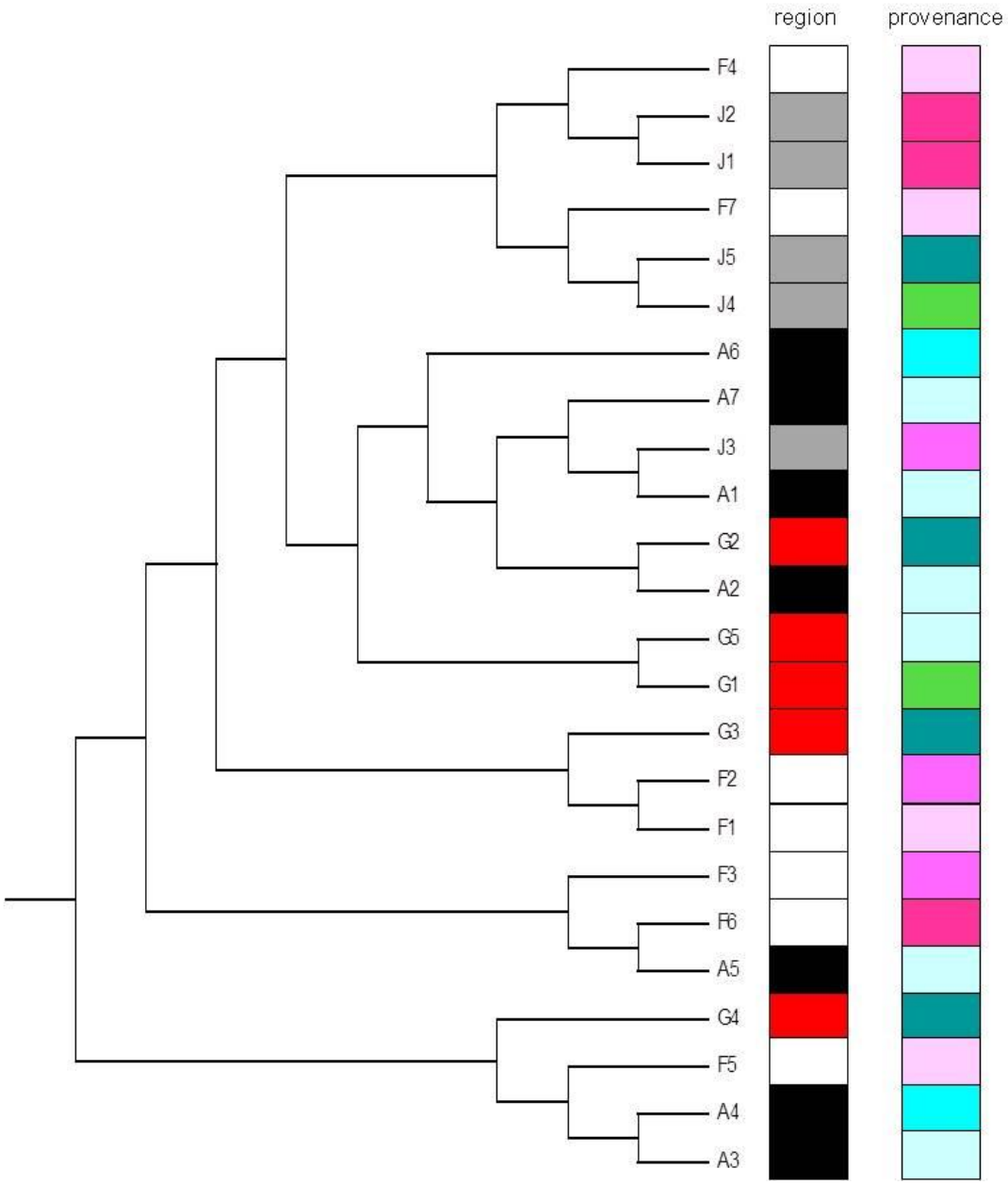


Fig. 21: UPGMA tree of 24 populations of *H. nobilis* in Bavaria. Populations are not grouped according to their geographic region or seed provenance zone (for affiliations see Tab. 6).

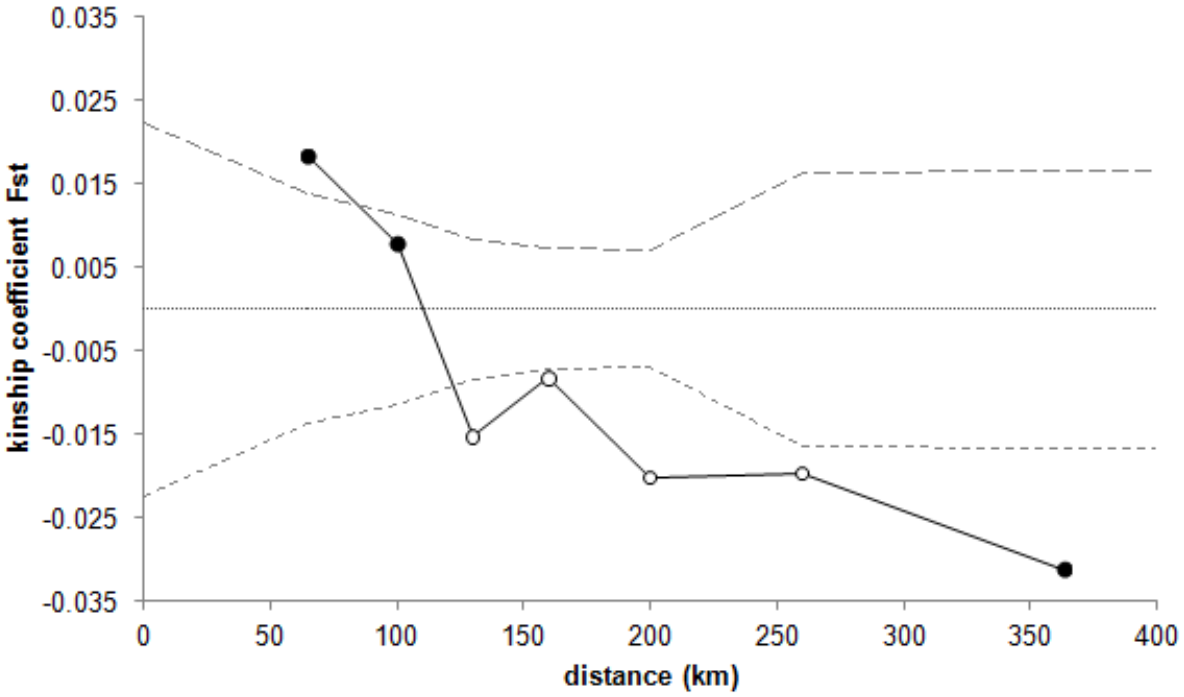


Fig. 22: Analysis of spatial genetic structure in seven distance classes of *H. nobilis* in Bavaria. Kinship coefficients are significant positive for the classes up to 100km, not significant or negative for the other classes.

DISCUSSION

Diversity within populations

Genetic diversity was high and mainly allocated within populations of *H. nobilis* (73%). Highest diversity within a population of *H. nobilis* was found in the Alps (70.28% PL in A5), while the population with smallest diversity was situated in Swabia (26.91% PL in J3). In the understorey herb *Anemone nemorosa* even 93% of diversity was located within populations (Stehlik & Holderegger 2000). Plant traits like pollen and seed dispersal, regeneration mode, germination and reproduction effort are main factors that influence diversity (Schmidt & Jensen 2000). Frequent gene flow between populations can further enhance diversity within populations (Després *et al.* 2002). High genetic diversity within populations is typical for outcrossing and perennial plant species (Hamrick *et al.* 1991). Diversity within populations was also high in other species belonging to the family of Ranunculaceae, e.g. *Aquilegia*

spec.: 78.9% (Zhu *et al.* 2011), *Pulsatilla vulgaris* (Hensen *et al.* 2005), *Ranunculus acris:* 89.03% (Odat *et al.* 2004), *Trollius europaeus:* 64% (Després *et al.* 2002). Therefore, high diversity within populations seems to be enhanced by traits within the family of Ranunculaceae.

As aforementioned, the modality of regeneration has a major influence on genetic diversity. In clonal propagating plants like *H. nobilis*, the ratio of clonal growth and seedling recruitment is decisive for genetic diversity of a population. The number of seedlings, even, when it is small, may enhance genetic diversity in a population (Soane & Watkinson 1979; Stöcklin & Winkler 2004; Watkinson & Powell 1993). Seedling recruitment was also reported for *A. nemorosa* after several studies questioned the influence of sexual reproduction (Cowie *et al.* 1995; Piroznikow 1994; Shirreffs & Bell 1984). A further example within the family of Ranunculaceae is *Ranunculus repens,* in which 3 % sexual recruitment is sufficient to maintain genetic diversity (Watkinson & Powell 1993).

Repeated seedling recruitment after colonisation of new sites has the same effect. This was described for 40% of the clonal species investigated by Eriksson (Eriksson 1989). Soil seed bank can further influence genetic diversity in plant populations (Listl & Reisch 2014; Zaghloul *et al.* 2013), but will have minor influence in *H. nobilis*, because seeds tend to be very short lived (Thompson *et al.* 1997).

A study already reported seedling recruitment in *H. nobilis* in 1948 (Tamm 1948), but the proportion of seedling recruitment and clonality is very much depending on the age of the plant community and several other factors (Chung 2008; Tague & Foré 2005). In contrasting habitats (moist and productive ash-poplar forests vs. dry and less productive mixed forests) genetic diversity of *Paris quadrifolia* differed, too (Jacquemyn *et al.* 2005). Genets were smaller and seedling recruitment more successful in moister forests resulting in higher genetic diversity in these habitats.

Another factor that has been shown to have major influence on seedling recruitment in forests is disturbance of the forest layers and the soil (Jensch 2004). While moderate damages of the forest promote coverage in *H. nobilis* (Ilisson *et al.* 2006), it was also shown that disturbance of woodland sites was responsible for reduced genetic diversity in populations of *A. nemorosa* (Rusterholz *et al.* 2009). We did not assess indications of disturbances of the populations and, therefore, cannot exclude these factors, but during sampling we could not find any conspicuous levels of disturbances in our sites.

We did not find any clones and, therefore, assume that sexual reproduction is occurring and clones have only small dimensions (< 2m). Additionally, external factors like anthropogenic (e.g. planting) and climatic events (e.g. dry spells, heavy rainfall or storm) may influence plant populations. Loveless & Hamrick (1984) already stated that pollen and seed dispersal effects on genetic structure are often masked by ecological factors. Therefore, we assume that a multitude of internal and external factors may influence the level of genetic diversity in the investigated populations of *H. nobilis*.

Populations had none or only few private markers (1-2), what would indicate that populations are rather recently established (Schönswetter *et al.* 2002; Wróblewska 2008). Young forest sites are characterised by *H. nobilis* in two studies comparing young and old sites (Aavik *et al.* 2009; Elofsson & Gustafsson 2000). In contrast, high levels of rare fragments and high genetic diversity within populations demonstrate the opposite. Because of founder effects populations in young forests would have reduced levels of diversity within populations compared to older stands (McCauley 1991). In *P. elatior* diversity was especially high in forests older than 35 years compared to younger ones (Jacquemyn *et al.* 2004). Similar results were found for *Trillium grandiflorum* with higher diversity on sites that have never been cleared for agriculture and are, therefore, older than 100 years (Vellend 2004). In *H. nobilis,* which is an indicator of historically old forests according to Wulf & Kelm (1994), the investigation of old stands in our study can further contribute to high levels of diversity within populations.

This corresponds to the assumption that populations of *H. nobilis* in Bavarian forests are mostly rather old and persistent. Several studies support the assumption that *H. nobilis*, where once it is present, remains very constantly and individuals get very old (Falkengren-Grerup & Eriksson 1990; Hermy 1994; Hermy *et al.* 1999; Sikorska *et al.* 2008; Tamm 1948).

Genetic diversity or rarity of fragments was not high or low in specific geographic regions or provenances –contrasting to what was shown for other species (Bylebyl *et al.* 2008; Després *et al.* 2002). Even the populations of *H. noblilis* in transition zones (G1-G5 in the prealpine area) had no extraordinary high or low levels of diversity or rarity of fragments. Therefore, we assume similar habitat conditions and population ages in all investigated regions.

Differentiation and spatial structure

Differentiation between populations was moderate in *H. nobilis* (Φ_{PT} = 0.269). Comparable values of differentiation have been identified in *A. nemorosa*: mean Gst = 0.29 (comparable to Fst) (Stehlik & Holderegger 2000)

Other species within the family of Ranunculaceae have similar values of differentiation. In *Ranunculus glacialis* Fst ranged from 0.33 between populations in central and eastern Alps to 0.62 in southwestern and western Alps (Schönswetter *et al.* 2004). In *Trollius europaeus* Fst ranged from 0.24 in the Alps to 0.39 in the Pyrenees (Després *et al.* 2002). Compared to other long-lived outcrossing perennials the values of *H. nobilis* are typical (Nybom 2004; Reisch & Bernhardt-Römermann 2014).

There was no correlation between genetic and geographic distances between populations of *H. nobilis* in Bavaria with Mantel test, but populations separated less than 100km are more related to each other than further apart. For the tree *Sorbus terminalis* the spatial genetic structure occurred only within 150km and was most likely explained by high rates of local extinction and recolonisation within these distances (Demesure *et al.* 2000). There is no significant correlation of genetic and geographic distances between populations of many other Ranunculaceae e.g. *Ranunculus acris* (Odat *et al.* 2004), *Ranunculus reptans* (Fischer *et al.* 2000), *A. nemorosa* (Stehlik & Holderegger 2000) and *T. europaeus* at regional scales in the Alps and Pyrenees (Després *et al.* 2002). Furthermore, gene flow between sites was very low (max. Nm = 3.60, average = 0.76). For populations of *A. nemorosa* gene flow was similar, with the highest rate of Nm = 4.34 between two sites in a distance of 1.5km. In our study population distances were much higher (min. 19.6km). Therefore, low gene flow between populations can be ascribed to pollination and seed dispersal traits in the family of Ranunculaceae.

Gene flow via pollen is likely and was shown before in *H. nobilis* (Żuraw *et al.* 2013). Given the non-significant spatial genetic structure we found, it is likely that the distance of current gene flow via pollen and seed is lower than the between population distances in this study. Neither pollen nor seeds can frequently overcome the distances between populations. Seeds were dispersed only in very short distances in several other myrmecochorous species. Significant differentiation of patches was e.g. found within a few metres for North American understorey herb *Trillium* spec. (Webster & Jenkins 2008). For *A. nemorosa* Moran's I was significant and positive only in a distance class of 0-0.5m, but not significant and negative in the following distance classes (Stehlik & Holderegger 2000). Similar results were shown for

myrmecochorous dispersed seeds of *Hepatica acutiloba* in North America, where seeds are highly aggregated within few meters (Smith *et al.* 1989). Clonal growth can further influence the spatial genetic structure and even enhance genetic relatedness at small scales (Reisch *et al.* 2007). This will also be essential within the investigated populations of *H. nobilis*.

Nevertheless, because relation between populations was significant within 100km, genetic diversity was high, and differentiation between sites moderate, gene flow has to occur also between populations. Additionally to prevalent pollen or seed dispersal in short distances within populations, occasional “long-distance” dispersal events (used for leaving out several neighbour populations) can also explain the missing spatial relation between populations (Cain *et al.* 2000). Stochastic variation in gene flow due to attachment of seeds to animals was shown e.g. for *Sanicula odorata* (Williams & Guries 1994). For *H. nobilis* the distribution by rodents is possible, but for *Trillium nivale* and *H. acutiloba* rodents did not influence seed dispersal at all (Smith *et al.* 1989). Moreover, for *H. nobilis* anthropogenic influence on gene flow, like replanting of individuals in gardens, is rather possible (Żuraw *et al.* 2013).

All our results support the theory Cain *et al.* (2000) suggested for woodland herbs: seed dispersal curves may explain best the distribution at spatial scale, while at intermediate, regional scale the island model and at large scale possibly a stepping stone model could fit. Populations in the regional scale cannot form a genetic relation according to the stepping-stone model between each other, if gene flow is occurring mainly within a few meters in populations and long distance dispersal is an occasional event.

Conclusions for in-situ and ex-situ conservation

For ex-situ conservation, diversity in the whole collection and differentiation between populations should be as high as possible. We expected a clear differentiation of geographic regions in *H. nobilis*. Our results show that most diversity can be found within the populations. None of the tested delineations (geographic regions, provenances, production areas) showed significant differentiation. Nevertheless, geographic regions showed high consensus with the distribution of genetic diversity. Moreover, we could detect ecological significant units within 100km. For ex-situ conservation in botanical gardens or genebanks, collections should be undertaken at least in all geographic regions of distribution.

Replanting should be restricted within 100km, but better within the geographic region. Considering the high differentiation between populations, we recommend that transfer of plant material should be done as close as possible to the donor populations.

H. nobilis is difficult to breed and it is uncertain, if seeds are suited for storage in genebanks. Broad-scale in-situ conservation is important for species like that. For long-lived, outbreeding myrmecochorous *H. nobilis* it is important to preserve old woods with high proportion of deciduous trees. Bavaria is the north-western distribution area of *H. nobilis* and, therefore, it is important to preserve suitable forest regions within the present limited distribution areas.

Chapter 5

Genetic adaptation and environmental influences on seed quality and longevity

ABSTRACT

The storage of plant seeds in genebanks is nowadays a standard method to preserve maintenance of biodiversity and the guarantee for food production. The knowledge of the seeds' quality and longevity is crucial to predict the time for renewal of the lots. Several studies assumed that genetic adaptation, seed traits and environmental conditions have influence on seed quality and longevity under natural and genebank conditions.

We used x-ray analysis, artificial aging, and combined germination and Tetrazolium tests to compare different accessions of seeds. Seeds of *Atropa belladonna*, *Plantago lanceolata* and *Ranunculus acris* were collected from alpine and lowland populations and grown in a common garden. Seeds from common garden populations were compared with wild populations, alpine with lowland populations. Further, we collected seeds of 22 Central European and alpine species in different years and tested the influence of climatic factors with model approaches in R.

Seed weight and percentage of filled seeds showed neither differences between alpine and lowland populations nor between common garden and their wild ancestor populations. Maximal and initial germination rate tended to be higher in wild than in common garden populations, and higher in lowland than in alpine populations for *R. acris* and *A. bella-donna*. Longevity in artificial aging was higher in lowland (compared to alpine) and common garden (compared to wild) populations – except for one population of *R. acris*.

Differences in germination and seed longevity were significant between two years for alpine populations. In seeds from lowland populations the percentage of filled seeds was significantly different between 2010 and 2012. Mean annual temperature and total annual rainfall, and mean annual temperature together with total rainfall and total sunshine within 90 days prior to harvest explained some variation in our data. For alpine populations rain during maturation time (90 days) was most influential for seed longevity.

We could show that seed quality and germination differs between habitats and collection years, but maternal reaction to environmental conditions is very species-specific. Part of the

differences in seed longevity is genetically adapted and part is based in maternal factors, which occur during the growing of the mother plant and/or the maturation of the seeds. In general, warm, dry, and sunny climate seems to enhance seed longevity. In alpine populations further dryness during maturation is important to elongate the lifespan of seeds.

Keywords: artificial aging, alpine species, genetic adaptation, maternal effects, seed longevity

INTRODUCTION

7.4 million accessions of plant seeds are stored in about 1750 official genebanks worldwide. A main challenge for genebanks is to determine the time, when too many seeds of a collection are damaged and it is necessary to rejuvenate the accession by new collections or regeneration (FAO 2014a). Storage conditions have been optimised over the years with best practicable results for orthodox seeds, when they are dried (<15% relative humidity, RH) and cold-stored (-18°C) (FAO 2014a; Vertucci & Farrant 1995). However, seed longevity is very variable within plant families and within species and, therefore, predictions can hardly be made (Börner *et al.* 2014; Hay *et al.* 1997; Kochanek *et al.* 2011; Nagel & Börner 2010; Nagel *et al.* 2009; Probert *et al.* 2009; Schoeman *et al.* 2010; Walters *et al.* 2005).

Seed quality and longevity is strongly influenced by pre-storage factors. The stage of seed maturity and mass maturity (= filling of the seed) (Ellis & Filho 1992) and the duration and conditions (temperature, desiccation) between collection and storage (Probert *et al.* 2007; Vertucci & Farrant 1995) are known to be the most sensitive phases in collections for genebanks.

Initial quality of seeds, in other words the percentage of empty or damaged seeds in an accession, can be revealed with germination tests or x-ray analysis, and the number of viable seeds can be recorded before storage. Especially collections from wild species sometimes have low quality due to damage by insects or fungi (Probert *et al.* 2009), or differences in seed maturity within one population (Guttermann 2000).

Matters of particular interest for genebanks are wild collections of alpine species as their habitat is especially threatened by climate change (Kim & Donohue 2013; Parolo & Rossi 2008; Rosbakh & Poschlod 2015). Moreover, seeds of alpine species seem to be in general shorter lived than seeds from lowland populations (Mondoni *et al.* 2014; Mondoni *et al.* 2011).

Plants react to environmental conditions by triggering seed and germination traits and, depending thereon, the longevity of their seeds in soil (Saatkamp *et al.* 2014). To examine the reasons for differences in seed longevity between families, species and habitats, correlations of seed traits with longevity have been tested (e.g. (Walters *et al.* 2005; Way 2003): Several seed traits, e.g. seed coat thickness, seed weight and seed shape had influence on seed persistence in soil (Gardarin *et al.* 2010; Poschlod *et al.* 2013; Saatkamp *et al.* 2009). For ex-situ storage germination conditions themselves (type of dormancy,

optimal temperature and light conditions) are not relevant at first sight (Abedi 2013). On the other hand germination related seed traits may still influence longevity under genebank conditions. Artificial aging reflects storage under ambient conditions in genebanks and can be used to test the influence of specific traits to longevity (Abedi 2013; Merritt *et al.* 2014; Mondoni *et al.* 2011; Probert *et al.* 2009). It was demonstrated that thick seed coats enhance seed longevity in soil and under standardized conditions (Abedi 2013; Gardarin *et al.* 2010; Mohamed-Yasseen *et al.* 1994). Two studies – Abedi (2013) and Merritt with colleagues (2014) – found significant indication that seed weight and longevity were related, while Probert *et al.* (2009) found no such relation.

Seed weight and seed coat thickness can vary even within species, otherwise presence or absence of endosperm and endosperm-type is species- or even family-specific. It was shown that endospermic seeds are shorter lived in two studies (Merritt *et al.* 2014; Probert *et al.* 2009), while the third study found no influence of endosperm (Mondoni *et al.* 2011).

Besides morphological and anatomical seed characteristics, external factors may have influence through maternal effects (Baskin & Baskin 2001; Donohue 2009; Guttermann 2000; Mondoni *et al.* 2014; Roach & Wulff 1987). Environmental conditions to which mother plants are exposed to, e.g. temperature, moisture or photoperiod, can affect oil content and other chemical ingredients of seeds (Lajara *et al.* 1990; Rieger *et al.* 2008; Seemann *et al.* 2010). Further, size and germination of seeds differ depending on environmental conditions of mother plants (Galloway 2005; McWilliams *et al.* 1968; Munir *et al.* 2001; Schimpf 1977). As mentioned above, these traits can influence seed longevity under natural, but also under artificial storage conditions.

Temperature and rainfall were correlated to seed longevity in Australian (Merritt *et al.* 2014) and European species (Mondoni *et al.* 2011; Probert *et al.* 2009). Warm and dry environments had positive influence on longevity during maturation of seeds (Mondoni *et al.* 2011). So far, none of the studies did reveal, if the differences are based on general genetic adaptation to environment or on environmental conditions that mother plants are exposed to – maybe only at the particular time period of seed development. Except for one study on *Silene vulgaris*, which has shown that variance in seed longevity is partly based in genetic adaptations, partly based in maternal effects in this species (Mondoni *et al.* 2014).

However, most of seed traits may also depend on maternal environment before and during seed development, which was shown by the following studies: Kochanek and colleagues (2011) found in common garden studies that even pre-zygotic cool environment enhanced

seed longevity in *Plantago cunninghamii* by a factor of 2 (Kochanek *et al.* 2011). In another context, it was shown that phytochromes, which are necessary for germination, are dependent on temperature during seed maturation (Donohue 2009).

Reducing germination and viability tests are main factors to ensure the efficiency of genebanks. If longevity could be related to particular environmental conditions, regeneration times for each species or accession in genebanks could be adapted. The knowledge of particular factors for better seed longevity and quality can further help to optimise the planning of upcoming field trips. Therefore, there is an urgent need for studies to reveal the influence of environmental conditions on quality and longevity of seeds.

In the present study we aimed to answer, if seed longevity is genetically determined by long-term adaptation to the environment or if seed longevity is influenced by climatic conditions that mother plants are exposed to before or during seed development and maturation.

In a first experiment we collected *Atropa belladonna*, *Plantago lanceolata* and *Ranunculus acris* seeds from populations in Bavaria and Thuringia and searched for differences in seed quality (percentage of filled seeds, seed weight), initial and maximal germination and seed longevity under artificial aging…

… between alpine and lowland populations of the same species.

… between those alpine and lowland populations regrown in common garden.

… between the common garden populations and their wild ancestors.

In a second experiment, influence of specific weather conditions was tested between different years in collections from the same 22 populations of European species from lowland (Bavaria, Upper Palatinate) and alpine (Bavaria, foothills of the Alps) populations. Models based on former studies were applied and tested for their relevance for seeds collected from the same populations in different years.

MATERIAL AND METHODS

Seed collection and handling

Experiment 1: Comparison of seeds from wild and common garden populations of high and low altitudinal origin

14 species with orthodox storage behaviour were collected in high and low altitude populations (Tab. 8). Of each population plants were grown from seeds in a common garden

at the University of Regensburg. Only three species produced seeds in both, alpine and lowland populations: *Atropa bella-donna* L. (Solanaceae), *Plantago major* L. s. l. (Plantaginacae) and *Ranunculus acris* L. (Ranunculaceae). Seeds were collected at the same stage of ripeness, cleaned and stored in a dry chamber (30% RH, 20°C) for at least 4 weeks. 1000-seed weight for each population was extrapolated by taking three samples of 100 seeds. Afterwards seeds were dried to <15% relative humidity (RH) with silica gel, vacuum-packed and stored at -18°C until the start of the aging experiments.

Tab. 8: Characteristics of populations of *A. bella-donna* (AB), *P. lanceloata* (PL) and *R. acris* (RA): Identification (*Id*) with "l" for lowland and "h" for highland (=alpine) populations, source of seeds (*src*) from wild (*w*) or common garden (*cg*). For wild populations further geographical information (*latitude* (N), *longitude* (E), *height a.s.l.* (m)) and name of the locality (*loc*) is provided: BGL = Berchtesgadener Land, KEH = Kelheim, SÖM = Sömmerda, TÖL = Bad Tölz-Wofratshausen, WAK = Wartburgkreis. Seed traits and results of aging experiments are indicated as *1000-seed weight*, percentage of filled seeds in *xray*, percentage of initial germination (*Ki*), maximal germination rate (*maxK*) and longevity in days with standard error (*p50± s.e.*).

Id	***src***	***latitude (N)***	***longitude (E)***	***height a.s.l. (m)***	***loc***	***1000 seed weight (g)***	***xray (%)***	***Ki (%)***	***maxK (%)***	***P50 ± s.e. (d)***
AB093_l	w	50.92188	10.34559	355	WAK	1.13	32.3	75.0	100.0	7.8 ± 1.5
AB_l	cg					1.02	71.6	100.0	100.0	9.0 ± 1.2
AB146_h	w	47.55886	12.79916	1100	BGL	0.82	73.6	71.4	85.0	9.5 ± 1.4
Ab_h	cg					1.18	84.9	86.1	86.1	7.2 ± 0.5
PL276_l	w	48.91117	12.03623	423	KEH	1.29	92.2	100.0	100.0	13.8 ± 0.3
PL_l	cg					1.51	97.2	80.5	82.0	17.3 ± 2.1
PL420_h	w	47.67459	11.53860	1040	TÖL	1.28	85.5	46.5	46.5	9.7 ± 1.0
PL_h	cg					1.26	91.8	20.0	34.0	15.8 ± 5.9
RA378_l	w	51.23069	11.02986	181	SÖM	0.78	100.0	83.3	100.0	30.7 ± 1.0
RA_l	cg					1.30	100.0	76.0	100.0	32.3 ± 3.3
RA418_h	w	47.55886	12.79916	1103	BGL	1.37	100.0	100.0	100.0	21.4 ± 0.3
RA_h	cg					1.06	100.0	91.8	98.0	25.5 ± 1.1

Experiment 2: Comparison of seeds collected in different years from the same populations

We chose lowland and alpine populations of 22 species to investigate the influence of weather conditions on seed longevity (Fig. 23, Tab. 9). The same populations were harvested at the same stage of seed ripeness in two years: 2009 and 2010, 2009 and 2011 or 2010 and 2011. Post-harvesting procedures were conducted using the same methods as described above.

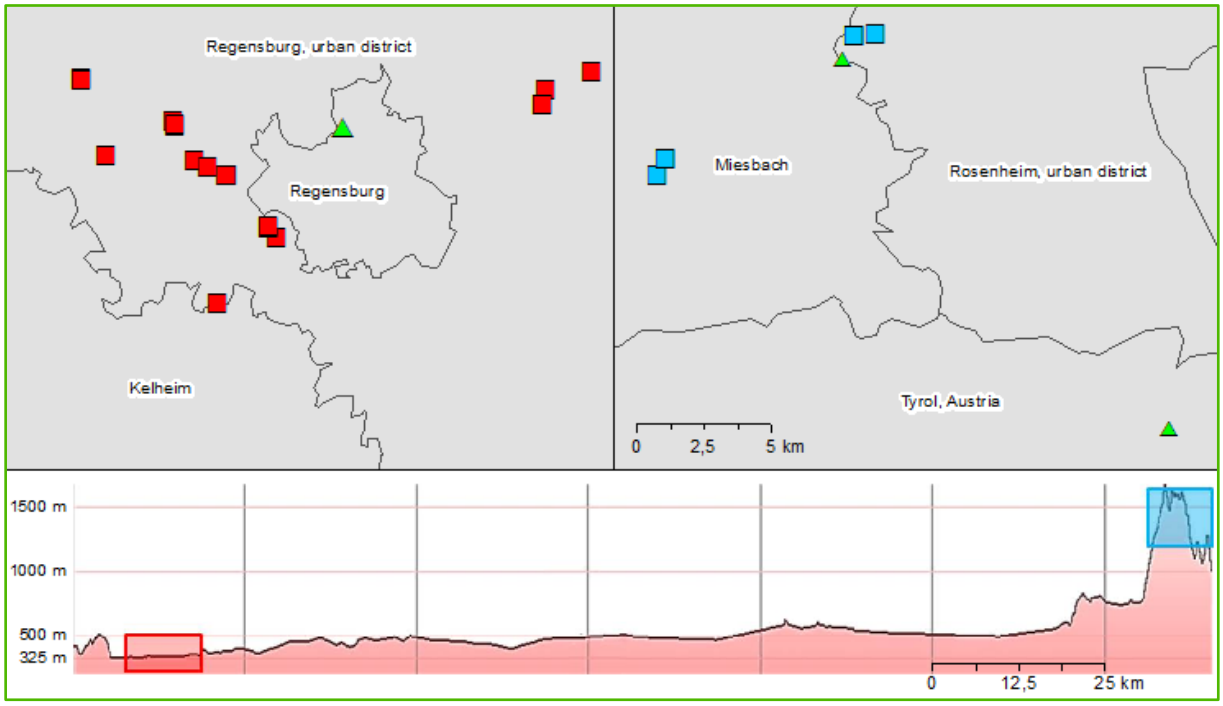


Fig. 23: Distribution of 22 investigated populations near Regensburg (low altitude, red) and Wendelstein / Kufstein (high altitude, blue), weather station are given in green triangles.

Climatic conditions were available through weather stations near the collection sites located in Regensburg (ID 4104, 365m) and Wendelstein (ID 5467, 1832 m)/ Kufstein (495m, AUT, for rainfall in high altitude) (Fig. 23) (available through Deutscher Wetterdienst: www.dwd.de). Mean annual temperature (°C), rainfall (cm^3) and sunshine (h) were extracted using monthly means from the time one year prior to collection date. To investigate the influence of maturation phase, we could not detect the exact starts of flowering periods for each population. Times of seed collections altered up to almost 8 weeks between years even for the same population. Therefore, we decided not to use the method described in Mondoni et al. (2011) to calculate ripening phases based on flowering and ripening times, but take 90 days prior to collection date.

Tab. 9: Table of populations used for comparison of different years: for each population botanical name, family, presence (*E*) or absence (*N*) of endosperm (*es*), endosperm-type, germination temperature and pretreatments, altitude (*meter a.s.l.*), germination requirement (*ger*: stratification, germination temperature) are given. For each collection: collection date (*colldate*), *seed weight* in gramm, percentages of filled seeds in xray analysis (*x-ray*), viability adjusted percentages of initial germination (*Ki*) and maximal germination (*Kmax*), longevity in days with standard error (*p50*) and climate data mean annual temperature and rainfall (*T mean ann*, *R mean ann*), total annual rainfall and total annual sunshine hours (*R Sann*, *SS Sann*), for 90 days before harvest total rainfall and total sunshine (*R S90*, *SS S90*).

Species*	***ID***	***Family (-aceae)***	***es***	***ger***	***height a.s.l (m)***	***colldate***	***seed-weight (g)***	***xray (%)***	***Ki (%)***	***maxK (%)***	***p50 ± s.e.(d)***	***T mean ann (°C)***	***R mean 90 (mm³)***	***R Sann (mm³)***	***SS Sann (hours)***	***R S90 (mm³)***	***SS S90 (hours)***
Angelica archangelica L.[a]	Aang1	Apia	E	22/14	334	08.2010	1.42	0.93	0.90	0.90	3.77 ± 0.91	8.9	3.3	721.6	1533.0	295.3	632.3
	Aang2					08.2012	2.71	0.97	1.00	1.00	6.37 ± 0.37	9.6	2.2	631.6	1784.6	198.5	648.5
Achillea clavennae L.	Acla1	Astera	N	22/14	1396	08.2011	0.42	0.92	0.92	1.00	20.87 ± 0.62	3.0	1.1	960.8	1797.8	442.7	261.2
	Acla2					09.2012	0.40	0.82	1.00	1.00	25.40 ± 0.02	3.1	5.3	462.9	1455.5	554.5	476.7
Arctium lappa L.	Alap1	Astera	N	22/14	356	07.2010	11.35	0.88	1.00	1.00	29.07 ± 0.16	9.1	3.0	639.4	1632.9	273.4	606.1
	Alap2					09.2012	12.58	0.82	1.00	1.00	29.81 ± 0.27	9.5	2.0	606.7	1793.3	178.3	602.5
Anthyllis vulneraria L. s.l.	Avul1	Faba	N	22/14	1639	08.2011	4.61	1.00	1.00	1.00	27.54 ± 1.94	3.0	1.7	960.8	1797.8	455.1	288.2
	Avul2					08.2012	7.94	1.00	1.00	1.00	30.90 ± 1.22	3.8	5.6	545.8	1634.6	475.4	502.3
Campanula patula L.	Cpat1	Campanula	E	22/14	433	07.2011	0.03	0.97	1.00	1.00	11.87 ± 0.57	8.7	3.0	710.8	1651.9	270.8	632.4
	Cpat2					07.2012	0.09	0.93	1.00	1.00	12.32 ± 0.23	9.6	2.2	631.6	1784.6	202.3	659.1
Campanula persicifolia L.	Cper1	Campanula	E	22/14	359	08.2010	0.20	0.99	1.00	1.00	11.05 ± 0.26	8.9	3.0	721.6	1533.0	272.8	637.9
	Cper2					09.2011	0.07	0.98	1.00	1.00	15.02 ± 1.65	9.1	3.2	702.7	1747.4	287.2	561.7
Digitalis purpurea L.	Dpur1	Scrophularia	E	22/14	397	08.2010	0.09	0.96	1.00	1.00	34.31 ± 1.20	8.9	3.0	721.6	1533.0	272.8	637.9

	Dpur2					08.2011	0.07	0.98	1.00	1.00	24.63 ± 1.94	8.8	3.6	690.8	1738.4	324.6	571.8
	Dpur3					08.2012	0.08	0.87	0.92	1.00	43.15 ± 5.63	9.7	2.3	621.3	1794.5	204.6	646.7
Fragaria vesca L.	Fves1	Rosa	N	22/14	418	07.2010	0.31	0.93	1.00	1.00	33.05 ± 0.82	9.1	2.3	639.4	1632.9	207.7	572.1
	Fves2					06.2012	0.26	0.86	0.94	0.94	34.72 ± 0.49	9.5	1.9	653.9	1763.7	167.1	581.4
Gentiana cruciata L.[b]	Gcru1	Gentiana	E	22/14	389	09.2010	0.15	0.99	1.00	1.00	17.56 ± 0.10	8.7	2.5	724.5	1501.7	225.0	591.2
	Gcru2					08.2011	0.15	0.99	1.00	1.00	21.61 ± 0.04	8.8	3.6	690.8	1738.4	327.1	563.5
Gentiana verna L.[c]	Gver1	Gentiana	E	22/14	387	06.2010	1.18	0.94	0.83	1.00	4.00 ± 0.91	9.0	2.3	649.9	1533.2	207.0	544.6
	Gver2					06.2012	0.06	0.89	0.93	0.93	7.72 ± 0.33	9.5	1.8	653.9	1763.7	161.3	589.3
Helianthemum nummularium (L.) Mill. s.l.[a]	Hnum1	Cista	N	22/22	1394	08.2011	1.21	0.98	0.90	1.00	52.09 ± 2.18	3.0	1.1	960.8	1797.8	442.7	261.2
	Hnum2					09.2012	1.41	1.00	1.00	1.00	67.65 ± 5.38	3.1	5.3	462.9	1455.5	554.5	476.7
Linum catharticum L.[c]	Lcar1	Lina	E	22/22	1390	08.2011	0.16	1.00	0.80	0.90	22.36 ± 6.23	3.0	1.1	960.8	1797.8	442.7	261.2
	Lcar2					09.2012	0.13	0.99	1.00	1.00	37.81 ± 1.60	3.1	5.3	462.9	1455.5	554.5	476.7
Lychnis viscaria (L.) Borkh.	Lvis2	Caryophylla	E	22/14	354	07.2011	0.05	0.98	1.00	1.00	52.98 ± 2.00	8.7	2.5	710.8	1651.9	222.1	688.9
	Lvis2					07.2012	0.07	0.96	1.00	1.00	25.64 ± 0.79	9.6	2.2	631.6	1784.6	202.3	659.1
Medicago lupulina L.	Mlup1	Faba	N	22/14	402	07.2011	1.56	1.00	1.00	1.00	23.01 ± 0.00	9.1	3.0	639.4	1632.9	273.4	606.1
	Mlup2					08.2012	2.50	1.00	1.00	1.00	33.67 ± 0.46	8.8	3.6	690.8	1738.4	324.6	578.3
Melilotus albus Medik.	Malb1	Faba	N	22/14	334	07.2010	1.44	1.00	1.00	1.00	58.48 ± 0.76	8.7	3.0	710.8	1651.9	270.8	632.4
	Malb2					08.2011	1.46	1.00	1.00	1.00	71.20 ± 0.07	9.6	2.2	631.6	1784.6	198.5	648.5
Origanum vulgare L.[b]	Ovul1	Lamia	E	22/14	341	09.2010	0.22	1.00	0.72	0.79	27.53 ± 0.63	8.7	2.2	724.5	1501.7	198.8	615.5
	Ovul2					09.2012	0.12	1.00	0.98	1.00	37.27 ± 0.85	9.5	2.0	606.7	1793.3	178.3	602.5
Plantago major L.	Pmaj1	Plantagina	E	22/14	335	07.2010	0.30	0.99	1.00	1.00	26.58 ± 0.00	9.1	3.0	639.4	1632.9	273.4	606.1

subsp. *major* [b]	Pmaj2					08.2011	0.27	0.82	0.98	1.00	25.61 ± 0.80	8.8	3.6	690.8	1738.4	324.6	578.3
Saxifraga rotundifolia L.	Srot1	Crassula	E	22/14	1252	08.2011	0.25	0.96	0.81	1.00	13.32 ± 1.04	8.8	3.7	690.8	1738.4	331.1	560.3
	Srot2					09.2012	0.05	0.99	0.93	1.00	15.36 ± 1.30	9.6	2.2	631.6	1784.6	202.3	659.1
Sedum album L.	Salb1	Solana	E	22/14	439	08.2011	1.44	0.96	1.00	1.00	20.49 ± 1.14	8.7	2.2	724.5	1501.7	199.3	609.0
	Salb2					07.2012	1.11	0.86	0.74	1.00	15.55 ± 1.91	9.7	2.3	621.3	1794.5	208.9	641.9
Sedum maximum (L.) Hoffm.	Smax1	Crassula	E	22/14	430	09.2011	0.07	1.00	0.98	0.98	15.57 ± 0.71	9.1	2.8	702.7	1747.4	255.1	559.1
	Smax2					10.2012	0.12	1.00	0.83	0.94	11.17 ± 0.71	9.5	1.5	606.4	1734.4	136.8	514.3
Solanum dulcamara L.[a]	Sdul1	Saxifraga	E	22/14	334	09.2010	0.05	0.99	0.89	0.93	5.47 ± 0.34	3.0	1.1	960.8	1797.8	442.7	261.2
	Sdul2					08.2012	0.07	0.98	1.00	1.00	10.91 ± 1.73	3.1	5.3	462.9	1455.5	554.5	476.7
Verbascum densiflorum Bertol.	Vden1	Scrophularia	E	22/14	344	09.2010	0.11	1.00	0.87	0.94	43.50 ± 1.86	8.7	2.5	724.5	1501.7	225.0	591.2
	Vden2					09.2012	0.11	1.00	0.48	0.81	40.2 ± 5.75	9.5	2.0	606.7	1793.3	178.3	602.5

* Botanical Names according to Wisskirchen & Haeupler (1998)

[a] Pre-sowing treatment = cold stratification at 4°C for 6 weeks

[b] Pre-sowing treatment = cold stratification at 4°C for 12 weeks

[c] Germination with 0.25% Giberrelic acid

[d] Pre-sowing treatment = scarification with sandpaper or seed coat chipped with scalpel)

Preliminary testing of germination requirements

Frozen seeds in aluminium bags were stored at 20°C for at least one day and rehydrated to 30% RH for another three days before processing was continued under room conditions (~40% RH, 21-25°C). Of each accession 12 samples of seeds were separated and stored in glass containers. All samples were x-rayed to examine the percentage of filled seeds.

Dormancy breaking and optimal germination conditions were evaluated before starting the ageing experiment. We used existing data from other studies (P. Poschlod, S. Rosbakh, S. Tausch, unpublished data) and databases (e.g. (ePIC 2014; Poschlod *et al.* 2003) to narrow down optimal conditions and performed preliminary tests on each population. Mechanical scarification with scalpel or sandpaper was used to open seed coats (Fabaceae, Lythraceae). To break physiological dormancy seeds were undertaken cold stratification for 6 or 12 weeks in a 4°C climatic chamber. For *Gentiana verna* we could not find conditions leading to germination rates >80% in time and, therefore, watered with 0.25% Giberrelic acid (Sarstedt, Germany) at the beginning of the treatment to break dormancy. Seeds were placed on paper in Petri dishes, watered with deionised, sterilized water and placed in climate chambers (RUMED 1301, RubarthApparate GmbH, Laatzen, Germany). Standard temperatures were 14/10°C, 22/14°C (both diurnal fluctuating) or 22 °C (constant). Light was provided for 14 h each day by white fluorescent tubes (3800 Lux). Germinated seeds were counted and removed twice a week. Seeds that have not germinated after 6 weeks were tested for viability with Tetrazolium tests according to ISTA recommendations (1999). Viability adjusted germination (vag) was calculated by dividing the germination rate in each sample by the percentage of x-ray viability in the same sample. Results of germination were further proved by Tetrazoliumtests of not germinated seeds in each sample.

Controlled ageing

Controlled ageing was conducted following the protocol of Millenium Seed Bank (Davies & Probert 2004; Newton *et al.* 2009; Probert *et al.* 2009). For rehydration seeds were deposited in open glass containers and stored in electrical enclosure boxes (ASYNTEK, Electrotechnik GmbH, Egesheim, Germany) with non-saturated LiCl (47%) at 20°C. Relative humidity of LiCl-solution and seeds was measured with AW-Dio Hygrometer (Rotronic GmbH, Ettlingen, Germany). After 14 days all seed samples were rehydrated. The first seed sample of each population was subjected to a germination experiment; all other samples were transferred an

enclosure box with 60% RH and 45°C. After day 1, 2, 5, 9, 20, 30, 50, 75, 100, 125 and 150 one container of each accession was removed and germination experiments were conducted with species specific optimal conditions.

Computations

We used probit regression analysis with drc package in R (R Development Core Team 2014; Ritz & Streibig 2005) to calculate the time for viability to fall to 50% (P50) and 85% (P85). Calculations were based on the equation of seed viability after day p: vp= Ki-p/σ (Ellis & Roberts 1980), where Ki is the initial viability (= upper limit) and σ is the standard deviation of the normal distribution of seed deaths and at the same timethe negative inverse of the slope of the probit regression line.

Experiment1: We compared seeds from high and low altitudes, and both wild and common garden origin. Differences in quality were reveled by comparison of percentages of filled seeds, initial and maximal germination. Aging performances were analysed via graphical plotting of germination to aging period (days) for each species.

Experiment 2:
Unpaired t-tests were used to compare seed longevity of species depending on endosperm (present/absent) in R. We also used unpaired t-tests to evaluate differences of p50, initial germination and percentage of filled seeds in x-ray between alpine and lowland populations. Paired t-tests were used to reveal differences in p50, initial germination, percentage of filled seeds in xray, seed weight and climatic conditions between the years in the same species: 2011 vs. 2012 (alpine), 2010 vs. 2011 (lowland), 2011 vs. 2012 (lowland) and 2010 vs. 2012 (lowland). Aging performances of seeds from different years were analysed via graphical plotting of germination to aging period (days) for each species.
As we had repeated measures design we tested for normal distribution of differences between the years (Field *et al.* 2012). Linear mixed-effects models (lme) with nlme package in R were examined (Pinheiro *et al.* 2014; R Development Core Team 2014), because within-group (within species) errors are allowed to be correlated and intercepts can vary across groups (=species). Therefore, models are only based on variance in seed longevity (p50) that is explained by the fixed factors in different years - and not the variance explained by the differences across species.

Our models were based on literature and contained seed weight, mean annual temperature, total annual rainfall, mean annual rainfall, total annual sunshine, total rainfall, and total sunshine 90 days before harvest in different combinations (Tab. 10). We took R^2 as measure for model fitting (Nakagawa & Schielzeth 2013; Xu 2003) and tested models against each other using delta AICc and Akaike weights in the package AICcmodavg in R (Burnham & Anderson 2004; Mazerolle 2006). Climatic conditions in both habitats (alpine and lowland) are very different, that was why we also tested models for both habitats separately.

Tab. 10: Models, which were tested on our dataset included the factors mean annual temperature (T mean ann), sum of annual rainfall (R Sann), sum of rainfall within 90 days prior to harvest (R S90), mean annual rainfall (R mean ann), seed weight in log scale (log(weight)), sum of sunshine hours within 90 days prior to harvest (SS Sann) and interactions of those factors.

model	***factor 1***	***factor 2***	***factor 3***	***factor 4***	***factor 5***
null model					
Mod2 [1)]	T mean ann	R Sann			
Mod3 [1)]	T mean ann	R Sann	log(weight)		
Mod4 [2)]	T mean ann				
Mod5 [2)]	R S90				
Mod6 [3)]	T mean ann	R mean ann	log(weigth)	(log(weight) * R mean ann)	T mean ann * R mean ann
Mod7 [4)]	T mean ann	R Sann	log(weight)	SS Sann	
Mod8 [5)]	T mean ann	R S90	SS S90		

[1)] Probert et al. 2009
[2)] Mondoni et al. 2011
[3)] Merritt et al. 2014
[4)] own model based on Probert et al. 2009 + sunshine
[5)] own model with high relevant factors from Probert et al. 2009 and Mondoni et al. 2011 + sunshine

RESULTS

Experiment 1:

Seed quality in terms of percentage filled seed in x-ray analysis tended to be not different or higher in common garden populations compared to wild populations (Tab. 8). Comparing populations from alpine and lowland habitats differences in seed filling were disparate: There were no differences in populations of *R. acris*; the *P. lanceolata* population from lowland contained more filled seeds than the population from the alpine stand, whereas for *A. belladonna* it was the other way around. Neither lowland vs. alpine seeds nor wild vs. common garden seeds showed differences in seed weight. Maximal germination was not different or higher in lowland than in alpine populations - and not different or lower in common garden than in wild populations. Regarding initial germination rates an obvious trend of higher rates in lowland populations was disproved by *R. acris,* which showed higher initial germination in alpine populations (wild and common garden). Initial germination rates were higher in wild populations of *P. lanceolata* and *R. acris*, but lower in wild populations of *A. belladonna* compared to common garden.

Seed longevity tended to be higher in populations from low altitudes both in wild populations and common garden populations (Tab. 8). An exception is population RA378_h, which is wild and from high altitude, and even though had extraordinary high longevity - higher than the lowland population and both populations from common garden (Fig. 24). Differences between longevity of high and low altitude populations were smaller in seeds from common garden than wild populations. Differences between wild and common garden seeds were in general higher in populations from high altitudes.

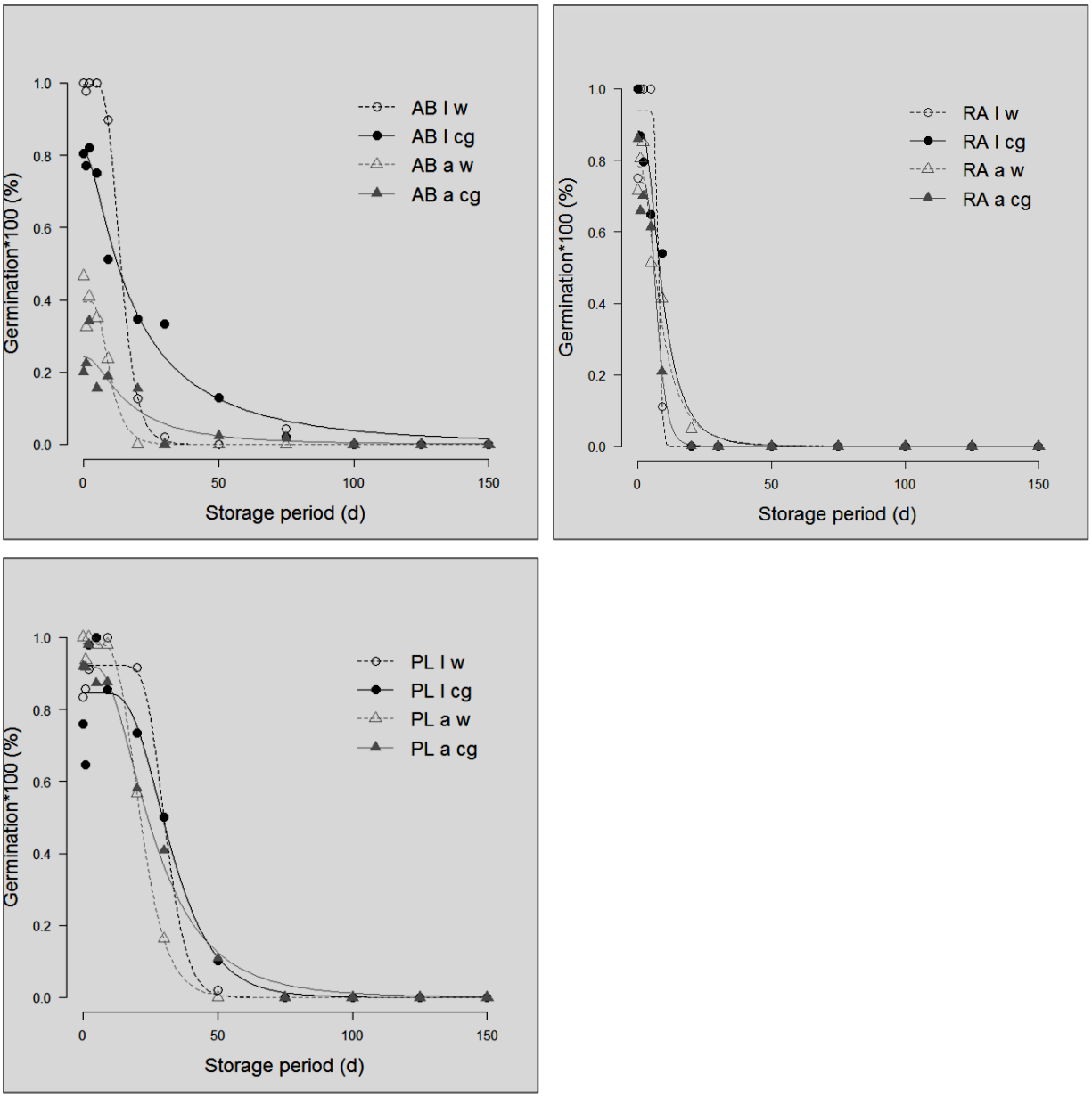


Fig. 24**:** Survival curves for wild (w) and common garden (cg) populations of *A. bella-donna* (AB), *P. lanceolata* (PL) and *R. acris* (RA), curves are fitted with probit analysis. Accessions are named according to Tab. 8.

Experiment 2:

General traits, germination and longevity data

Aging performances differed in 22 European species, but showed similarities within species and families (Fig. 25). The time taken for the viability to fall to 50% (p50) varied between 3.77 days for *Angelica archangelica* and 71.20 days (± 0.07) for *Melilotus albus* (Tab. 9).

We could not detect significant differences of p50, initial germination and seed filling in x-ray between lowland and alpine species ($p>0.05$). Differences of p50 and initial germination were significant for alpine species comparing the years - 2011 and 2012 (p50: $t = -3.313$, $p = 0.030$ and Ki: $t = -3.130$, $p = 0.035$, respectively). For populations collected in lowland only significant differences could be detected for xray filling compared between 2010 and 2012 ($t = 2.491$, $p = 0.042$). Differences in germination performances during the first days of the aging experiment were very high in the collections of *Linum catharticum*, *Origanum vulgare* and *Verbascum densiflorum* (Lcar, Ovul, Vden in Fig. 25). The variation of longevity within populations between different years was lowest in *Campanula patula* with a difference in p50 of 0.45 days and highest in *Lychnis viscaria* with a difference of 27.34 days (Fig. 25). Seeds without endosperm had significant higher longevity, higher initial germination rates and higher seed weight than endospermic seeds (p50: $t = -4.378$, $p < 0.001$; Ki: $t = -2.590$, $p = 0.013$, seed weight: $t = -2.702$, $p = 0.018$).

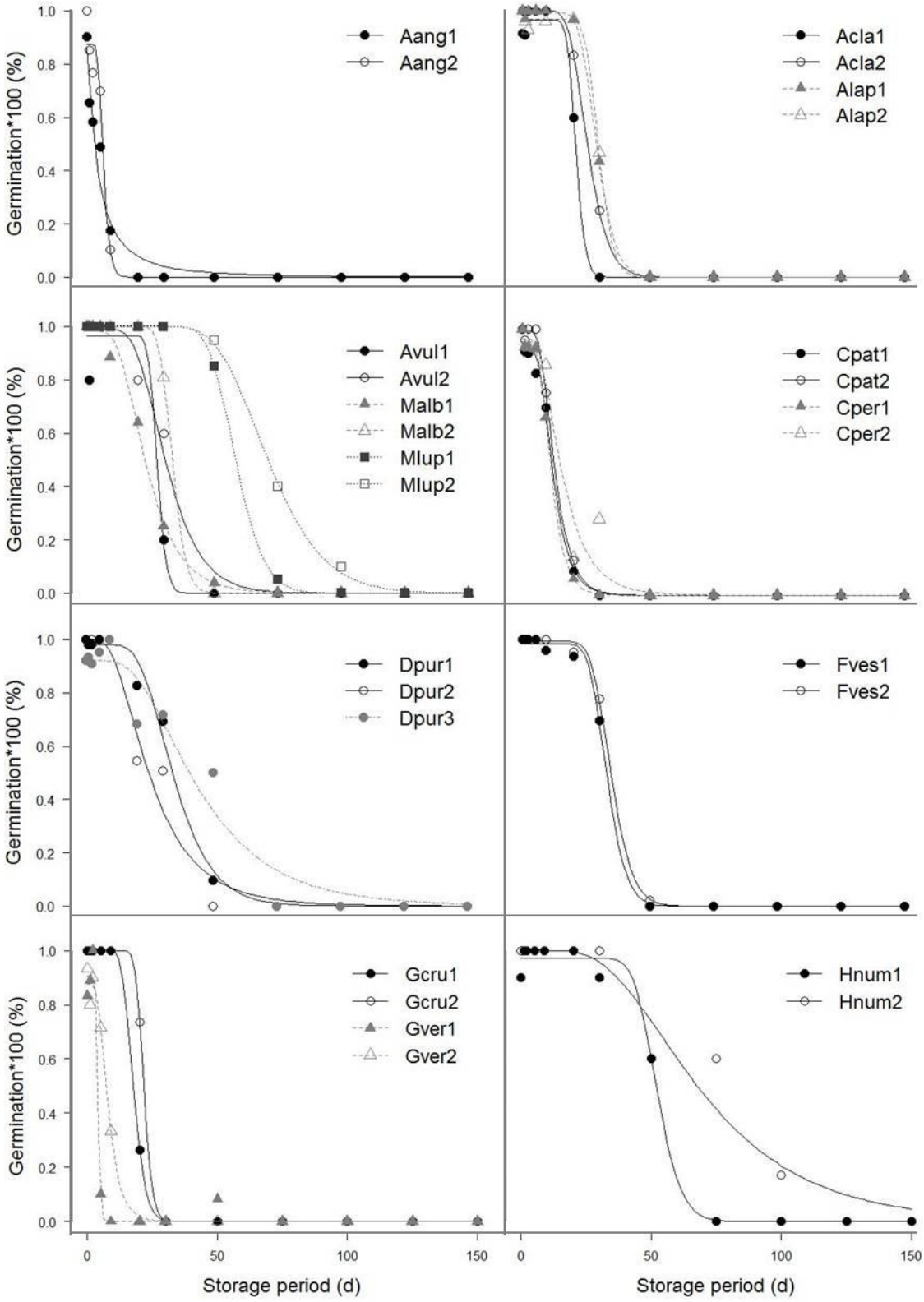


Fig. 25: Survival of seeds during aging experiments; curves fitted by Probit analysis. Accessions are named according to Tab. 9.

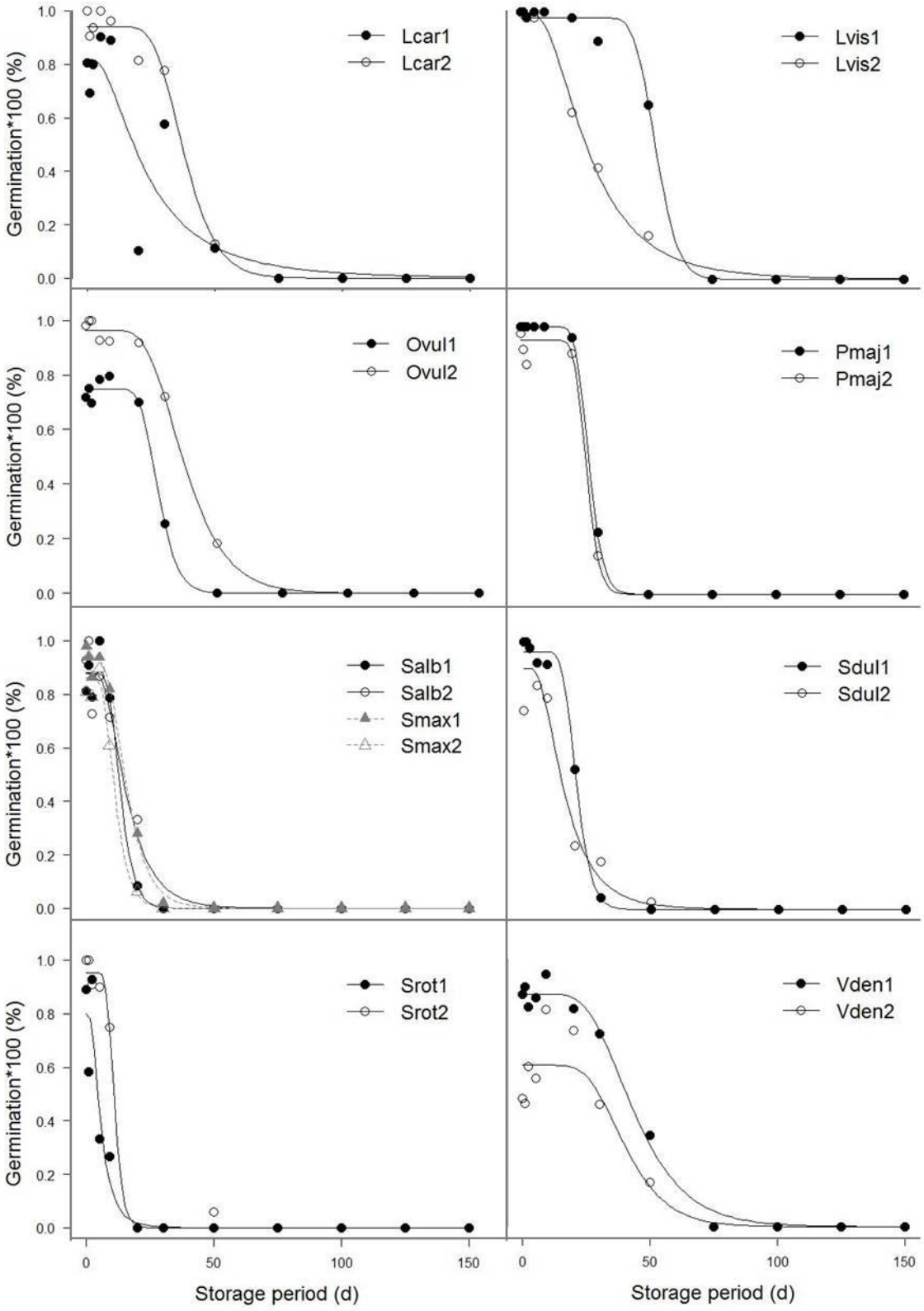


Fig. 25: continued

Correlations of longevity and climate

Comparison of relevant models revealed that most of the models explain the variation not as good as the null model that indicates no influence of any of the investigated factors. Overall the explanatory power of all models should be handled carefully, because they are only exiguously better than the null model (Tab. 11). The model for alpine species is moreover degraded by few repeats. For the whole dataset two models had better fitting than the null model: model 2 shows influence of mean annual temperature and total rainfall, model 8 explains the data with mean annual temperature, total rainfall and total sunshine 90 days before harvest. Data of alpine populations were explained best by rainfall within 90 days before harvest indicating high influence of rainfall within the reproduction period in the Alps. For seeds originating from lowland populations longevity could not be related to any model.

Tab. 11: Model selection table for candidate models based on literature research: given is second order Akaike's information criterions (AICc), AICc distance to best model (Delta_AICc), quasi-likelihood of each model (AICcWt) and quasi-likelihood of the sum of models in each step (cum_Wt), further shown are variations explained by fixed and random and fixed effects (marginal and conditional R^2).

alpine and lowland populations	***K***	***AICc***	***Delta AICc***	***AICcWt***	***Cum.Wt***	***R^2 Marginal***	***R^2 Conditional***	***AIC***
Mod 2*: T mean ann + R Sann*	5	-5.5	0	0.28	0.28	0.03	0.89	-7.04
Mod 8*: T mean ann + R S90 + SS S90*	6	-5.0	0.54	0.21	0.49	0.03	0.90	-7.17
null model	3	-4.8	0.71	0.19	0.68	0.00	0.86	-5.38

alpine populations	***K***	***AICc***	***Delta AICc***	***AICcWt***	***Cum.Wt***	***R^2 Marginal***	***R^2 Conditional***	***AIC***
Mod 1*: R S90*	4	6.49	0	0.74	0.74	0.08	0.97	-1.51
null model	3	8.81	2.33	0.23	0.97	0.00	0.82	4.81

DISCUSSION

It is an ardent and ongoing question for genebanks, which factors determine the quality and longevity of cold stored seeds. In the present study we could detect differences in percentages of filled seeds per population between different growth environments - on wild stands and in common garden - and between collection years 2010 and 2012 in lowland habitats. Initial and maximal germination were influenced by origin and growth habitat, but in different ways for each species. We could show that seed longevity was positively related to common garden and lowland habitats. Models emphasised the influence of annual temperature, rainfall and sunshine on seed longevity between collection years in our study.

Common garden approach

Neither lowland vs. alpine seeds nor wild vs. common garden seeds showed differences in seed weight. This is a contrast to a study of Stöcklin *et al.* (2009), where seed weight was higher in alpine than in lowland species. However, we found that the percentage of filled seeds was always higher or not different in common garden populations compared to wild collections. Environment of mother plants can influence the percentage of matured and filled seeds. For *Hordeum vulgare* Filho & Ellis (1991) have shown that filling of seeds differed between collections in two consecutive years. Seed development and seed quality was also influenced by water availability of the mother plants in *Brassica campestris* (Sinniah *et al.* 1998). The controlled watering and moderate temperatures in common garden populations could have ensured or enhanced maturation and, therefore, mass maturity and the number of filled seeds.

Initial and maximal germination of *R. acris* and *P. lanceolata* seeds were higher in wild than in common garden populations. Filling of seeds was taken into account for germination data (see Material and Methods section) and, therefore, does not play any role for germination data here. Germination of seeds under applied laboratory conditions was optimised to the seeds from wild habitats of *R. acris* and *P. lanceolata*, while germination conditions were maybe not optimised for common garden seeds. For *A. belladonna* it was the other way around: common garden seeds had even better germination rates than wild collected seeds. Depending on the weather and soil conditions at the wild habitats, it was possible that the common garden environment induced dormancy of seeds – or reduced it in the case of *A. belladonna.* For *Bromus tectorum* e.g. it was shown that water stress reduced dormancy of

seeds within one generation (Meyer & Allen 1999). Further, in *Arabidopsis thaliana* maternal photoperiod influenced the germination conditions of the seeds (Munir *et al.* 2001). Which parameters are most influential for which trait is species-specific and also depends on the limiting factors of the habitat, e.g. water, temperature, light or pH gradient (Kochanek *et al.* 2011; Meyer & Allen 1999).

Lowland populations had longer lived seeds from wild and common garden plants, with the exception of one wild population of *A. belladonna* from the Alps (AB146_h). It was already proofed for several species that seeds originating from higher altitudes had shorter longevity (Mondoni *et al.* 2014; Mondoni *et al.* 2011; Probert *et al.* 2009). The most highlighted reason was the unstable, cold and wet environment. So far, only one study could proof that in *S. vulgaris* the differences in longevity are not only based on maternal effects but also on genetic adaptation (Mondoni *et al.* 2014).

In our experiment common garden populations had longer lived seeds than the wild ancestor populations (AB146_h excluded). With seeds from common garden populations, we could show that seed longevity can be enhanced by maternal effects only. This is further underlined by a study of Kochanek et al. (2011) showing that a prezygotic cold environment and coldness only during maturation enhanced longevity of *P. cunninghamii* seeds of the same origin. Maternal effects of weather also have major influence on dormancy in several grass species. It was suggested that interrelation of maternal storage tissue and embryo via hormones can induce or reduce dormancy (Simpson 1990). Similar interrelations can be suggested for maternal influences on longevity.

On the other hand genetic adaptation in the Alps is caused by differences in precipitation and temperature (Manel *et al.* 2012). Differences in longevity of common garden seeds between populations that originate from alpine and lowland populations were smaller than among their wild ancestor populations, but still present. Therefore, we could also show that part of the variation in seed longevity is obviously determined by genetic factors.

Different years approach

In the present study seed longevity and initial germination only differed significantly between years of seed collection from the same populations of alpine species. Seed filling differed only for lowland species and only, when collection years 2010 and 2012 were compared. Seeds without endosperm lived significantly longer, while seed weight had no relevance for longevity.

As we have shown in our common garden approach before, seed longevity, seed filling and initial germination are different for seeds of the same ancestor population, when mother plants were grown in different environments. The differences between years in one population can, therefore, also be related to the amount and combination of differences in the environment. Differences in seed filling were also noticed in *H. vulgare* seeds that were collected in consecutive years (Filho & Ellis 1991).

It was shown in several studies before that endospermic seeds were shorter lived (Merritt *et al.* 2014; Probert *et al.* 2009). In contrast Mondoni et al. (2011) found no significant differences in longevity between seeds with and without endosperm. Despite a balanced proportion of endospermic to non-endospermic seeds in all studies, the different results could be based in one specific embryo type: Merritt et al. (2014) found that one seed embryo type called “folded” - common in Fabaceae, Malvaceae, Myrtaceae, Spinadaceae - lived significantly longer compared to all 12 and 13 seed embryo types classified by Martin (Martin 1946) and Baskin &Baskin (Baskin & Baskin 2007), respectively. Our study, as well as the studies of Probert et al. (2009) and Merritt et al. (2014) contained several species with folded embryo type while Mondoni et al. (2011) only included one sample. Therefore, the differences in longevity between endospermic and non-endospermic seeds could be enhanced by the amount of extraordinary long living folded embryo types within the non-endospermic group.

Seed weight did not differ significantly between collection years and was not related in any of the relevant models. Seed weight was significantly related to maternal environment in greenhouse crossings of *Nemophila menziesii* (Platenkamp & Shaw 1993) and Merrit et al. (2014) found weak but significant correlation of seed weight and longevity. Alike our study Probert et al. (2009) found no effect of seed weight on longevity.

Models that should explain seed longevity on the basis of climatic parameters were mainly based on extreme weather conditions (mountains vs. lowland) and all had weak explanatory character (Merritt *et al.* 2014; Probert *et al.* 2009), which was the same for our dataset. Nevertheless, we found few parameters and models suggested in former studies that fit our data for longevity.

We could show that models, which were appropriate to indicate longevity of seeds collected in different years, included the factors mean annual temperature, total annual rainfall and total annual sunshine. For alpine populations only rainfall 90 days before harvest was relevant for longevity.

In Probert et al. (2009) and Mondoni et al. (2011) mean annual temperature was based on data from 1950 to 2000, in Merritt et al (2014) mean annual temperature was revealed for collection years. All of them found indications that mean annual temperature influences longevity in general, when habitats are compared. It was further shown that temperatures in the phase of seed ripening had very little or no relevance for longevity (Mondoni *et al.* 2011). Based on our models we ascertained that mean annual temperature is also relevant, when different years of collections from the same populations were tested. We found that warmer annual temperatures are to prefer, when collections in one habitat have to be made. In years with higher mean annual temperatures seeds tend to be longer lived.

It was suggested that higher rainfall reduces longevity (Merritt *et al.* 2014; Probert *et al.* 2009), what we could confirm based on two relevant models. Mondoni et al. (2011) specified that rainfall during reproduction period was more influential than annual data. Maturation is an important period with high effects in seeds traits and traits of offspring plants (Donohue 2009; Johnsen *et al.* 2005). Maturation of seeds and germination behaviour is influenced by water availability (Meyer & Allen 1999), which in turn is relevant for longevity (Sinniah *et al.* 1998). Drought at the end of the mass maturation period positively influenced the seed quality and longevity in *Brassica campestris (Sinniah et al. 1998)*. More rainfall during maturation time caused shorter lived seeds in our alpine populations, too.

Within the tested models sunshine hours had positive influence on seed longevity - in combination with temperature and rainfall. It was shown before that light availability of mother plants can influence seeds (Guttermann 2000; Munir *et al.* 2001). Climatic factors are often strongly related, what makes it further difficult to exclude them of each other (Mondoni *et al.* 2011). This is extraordinary relevant, when extreme events were compared. The differences of rainfall between lowland and alpine habitats for example will obviously go in hand with temperature and sunshine, which could also enhance the relevance of one of the factors in our model. As habitat conditions on wild stands are characterized by alternating and limiting factors, it is further difficult to realise the crucial parameter or time span that triggers differences in seed quality of the offspring.

However, to conclude we can say that - in general - years with high temperatures, low rainfall and much sunshine tend to produce longer lived seeds in European species. In alpine populations, which are characterised by short vegetation periods, moreover little rainfall during maturation would enhance longevity of seeds.

Chapter 6

General Discussion and Conclusion

Dispersal and history of plants is linked to human history since beginning of agriculture 6000 years ago (Poschlod 2015; Poschlod & Bonn 1998). Nevertheless, in the last decades species get lost 100 times faster than naturally, due to human influences on the environment (Pimm *et al.* 2014; Wilson 2003). Not only the extinction of several species, moreover the loss of areas and habitats that contain and form different varieties and adaptations is the biggest threat to biodiversity (FAO 2014b). Conservation of plants in-situ should be preferred, e.g. postulated by Maxted (2008) for crop wild relatives (CWR) containing landscapes. Nevertheless, adaptions in plants sometimes cannot keep up with fast global changes. Nowadays, genebanks are a necessary tool to preserve intraspecific biodiversity and genetic diversity in ex-situ collections (CBD 2002, www.cbd.int, press release).

In general, the storage of high genetic variation within populations on one hand, and highly differentiated populations on the other hand, is the best basis for stable and adaptable plant populations in future (Culley *et al.* 2002; Reed & Frankham 2003). With different levels of funds, the possibility and time to get background information is limited and, therefore, this declaration is far from practicable, especially for common species that are widely distributed (Guerrant *et al.* 2004).

The present study identified differentiation levels and genetic variation in populations of three common plant species within two geographic scales (chapter 2, 3, 4). Moreover, high phenotypic intraspecific variation in seed quality and longevity were identified (chapter 5).

HISTORY OF PLANT POPULATIONS

The history of a species is crucial for prioritisation of regions and populations for collections. High genetic diversity within a population can be the result of long history on site or of secondary contact zones. The latter are not necessarily adapted to their habitat (Walter & Epperson 2005) chapter 2. Furthermore, introgression of new established to indigenous populations is contributing to a complicated pattern of haplotype distribution and differentiation between populations.

In threatened species it was already suggested that population history has major influence on the genetic variation within and between populations (Ellstrand & Elam 1993). As crucial is the knowledge of population history for common plants like *Sedum album*, which are affected by anthropogenic influences recently.

PLANT TRAITS

The delineation of collection/study areas sometimes makes it impossible to get an overview of the species history. Through population genetic analysis it was possible to reveal differentiated areas and populations that can be preferred in collections of *Lathyrus pratensis* and *Hepatica nobilis* in Bavaria (chapter 3 and 4). To minimise the influence some life history traits on genetic structure, we tested two species with high similarities: perennial, outbreeding, insect pollinated, characterised as common in Bavaria – within a comparable geographic range (Hamrick *et al.* 1991; Nybom 2004; Reisch & Bernhardt-Römermann 2014; Stöcklin *et al.* 2009).

Both *L. pratensis* and *H. nobilis* showed diversity levels, which were in the range described for outbreeding perennials (mean SI = 0.21 and 0.29, respectively) (Hamrick et al. 1991). In both species differentiation between populations even exceeded findings for outbreeding, perennials at regional scale (Nybom & Bartish 2000). Nevertheless, genetic differentiation between populations and spatial genetic structure resulted in remarkable differences. While in *L. pratensis* 57% of genetic diversity was allocated between populations, in *H. nobilis* it was even 73%. *L. pratensis* is able to grow in ruderal and short-living habitats, and therefore, the distances between populations are smaller than in *H. nobilis*. The latter is limited to calcareous beech forests, with distances in between that can be too far for seeds or pollen to overcome. Seed dispersal vectors in *H. nobilis* are ants further leading to a seed dispersal that is limited to several square meters within populations.

Those differences can also be seen in the spatial genetic structures. *L. pratensis* has a clear relation of genetic and geographic distances up to 110km, while a spatial genetic structure is missing in *H. nobilis*. No isolation-by-distance effects can be detected between *H. nobilis* populations, gene flow between populations happens occasionally and on long-distance.

When distribution of genetic diversity within and between populations was compared with regionalised seed production revealed one production area was concordant in *L. pratensis*. Populations of *L. pratensis* showed differentiation in north and south clusters. In the case of

H. nobilis regionalised seed production provenances had no concordance and are, therefore, meaningless.

Postulated to some extent (Nybom & Bartish 2000; Reisch & Bernhardt-Römermann 2014), the studies of *S. album*, *L. pratensis* and *H. nobilis* further emphasise the importance of population history and species life history traits as most important factors for the geographic scale of genetic variation.

INTRASPECIFIC DIFFERENCES IN SEEDS

Seed traits, especially longevity, have been topic of recent studies, of which few revealed the genetic determination of the trait (e.g. see studies of (Donohue ; Kochanek *et al.* ; Mondoni *et al.*). In *Silene vulgaris* part of the differences in longevity within one species is genetically determined, but part is also specified by maternal effects (Mondoni *et al.* 2014). Our study of three different species enhances these findings.

Moreover, by a comparison of different models comprising climatic data, we could show that some models had at least some explanatory power on our data (Mondoni *et al.* 2011; Probert *et al.* 2009). Studies like Mondoni *et al.* (2014), maybe over a wider range of taxa and geographic scales, could give the basis for knowledge about the reasons for accelerated aging. For genebank management this kind of data is urgent and, therefore, should be linked with results from studies and genebank experience from all over the world.

STRENGTHS AND LIMITATIONS

Chapter 2 presented a picture of the history of *S. album* populations and the human influence on single populations. More populations, especially in sparsely populated areas (Kroatia, Greece), where it was hard to find populations, would give an even better picture of the plants history. The migration from East to Western and Northern Europe and the possible existence of glacial refugia in the East would become clear.

Even though, overall influence of sowings is implausible, it cannot be excluded for all populations of *L. pratensis* (chapter 3). We tried to sample populations, which were not sown or obviously influenced; nevertheless, former sawing and the input of nearby areas cannot be excluded. To test almost unaffected populations of a common and frequently sown species, nature reserves or populations with familiar history would have to be tested, which was not

included in the framework of the present study. A study comparing restoration zones and untreated areas is conducted at the University of Regensburg since 2014 (Kaulfuss & Reisch, pers. communication).

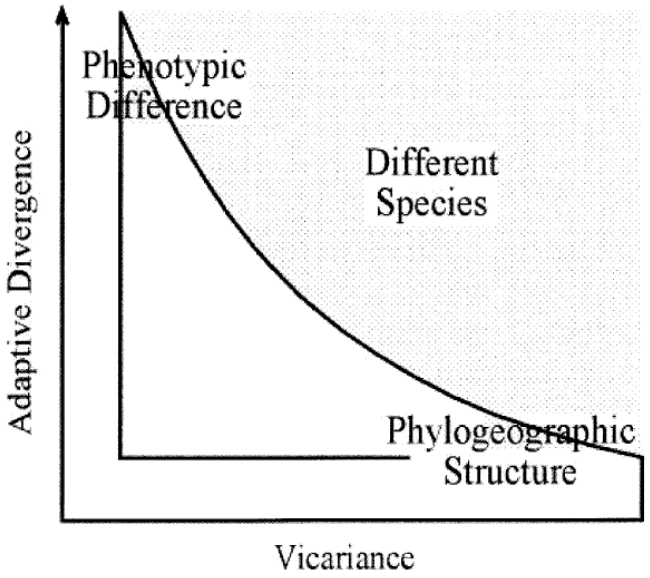


Fig. 26: Two driving forces of genetic diversity within the species level (Moritz 2002)

Genetic diversity and differentiation caused by vicariance through isolation processes can be revealed by AFLP markers (Fig. 26). Besides, selection pressure caused by environmental conditions can lead to adaptive divergence, which can result in strong phenotypic differentiation (Ehrlich & Raven 1969). Only single or few genes are concerned. Therefore, it is often criticised that adaptations to environmental conditions caused by selection pressure can mostly not be revealed by neutral markers (Parra-Quijano *et al.* 2012; Varshney *et al.* 2007). Quantitative trait loci or differences in fitness parameters would be better to reveal those adaptations (see chapter 5). For evaluation of genebank priorities concerning special adaptations (for breeding etc.) marker systems like SNP would also be appropriate (Varnish et al. 2007). Nevertheless, neutral marker systems are an effective tool in population genetic to reveal phylogeographic structure by neutral differentiation of regions and populations (Varnish et al. 2007). Moreover, studies already have shown that proportions of divergence correlates with markers under selection pressure and the evolutionary potential is reflected by neutral markers even better (Leinonen *et al.* 2008; McKay & Latta 2002).

Experiment 1 in chapter 5 gives an impression about the proportions of genetic and environmental influence on the quality and longevity of a species. This is strongly supported

by a recent study of *S. vulgaris* (Mondoni *et al.* 2014). Nevertheless, more species should be tested in the common garden experiment to get statistically stable results. Up to date, three more species have been grown in a common garden and tested in an aging experiment. The data have to be analysed.

CONCLUSION AND PERSPECTIVES

The maintenance of genetic diversity below the species level and, therefore, the understanding of all connected processes in ecosystems is urgent. With more knowledge about genetic diversity it is possible to understand persistence of species and stability of ecosystems (Moritz 2002). The necessity of genetic diversity within genebank collections is stated more and more in scientific and popular media, esp. for PGR species (FAO 2014b; Guerrant *et al.* 2004; Laarz 2014; Parra-Quijano *et al.* 2012).

Information on intraspecific variation for widely distributed plant species is often not accessible or neglected in studies and genebank collections (Michalski & Durka 2012; Wilkinson 2001; Williams 2007). For common species it could be suggested that differentiation is low because of frequent seed or pollen exchange and human influence on populations. Further, especially for common species the financial factor is opposed to the immediate necessity, because there is no obvious threat (van Jaarsveld *et al.* 1998). Conservation strategies - collections for genebanks included - which are only based on ecogeographic background are not sufficient for common species (Forwick *et al.* 2003; Michalski & Durka 2012). In genebank guidelines life history traits of species are mentioned, to which collections should be attuned to (ENSCONET 2009b; FAO 2014a).

Our study of three species further enhances the effect of population history, species traits and seed dispersal in genetic diversity and differentiation of populations. A classification of differentiation and genetic diversity based on biological traits would help to define more precise collection strategies for common species (Bussell *et al.* 2006). In combination with well documented restoration projects for single species, the success of restoration projects could be increased, too (Godefroid *et al.* 2011)

The influence of environmental conditions on seed longevity otherwise, seems to be universal and not species specific. This was shown in several studies concerning a number of plant species (Mondoni *et al.* 2014; Mondoni *et al.* 2011; Probert *et al.* 2009). With the collection of more data, future collection trips can be adjusted to best weather conditions.

Stored seeds would have good quality and longevity – and the efforts for testing, recollection and regrowth would be reduced.

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APPENDIX

Table S1. Polymorphic sites revealed by primer “Tab f” within trnL-trnF chloroplast regions of *S. album* are given, Genbank accession numbers are included.

name	*tab f*																*Genbank accession number*
	46	*107*	*160*	*217*	*238*	*251-252*	*282*	*290*	*330-331*	*311*	*339-350*	*335*	*378*	*374*	*382*	*447*	*tab f*
A	T	G	T	C	T	AT	T	T	C	CT	-	-	A	T	G	a	KT886498
AND	T	G	T	C	A	--	T	T	C	CT	-	-	A	T	G	a	KT886518
BE	T	G	T	C	A	AT	T	T	C	CT	-	-	A	T	G	a	KT886496
CZ	T	G	T	C	T	AT	T	T	C	CT	-	-	A	T	G	a	KT886500
D1	T	G	T	C	T	AT	T	T	C	CT	-	-	A	T	G	a	KT886501
D2	T	G	T	C	T	AT	T	T	C	CT	-	-	A	T	G	a	KT886502
D3	G	A	T	T	A	--	T	T	C	--	T	*[1]	C	T	G	a	KT886503
D4	T	G	T	C	T	AT	T	T	C	CT	-	-	A	T	G	a	KT886509
D5	T	G	T	C	T	AT	T	T	C	CT	-	-	A	T	G	a	KT886524
D6	T	G	T	C	T	AT	T	T	C	CT	-	-	A	T	G	a	KT886523
E1	T	G	T	C	A	--	T	T	C	CT	-	-	A	T	G	a	KT886504
E2	T	G	T	C	A	AT	T	T	C	CT	-	-	A	T	G	a	KT886517
E3	T	G	T	C	A	--	T	T	C	CT	-	-	A	T	G	a	KT886519
E4	T	G	T	C	T	AT	T	T	C	CT	-	-	A	T	G	A	KT886523
F1	T	G	T	C	A	AT	A	T	C	CT	-	-	A	T	G	a	KT886506
F2	T	G	T	C	A	AT	A	A	C	CT	-	-	A	A	G	a	KT886508
F3	T	G	T	C	A	AT	T	T	C	CT	-	-	A	T	G	a	KT886510
F4	T	G	T	C	A	AT	T	T	C	CT	-	-	A	T	G	a	KT886511

I1	T	G	T	C	T	AT	T	T	C	CT	-	-	A	T	G	a	KT886492
I2	T	G	T	C	T	AT	T	T	C	CT	-	-	A	T	G	a	KT886493
I3	T	G	T	C	T	AT	T	T	C	CT	-	-	A	T	G	a	KT886494
I4	T	G	T	C	T	AT	T	T	C	CT	-	-	A	T	G	a	KT886495
I5	G	G	T	C	A	--	T	T	C	--	-	*2	C	T	G	a	KT886513
I6	T	G	T	C	A	--	T	T	C	CT	-	-	A	T	G	a	KT886514
I7	T	G	T	C	T	AT	T	T	C	CT	-	-	A	T	G	a	KT886516
I8	T	G	T	C	A	--	T	T	C	CT	-	-	A	T	G	a	KT886525
PO	T	G	T	C	A	--	T	T	C	CT	-	*3	A	T	G	a	KT886515
S1	T	G	A	C	T	AT	T	T	C	CT	-	-	A	T	G	a	KT886497
S2	T	G	T	C	T	AT	T	T	C	CT	-	-	A	T	T	a	KT886512
SK	T	G	T	C	T	AT	T	T	C	CT	-	-	A	T	G	a	KT886499
SRB1	G	A	T	T	A	--	T	T	A	--	T	*1	C	T	G	a	KT886520
SRB2	G	A	T	T	A	--	T	T	A	--	T	*1	C	T	G	a	KT886521

*1 ATAATATATTAA, *2 A-------TTAA, *3 AAAAT-TATTAT

Table S2. Polymorphic sites revealed by primer “Tab c” within trnL-trnF chloroplast regions of *S. album* are given, Genbank accession numbers are included.

name	*tab c*														*Genbank accession number*
	467	*504*	*539*	*561-562*	*578*	*585 -594*	*599-600*	*604*	*626-628*	*637*	*647-648*	*686*	*742*	*767*	*tab c*
A	T	T	A	CG	A	*[4]	--	A	GTA	G	CA	A	T	A	KT886463
AND	T	T	A	CG	A	*[4]	--	A	GTA	T	Cg	A	T	A	KT886484
BE	T	T	A	CG	A	*[4]	--	A	GTA	G	CG	A	T	A	KT886461
CZ	T	T	A	CG	A	*[4]	--	A	GTA	G	CA	A	T	A	KT886465
D1	T	T	A	CG	A	*[4]	--	A	TAC	G	Ca	A	T	A	KT886466
D2	T	T	A	CG	A	*[4]	--	A	GTA	G	CA	A	T	A	KT886467
D3	T	T	-	TT	-	*[4]	--	C	GTA	G	AG	A	T	A	KT886468
D4	T	T	A	CG	A	*[4]	--	A	GTA	G	CA	A	T	A	KT886475
D5	T	T	A	CG	A	*[4]	--	A	GTA	G	CA	C	T	A	KT886488
D6	T	T	A	CG	A	*[4]	--	A	GTA	G	CA	A	T	A	KT886490
E1	T	T	A	CG	A	*[4]	--	A	GTA	T	Cg	A	G	A	KT886470
E2	T	T	A	CG	A	*[4]	--	A	GTA	G	Cg	A	T	A	KT886483
E3	T	T	A	CG	A	*[4]	--	A	GTA	T	Cg	A	T	A	KT886485
E4	T	T	A	CG	A	*[4]	--	A	GTA	G	Ca	A	G	A	KT886489
F1	T	T	A	CG	A	*[4]	--	A	GTA	G	CG	A	T	A	KT886472
F2	T	T	A	CG	A	*[4]	--	A	GTA	G	Cg	A	T	A	KT886474
F3	G	T	A	CG	A	*[4]	--	A	GTA	G	CG	A	T	A	KT886476
F4	T	T	A	CG	A	*[4]	--	A	GTA	G	CG	A	T	A	KT886477
I1	T	A	A	CG	-	*[4]	--	A	GTA	G	CA	A	T	A	KT886457
I2	T	C	A	CG	A	*[4]	--	A	GTA	G	CA	A	T	A	KT886458
I3	T	T	A	CG	A	*[4]	--	A	GTA	G	CA	A	T	A	KT886459

I4	T	T	A	CG	A	*[4]	--	A	GTA	G	CA	A	T	A	KT886460
I5	T	T	-	TT	-	--	--	C	GTA	G	AG	A	T	A	KT886479
I6	T	T	A	CG	A	*[4]	--	A	GTA	T	CG	A	T	A	KT886480
I7	T	T	A	CG	A	*[4]	--	A	GTA	G	C-	A	T	C	KT886491
I8	T	T	A	CG	A	*[4]	--	A	GTA	T	Cg	A	T	A	KT886483
PO	T	T	A	CG	A	*[4]	--	A	GTA	T	CG	A	T	A	KT886481
S1	T	T	A	CG	A	*[4]	--	A	GTA	G	CC	A	T	A	KT886462
S2	T	T	A	CG	A	*[4]	--	A	GTA	G	CA	A	T	A	KT886478
SK	t	T	A	CG	A	*[4]	--	A	GTA	G	CA	A	T	A	KT886464
SRB1	T	T	-	TT	A	*[4]	AT	C	GTA	G	AG	A	T	A	KT886486
SRB2	T	T	-	TT	A	*[4]	AT	C	GTA	G	AG	A	T	A	KT886487

*[4] AAGTTTGT